PART 3

Histochemical studies on the embryos of

带你 ornata treated with Cytochalasin H
INTRODUCTION

It has now been clearly established that in intact organisms the cells are held together by the properties of the cell surface. Cell surface is a broad term used to describe the cell plasma membrane and associated intra and extracellular coat. The intracellular coat being the microtubules and microfilaments and extracellular coat is made of carbohydrate rich material. The equivalent in animal tissues of the polysaccharide coat of the bacteria and the plant cells has until recently been considered to be represented in only a few animal cells. Until specialized histochemical methods were devised for the ultrastructural detection of carbohydrates most electron microscopists discarded the concept of the presence of the cell surface coat in multicellular organisms, though many scientists using histochemical techniques for light microscopy not only had accepted the presence of it but also had assigned some specialized functions to it (Holtfreter, 1943).

In 1963, Bennett proposed the comprehensive term 'glycocalyx' to describe a polysaccharide rich surface coating apparently present in many cells.
Electron microscope studies have demonstrated clearly, the presence of two types of surface layers associated with the periphery of the animal cells.

1) Cell coat
2) Basal lamina.

Cell coats have been demonstrated in a large variety of animal cells as carbohydrate containing components of the cell membrane. It is a thin coat of mucopolysaccharides and glycoproteins attached to the outer leaflet of the protein plasma membrane. The intimal relationship of the cell coats with the plasma membrane, their resistance to physical treatment and their relation to specific cell types favour the view that they are not merely inert films of the condensed ground substance or mucus, but integral components of the cell membrane responsible for some of the fundamental properties of the cell surface.

The importance of cell surface properties in normal cell differentiation is evidenced by changes in the adhesiveness of the cells during morphogenetic movements e.g. the transition from blastula to early gastrula in teleost egg is accompanied by an increased adhesiveness of gastrula cells which flatten extensively on the glass substratum whereas blastula cells remain spherical. During cellular
differentiation and tissue organization significant changes take place at the cell surface. Cell surface coat changes according to the functional requirements of the cells.

Cell coats in general may be related to some fundamental properties of the cell surface such as cell adhesiveness.

Selective cell adhesion has been well documented by Weiss (1958, 1960). Moscona (1962) has shown that intercellular material plays a role in cell adhesion. Many observations like morphological identification of the intercellular material (Gersch and Catchpole, 1949) or enzymatic dissociation of cells (Moscona, 1952) have indicated the presence of intercellular material (Rinaldini, 1958). Materials enhancing cell aggregation in sponges have been described as protein or mucopolysaccharide. (Humphreys, 1963; Lillian and Moscona, 1967). The factors demonstrated in the reconstruction of sponge cell adhesion fits the historical expectations of an intercellular cement.

On the basis of all the studies it has been suggested that histogenetic attachment of cells is mediated by specific cell products that function at the cell surface or inbetween cells and their molecular characteristics play a role in conferring upon embryonic cells the properties of selective
affinity and surface specialities that are essential for histogenesis. The validity of this general concept was supported by the isolation and characterization of cell products which specifically enhance the aggregation of dissociated sponge cells (Humphreys, 1963 and Moscona, 1963).

The drug Cytochalasin has an effect on cell sorting and reaggregation of cells has been shown by several investigators using different cells.

Armstrong and Parenti (1972) showed that CB inhibits cell sorting in heterotypic cell aggregates of chick heart and pigmented retina cells but considerable amount of sorting occurred in neural retina cells and pigmented retina cells.

CB disrupted the formation of the characteristic pattern of islands of heart cells of chick embryo within a retinal continuum was reported by Maslow and Mayhew (1972). They attributed this to the effect of CB on the microfilaments which inhibit the movement of cells within aggregates. Steinberg and Wiseman (1972) confirmed this using intermixed embryonic cells of chick.

Appleton and Kemp (1974) and Maslow and Mayhew (1974) have also shown the same using CB.

Jones and Partridge (1974) used CB on Limpet haemocytes. Aggregation was totally but reversibly inhibited.
CB according to them acts on the early stages of contact formation between the cells.

Jones (1975) suggested that the effects of CB on cell sorting and reaggregation of cells are quickly reversed on restoration of fresh medium. They interpreted this by stating that effects of CB might be on the cell surface.

