ABSTRACT OF THESIS

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Degree Doctor of Philosophy. *Date* October, 1957

Title of Thesis SOME BIOPHYSICAL STUDIES OF DEVELOPMENTAL PROCESSES.

The nature of the mechanism of adhesion of cells in amphibian embryos has been examined. It has been investigated in terms of the phenomena of disaggregation and reaggregation, which represent the loss and regaining of adhesion by these cells. The main conclusions of the work described in the thesis are:

The loss of adhesion between cells is connected with the removal of calcium from the cells, and only occurs in certain pH ranges. The readhesion of cells of blastula or gastrula stages is preceded by an uptake of calcium, whose kinetics suggest surface adsorption. Cells of these stages form their first adhesions without regard to the types of cell that they are in contact with. A ribonucleoprotein material is concerned with the readhesion of the cells of these stages. It is suggested that it may be concerned with the binding of calcium to the cell surface. The cell surface of preblastular stages is not stable in the absence of calcium but becomes stable to such conditions during blastular stages. In neurular stages the ability of the cell surface to adsorb calcium ceases. This failure of calcium adsorption occurs at the same time as the mechanism of cell adhesion appears to become selective, so that only cells of like type adhere to one another. In order to account for the phenomena of cell adhesion occurring in blastular and gastrular stages it is suggested that cell adhesion may be due to the attraction which exists between surfaces when they carry little charge. The nature of the uptake of calcium, the pH thresholds for disaggregation and reaggregation, the dependence of adhesion on the presence of calcium ions and other phenomena have been shown to fit the expectations of such a theory.

Use other side if necessary.

SOME BIOPHYSICAL STUDIES OF DEVELOPMENTAL PROCESSES.

by

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Submitted to the
University of Edinburgh
as a thesis for the degree of
DOCTOR OF PHILOSOPHY.

Institute of Animal Genetics,
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October, 1957.

FOREWORD.

I should like to thank Professor C.H. Waddington and Dr. G.G. Selman for their advice and constant encouragement during the course of this work. I also thank Professor J.N. Davidson of the University of Glasgow for his advice on techniques in the investigation of nucleic acids. Mr. J. Dainty of the University of Edinburgh gave me advice on the investigation of the ionic relations of living cells for which I am most grateful. I thank Dr. U. Mittwoch of University College London for her advice on statistical methods.

Dr. S. Brahma, Mr. E. Lucey and other members of the Institute of Animal Genetics gave me invaluable technical assistance for which I am most grateful. The work was carried out in the Institute of Animal Genetics, University of Edinburgh, by kind permission of Professor C.H.Waddington. The receipt of a maintenance grant from the Department of Scientific and Industrial Research during the period of this work is gratefully acknowledged.

CONTENTS.

Introduction

The higher animals are composed of many cells of a wide variety of types. The organisation of these cells into an animal body requires their assembly in a definite and exact relationship with one another. This relationship is such that the cells are able to assist one another in forming and maintaining the tissues of the body and their functions, and in turn enables these to be integrated into the whole animal. It is consequently the expression of a most delicate harmony held amongst the cells, which persists until age and death destroy it. In the mature animal this relationship between the cells appears to be of a nature only changed by the action of senescence, although we know that this is only an illusion of constancy, for cells and their components are constantly being replaced by others. During the development of an animal the relations between the cells change extensively for the population is continually enlarging and its members are changing in type. As the animal matures these changing relations slowly settle down into the more constant ones characteristic of maturity. One of these relations is observed in the adhesion of cells to one another: without it there would be no multicellular animals.

As the animal develops cell division causes the production of an increasing number of cells which usually adhere to one another. If the cells remain stationary or are merely moved

in an unorganised manner by the outpushing of cells due to their own proliferation it is not necessary that there be differences in adhesion between the cells. If there were differences in adhesion between the various types of cells the outpushing due to proliferation would produce a body of some definite structure. But the movements of morphogenesis cause united groups of cells to move in a most directed manner. These groups sever their connection with one group of cells, pass between others without adhering to them and may finally settle down elsewhere and form firm adhesions to yet other types of cell. In these three stages of their movements there are demonstrated differences in adhesion between various sorts of cells. Later in growth the organs of the body become steadily more discrete from one another, and in part this is merely an expression of the stronger adhesion of the cells of an organ to one another than to those of other organs around. Furthermore it has been suggested (Holtfreter, 1944a) that the morphogenetic movements may have their cause in the differential adhesion of cells to one another. With these ideas in mind the mechanisms of adhesion and the differences in adhesion between cells, were examined in the embryos of the anuran, Xenopus laevis (Daudin). This material was chosen in preference to other amphibians because the eggs may be obtained at any time of the year.