On the basis of electron microscopic observations Greenberg et al. (1973) suggested that effects of CB is on the cell surface and it interferes with intercellular adhesive process thereby inhibiting reaggregation in Micromonas. In 1977, Greenberg et al. stated that CA, CB and CE inhibit reaggregation of dissociated sponge cells. The inhibition is graded varying with the dose and duration of the treatment. CE is more potent than CA and CB.

The observation that the effects of Cytochalasin is on cell surface and therefore it inhibits reaggregation of cells agrees with the observations stated earlier where it has been concluded after several observations that cell adhesiveness is the major role played by cell surface material which is rich in carbohydrate.

Cell adhesion, cell reaggregation and cell sorting are the processes which are related to the function of the mucopolysaccharide-glycoprotein surface coat. Any alteration
in the cell surface can affect the cell adhesion thereby bringing about disaggregation of cells and inhibition of cell reaggregation and cell sorting.

Cell adhesion has an important role in morphogenetic movements in embryonic development. Gastrulation which involves morphogenetic movements is a composite of processes involving changes in cellular adhesion, movement and induction (Nieuwkoop, 1973), all of which have been functionally related to cell surface material and their changing temporal pattern.

All the available data indicates that Cytochalasin has some effect on cell adhesion, cell reaggregation, cell sorting and it has been proved that these processes are the properties of the cell surface coat. Therefore it can be said that Cytochalasins affect the cell surface. This has been proved by several workers. (Please see General Introduction).

It has been observed in Part I and Part II that CH brings about disaggregation of embryonic cells in Microrylora ornata. This is the primary effect of the drug which causes inhibition of morphogenetic processes and so gastrulation does not proceed. There is sufficient evidence to indicate that both disaggregation of cells and inhibition of morphogenetic movements can be caused due to altered cell
surface. It would not be inappropriate therefore to test whether CH has any effect on the cell surface using histochemical techniques to stain the carbohydrate rich cell surface in the embryos of *Microhyla ornata* with and without the treatment of CH.
MATERIALS AND METHODS

To observe the effects of the drug CH on the cell surface material viz. glycoproteins and mucopolysaccharides, it was thought desirable to study the histochemistry using PAS and Alcian blue staining techniques.

The experiment was carried out as usual on the embryos in neurula stage without the vitelline membrane using 1.5 µg/ml concentration of CH. The treatment was given for 1½ hrs. during which the embryos showed disaggregation. After the treatment some of the embryos from experimental along with the corresponding controls were fixed for histochemistry.

The embryos were fixed in three different fixatives viz. Lillie's fixative, calcium acetic formol (CAF) and formol alcohol. The embryos were fixed for 3 to 6 hours at 0-4°C for the better preservation of cell surface material. They were washed at 4°C with distilled water, dehydrated using alcohol grades and were embedded in paraffin wax as for routine histology.

For the staining of neutral polysaccharides Periodic acid Schiff's reaction (PAS) after McManus as given by Pearse (1968) was used. For this technique all three fixatives gave good results.
The 6 μm thick sections were brought to water and then were oxidized for about ten minutes in 1% aqueous periodic acid. They were washed in running water for five minutes and stained in Schiff's reagent for ten minutes, again washed in running tap water for five minutes dehydrated in alcohol and cleared in xylene and mounted in DPX. The sections were photographed.

For Alcian blue (AB) reaction formal alcohol was found to be the most suitable fixative. The technique used was after Steedman as given by Pearse (1968). This was used to illustrate the presence and distribution of acid mucopolysaccharides. AB solutions at pH 2.5 and 1.0 were used to distinguish between the non-sulphated and sulphated mucopolysaccharides respectively. AB was a 1% solution. The pH was maintained at 2.5 by 3% acetic acid and at 1.0 by 0.1 N HCl. The sections were stained in AB solution for 30 minutes, washed in distilled water for five minutes and dehydrated in alcohol grades. They were cleared in xylene and mounted in DPX. The sections were photographed.
RESULTS

The use of histochemical techniques at the light microscopic level indicate the presence of carbohydrate rich material in most of the cells.

Two techniques are very commonly used to stain the mucopolysaccharide—glycoprotein rich material. They are Periodic acid Schiff's staining (PAS) and Alcian blue staining.

Periodic acid breaks carbon-carbon bonds in various structures where they are present as adjacent hydroxyl (CHOH—CHOH) groups or adjacent hydroxyl and amino groups (CHOH—CHNH₂) converting them to dialdehydes (CHO—CHO). These aldehydic groups are then detected by Schiff's reagent which gives a purple colour when it is combined with aldehyde.

Our results in the present investigation indicate that the control embryo shows a positive reaction for PAS staining (Plate XIV, Fig. 31). Dark stain is observed at the outer layer of the section indicating that the ectodermal cells and the neural plate cells have taken up the stain. The stained region indicates the presence of neutral mucopolysaccharides.

The section of the experimental embryo (Plate XIV, Fig. 32) at the same magnification as that of the control
section (i.e. x 100) appears swollen. This can be due to the
disaggregation of the cells, because of which the cells fall
apart. Therefore the compact arrangement that is seen in the
control embryo is not seen in the experimental embryo. The
intensity of the PAS staining is considerably reduced in the
treated embryo. This shows that the drug CH affects the
neutral mucopolysaccharides.

Cationic dyes like Alcian blue are positively charged
molecules which consequently are able to bind and precipitate
polyanions. The binding of cationic dyes is electrostatic and
therefore depends on the pH of the staining medium. Thus when
AB is used below 2.5 pH it gets selectively bound to sulphate
group of acidic carbohydrates. Lowering the pH increases the
staining specificity which is then restricted to the sulphate
residue.

The control embryos (Plate XV, Fig. 33) stained at
pH 1.0 with AB does not show any stain in the section
indicating that the sulphated mucopolysaccharides are not
present at all or they are not present in sufficient quantity
so as to take up the stain in the control embryos of Microhyla
ornata in neurula stage.

The sections of the experimental (Plate XV, Fig. 34)
embryos do not show any difference as far as AB staining at
pH 1.0 is concerned. The increase in the size of the embryo after treatment with CH is noteworthy. The section of the experimental embryo is almost double the size as compared to that of the control embryo at the same magnification (i.e. x 100).

In the control embryos (Plates XVI, XVII, Figs. 35, 37) stained with AB at pH 2.5 it can be seen that the cells have taken up the stain. Only yolk cells do not take up any stain. All the structures typical of the stage are clearly seen. AB stains the non-sulphated mucopolysaccharides at pH 2.5. The stained sections therefore reveal the presence and distribution of the non-sulphated mucopolysaccharides in the early neurula of *Microchyla ornata*.

The sections of the experimental embryos (Plates XVI, XVII, Figs. 36, 38) treated with CH (1.5 μg/ml) for 1½ hrs do not show any tissue stained with AB at pH 2.5. This clearly shows that the non-sulphated mucopolysaccharides in the neurula are affected by the treatment of CH and therefore do not get stained.

The rest of the features in the experimental embryos are similar. There is a considerable increase in the size of the embryo after the treatment.
DISCUSSION

The results indicate that due to the treatment of the chemical Cytochalasin H, the neutral mucopolysaccharides in the embryos of *Microhyla ornata* in neurula stage are affected. This is clearly seen with PAS staining. The treated embryo shows less intensity of staining (Plate XIV, Figs. 31, 32).

Similarly as evidenced by AB staining the treatment of CH affects the non-sulphated mucopolysaccharides. The sulphated mucopolysaccharides are not present in the control embryo and the treatment of CH does not have any effect on it. This conclusion is drawn from the results where AB does not stain the sections of even control embryos at pH 1.0 at which it gets selectively bound to sulphated mucopolysaccharides (Plate XV, Figs. 33, 34) and at pH 2.5 the sections of control embryos get stained but those of the treated embryos do not show any stain indicating that the non-sulphated mucopolysaccharides are present in the control embryo of neurula stage and the treatment of CH affects them (Plates XVI, XVII).