General Methods

Eggs of *Xenopus laevis* were obtained from matings induced by injection of chorionic gonadotrophin. Such eggs were allowed to develop at 18° - 23°C in tap water until they were a few Nieuwkoop stages from the stage required. (Nieuwkoop and Faber, 1956). They were then transferred to the saline solution known as Holtfreter solution diluted ten times with water. The jelly coats were removed with forceps. Once the outer hard coat of jelly is punctured the inner jelly softens and swells on exposure to the weak saline, and soon the entire jelly may be easily removed. The chemical decapsulation method of Spiegel (1951) was tried but it was found that the calcium permeability of the cells was altered even if subsequent development was not visibly impaired. The embryos were then placed in full strength Holtfreter solution for removal of the vitelline membrane and for other operations. Removal of the vitelline membrane with forceps is frequently accompanied by damage to the embryo. The main reason for this appears to be that on puncturing the membrane the embryo begins to swell out of the hole. If the puncture is made on the dorsal side of the embryo the blastocoel or archenteron roof bursts open with a rupture of some of the cells. In cleavage stages the cell wall is apt to rupture. I find that if care is taken it is possible to remove the membrane without visible damage to the embryo. In those experiments made in order to see whether or no certain chemicals

would dissolve the surface coat and disaggregate the embryo, it is necessary to have an undamaged coat initially, for it may be that the coat can prevent the entry of the chemical into the embryo ; although the chemical if once inside would cause disaggregation. In such experiments great care was taken to ensure that the embryos were not damaged during the removal of the vitelline membrane. On other occasions a very slight damage at most was allowed to be made in the removal of the vitelline membrane ; such a wound healing rapidly. Embryos which were more extensively damaged were not used.

Whole embryos or portions of them freed of their membranes in this manner were then used in experiments of cultured as controls. Normal culture was done in Holtfreter's solution (Holtfreter,1931), buffered to between pH 6•9 and 7•1 with the phosphate buffer (0•0084M in phosphate ion) introduced by Deuchar (1953) or with 2-amino-2-hydroxymethyl-1,3-propanediol (otherwise known as tris) 0•001M and hydrochloric acid. Flickinger (1952) has shown that tris has no harmful effect on amphibian cells ; by its use one is able to avoid the interference which the phosphate buffer gives when estimating the calcium content of the Holtfreter solution. The pH of the culture medium is much lower than that of the solution originally decribed by Holtfreter,which had a pH of about 8 and which contains bicarbonate ions. It has been shown by Flickinger (1954)

and Trinkhaus and Drake (1956) that the bicarbonate ion assists in the nutrition of the embryo. In comparing the results of the experiments to be described with others it should be remembered that there are these differences in the culture medium.

The precautions taken to maintain asepsis were the autoclaving or sterilisation by filtration of all solutions and the heat sterilisation of glassware and instruments. Experiments were done at 19°- 21° C unless otherwise stated. All solutions were made up from glass-distilled water, and for those experiments involving calcium determination the water was further purified by passing it through a mixed bed ion exchange column. From time to time the distilled water was analysed for iron and calcium. Calcium analysis was done by the method described on page 80, the analysis for iron was done using α-α'-dipyridyl. Chemicals were of 'analar' grade whenever possible. The pH of the solutions was measured with a valve-voltmeter type pH-meter to an accuracy of ± 0·01 unit: fresh M/20 potassium phthalate was used as a standard.

On occasion embryos of Triturus alpestris (Laur), palmatus (Razoumoski), cristatus (Laur) and vulgaris (Linn) were used. They were cultured in the same manner as the Xenopus embryos, save for small differences in the technique of removing the jelly coats.

Introduction to the experiments

Disaggregation, also known as dissociation, is the process of parting multicellular animals into individual separate cells. If these cells are comparatively undamaged their reaggregation into multicellular structures may be brought about in certain circumstances with certain types of cells. The separation of the cells from one another must be due either to a stimulation to the cells to actively move themselves away from one another, or to a loss of adhesion between one another, or to the removal of a mutual cement. Any combination of these causes may produce disaggregation. However there is little evidence for active movement of the cells pulling themselves apart from their neighbours producing disaggregation. The two remaining causes are concerned with the processes that cause cells to adhere in multicellular structures. Therefore a study of the processes of disaggregation should provide evidence about the nature of adhesion between cells. The first part of this account of the work describes the various means of disaggregating embryos by chemical means. This will show that calcium ions are of great importance in maintaining cell adhesion during early stages of development.