In Part 1, it has been shown that the treatment with CH causes disaggregation of the embryonic cells in *Microhyla ornata* (Plates II, V, Figs. 6, 16, 20). In Part 2 it has been confirmed that disaggregation of cells is the primary effect of the chemical.
The role of mucopolysaccharides and glycoproteins which are the two components of the cell surface material has been clearly established in cellular adhesion by several workers. Most of this work has been carried out using either sponge cells or chick embryonic cells (Ito, 1965; Rambourg et al., 1968; Moscona, 1968 and 1974; Roseman, 1970; Roth et al., 1971 and Pratt and Hassell, 1975). They have also assigned the important role of cellular recognition to this protein-carbohydrate complex.

It is quite likely therefore that any change in the cell surface material will result in affecting cellular adhesion and might lead to disaggregation of cells.

Sanger and Holtzer (1972a) have shown that CB inhibits the incorporation of ($^{14}$C) glucosamine into total mucopolysaccharides and glycoproteins by over 50% in cultured myoblasts, fibroblasts and non-sulphated chondroblasts.

CB has been shown to affect the transport of glucose, glucosamine and deoxyglucose in Novikoff rat hepatoma cells by Estensen and Plagemann (1972).

From the above mentioned references it can be seen that either the transport of the essential components like glucosamine required for the synthesis of the mucopolysaccharides or its incorporation into mucopolysaccharides-glycoproteins is
affected by CB treatment. The net result of both is inhibition of the synthesis of these products or the slow down in the rate of its synthesis.

CB has been shown to cause disaggregation of gastrulae cells in *Ella nigriana* by Schaeffer *et al.* (1973b) with increasing rapidity at higher concentrations. The tissues of the gastrula showed differential responsiveness, the ectoderm being the most resistant. They showed that CB produces a significant reduction in the electrophoretic mobilities of the disaggregated endoderm and chordamesoderm. The cell separation was brought about due to the altered surface charge of the endoderm and chordamesoderm as a result of the treatment.

These are the two views regarding the mode of the action of CB in causing disaggregation of cells. It was observed by Schaeffer *et al.* (1973b) that ectoderm disaggregates at high concentrations of CB but still exhibits no significant change in the electrophoretic mobility. In our experiments also we have observed the disaggregation of ectoderm at higher concentrations of CB viz. 10 μg/ml, which can be seen in the form of white patches on the ectoderm which become loose and burst releasing cells in the medium. The disaggregation of the ectoderm cannot be explained on the basis of the reasoning used by Schaeffer *et al.* (1973a, b). If the
ectodermal cells do not exhibit altered surface charge and therefore change in electrophoretic mobility as a result of the treatment it cannot be the cause of disaggregation of cells.

It seems probable therefore, that CB must be acting on the surface mucopolysaccharides and glycoproteins which is the cementing material for the cells.

Joshi and Mulherkar (1977) have shown that CH causes disaggregation of gastrulae of Microhyla ornata and these effects are reversible on removal of the drug from the medium up to a certain duration of exposure to the drug. The reversible effects have also been shown in case of CB by Schaeffer et al. (1973b) and by other workers like Jones (1975), Greenberg et al. (1973). It was observed by these investigators that the effects of both CB and CH are quickly reversed on restoration of drug free medium. Based on these observations it can be said that the effects of the drug are on the cell surface.

Our histochemical observations with PAS and AB staining indicate that CH affects neutral mucopolysaccharides and non-sulphated mucopolysaccharides in particular. These are the major components of the cell surface material which is responsible for the close attachment of cells; therefore we conclude that CH treatment has an effect on the cell surface.
In the present investigation it could be seen that the sulphated mucopolysaccharides are either absent or are present in such minute quantity that they do not get stained with AB at pH 1.0. However our observations do not tally with the observations of Sugiyama (1972), Karp and Solursh (1974) who have emphasized the importance of sulphated mucopolysaccharides in the development of the blastula of sea urchin. Using radioactive sulphates Kosher and Searls (1973) have shown its incorporation into mucopolysaccharides of the invaginating cells of the frog gastrula. Continued mucopolysaccharide synthesis was also apparent in developing neural tissue.

The non-sulphated mucopolysaccharides get affected due to the treatment. Whether CH inhibits the synthesis of the mucopolysaccharides or brings about some chemical change in the cell-binding material by getting adsorbed on it, is not clearly known. It still remains a mystery in case of CB also.

Our observations using histochemical techniques for the staining of mucopolysaccharides and glycoproteins just make it clear that the total content of non-sulphated mucopolysaccharides is reduced due to the treatment of CH.

To support and confirm the fact that the cell surface
material gets affected after the treatment we thought of observing the effects of the drug at ultrastructural level using a specific marker for the cell surface material viz. colloidal Lanthanum. This is discussed at length in Part 4.
SUMMARY

1. Using PAS staining technique it is found that the total content of neutral mucopolysaccharides is reduced in the neurulae of *Microchlya ornata* on treatment with CH. This is indicated by the fact that the intensity of staining is reduced in the treated embryos as compared to the control embryos.

2. Sulphated mucopolysaccharides are either absent or are present in traces so as not to take up the AB stain at pH 1.0 even in the control embryos. The treatment of CH does not bring about any change in this.

3. The non-sulphated mucopolysaccharides are reduced after the treatment as is seen by AB staining at pH 2.5. The control embryos show the stain whereas no tissue from the experimental embryo takes up the stain.

4. After the treatment there is considerable increase in the size of the embryo as compared to the size of the control at that stage probably due to the disaggregating effect of the chemical.
<table>
<thead>
<tr>
<th>Plate Nos.</th>
<th>Figure Nos.</th>
<th>Histochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>XIV</td>
<td>31</td>
<td>Section of neurula of <em>Microchyla ornata</em> stained with PAS indicating presence of neutral mucopolysaccharides.</td>
</tr>
<tr>
<td>XIV</td>
<td>32</td>
<td>Section of the embryo treated with CH stained with PAS indicating reduced intensity of staining.</td>
</tr>
<tr>
<td>XV</td>
<td>33</td>
<td>Section of the control embryo stained with AB at pH 1.0 shows absence of sulphated mucopolysaccharides.</td>
</tr>
<tr>
<td>XV</td>
<td>34</td>
<td>Section of the experimental embryo stained at pH 1.0 with AB shows no effect due to treatment.</td>
</tr>
<tr>
<td>XVI, XVII</td>
<td>35, 37</td>
<td>Sections of the control embryos stained at pH 2.5 with AB indicating presence of non-sulphated mucopolysaccharides.</td>
</tr>
<tr>
<td>XVI, XVII</td>
<td>36, 38</td>
<td>Sections of the experimental embryos stained with AB at pH 2.5 show reduction in content of non-sulphated mucopolysaccharides due to the treatment.</td>
</tr>
</tbody>
</table>
Stage at treatment - Neurula of *Micromya ornata*

Concentration of CH - 1.5 μg/ml

Stain - PAS

**Fig. 31** - Section of control embryo showing dark PAS staining at ectoderm and neural plate region (x 100).

**Fig. 32** - Section of experimental embryo showing enlarged size and reduced intensity of staining (x 100).
Stage at treatment - Neurula of *Microhyla ornata*
Concentration of CH - 1.5 µg/ml
Fixative - Alcohol formol
Stain - Alcian blue at 1.0 pH

**Fig. 33:** Section of control embryo. The cells do not show any staining with AB at 1.0 pH indicating absence of sulphated mucopolysaccharides (x 100).

**Fig. 34:** Section of the experimental embryo like control does not show the staining of AB at 1.0 pH (x 100).
Stage at treatment - Neurula of *Microhyla ornata*

Concentration of CH - 1.5 μg/ml

Fixative - Alcohol formol

Stain - Alcian blue at 2.5 pH

**Fig. 35:** Section of the control embryo which has taken up the stain at 2.5 pH indicating the presence of non-sulphated mucopolysaccharides (x 100).

**Fig. 36:** Section of the experimental embryo. Note that no tissue has taken up the stain at 2.5 pH. The non-sulphated mucopolysaccharides get affected due to the treatment (x 100).
PLATE XVII

Stage at treatment - Neurula of *Microhyla ornata*
Concentration of CH - 1.5 μg/ml
Fixative - Alcohol formol
Stain - Alcian blue at 2.6 pH

Fig. 37 : Section of another control embryo as in Fig. 35. Note the dark stain due to Alcian blue in ectoderm and neural plate. The yolk cells do not show any stain (x 100).

Fig. 38 : Section of another experimental embryo indicating absence of stain after the treatment (x 100).