The processes of reaggregation involve the readhesion of the cells. In consequence a study of this has been made with

direct observation and with time-lapse films, and experiments have been done to test the effects of various chemicals and physical conditions on reaggregation. In the third portion of the work the results of a closer study of the effects of calcium on cell adhesion are given. The location of the calcium responsible for adhesion has been investigated, and some suggestions as to the nature of the chemical groups binding calcium to the cell are given. Lastly an investigation was made to see whether there was any form of cement concerned in cell adhesion in these embryos: some evidence for the presence of a ribonucleoprotein cement is given.

Disaggregation of embryos

A variety of methods for the separation of cells from one another have been tried. They are either mechanical ones in which adhering surfaces are torn apart, or chemical ones in which the adhesive properties of the cells are so weakened that the slightest mechanical action, such as gravity, separates the cells. Those who have worked on sponge or coelenterate tissues have favoured the mechanical methods. (Wilson, 1907; Galtsoff, 1925; Lehn, 1953). However such methods produce an assortment of fragments of the animal, which range from small groups of cells down to various cytolytic products. The ideal method of disaggregation would produce a complete separation of the animal

into single cells in an undamaged condition.

Chemical methods of disaggregation have the advantage that deductions may be made from their results about the chemical nature of the structures involved in cell adhesion. On the other hand although cells may not be visibly damaged there may be subtler forms of damage. If the cells can readhere and continue an organised life there is evidence that comparatively little damage has been done. But it is possible that even then their capacities for differentiation may have been affected by exposure to the disaggregating chemical. To add to the complexity of the problem it is possible that mere removal of the cells from their normal environment for a short while may permanently damage them or alter their potentialities for development, even though the chemical itself does not damage them (Trinkhaus, 1956). Thus it becomes an extremely difficult if not impossible task to detect small changes in the nature of the cells caused by the disaggregation procedure.

With these points in mind it was decided to use chemical methods of disaggregation. Roux (1896), Herbst (1900) and many others have noticed the importance of calcium ions for the maintenance of adhesion of the cells in embryonic material, and that the absence of calcium ions from the culture medium

tends to cause disaggregation. Some tissues require magnesium ions to maintain cell adhesion as was found for Mytilus gill by Gray (1926), but the amphibian embryonic material does not appear to rely on the action of magnesium to any great degree as will be seen from the results of experiments to be described. These studies suggest that calcium ions and in certain cases magnesium ions as well, either cause the cell surface to become adhesive or maintain the stability of an intercellular cement. Thus removal of calcium causes loss of adhesion between cells. The methods for extracting calcium ions from embryos which depend on washing in calcium-free media are not efficient. The reason for this is that the external medium soon shows an appreciable concentration of calcium derived from the embryos. Even if a number of extractions of calcium are done in succession, extraction will be slow unless the equilibrium constant k_1 is of fairly low value :

$$k_1 = \frac{(\text{conc. of calcium bound to the cells})}{(\text{conc. of calcium in external medium})(\text{conc. of free binding sites})}$$

It should be pointed out that this equilibrium constant has a value which probably will vary greatly with pH. In consequence, merely placing the embryos in calcium-free media will not remove all the calcium involved in cell adhesion, and sufficient might remain for considerable adhesion to be maintained.

Fairly recently the chemicals which complex calcium have been used for disaggregation, (Zwilling, 1954; Anderson, 1953; King & Briggs, 1955). It seems that these agents were chosen because of their considerable affinity for ionic calcium with which they react to form non-polar compounds; thus removing all the calcium ions from a solution into which they have been introduced. This affinity for calcium ions is expressed in the value of the equilibrium constant k_f:

$$k_f = \frac{\text{(conc. of the complexing agent in calcium form)}}{\text{(conc. of the complexing agent not binding calcium)} \times \text{(conc. ionic calcium)}}$$

see Schwarzenbach, 1954.

This equilibrium constant is also known as the stability constant or as the formation constant. It is usually expressed as a logarithmic value. Thus if a complexing agent is used to remove ionic calcium from the embryos, at equilibrium there will be a very small amount of ionic calcium in the medium. And unless the equilibrium constant for the holding of calcium by the cell or cement is for similar or greater value the amount of calcium held by the embryo will be small.

The complexing agents which have been mostly used to remove calcium and magnesium ions in order to bring about disaggregation are ethylene-diamine tetracetate as the sodium salt (this is also known as versene, or as EDTA, which abbreviation will be used

subsequently) and the citrates. In this work nitrilo-triacetate (also known as ammonia triacetate or NTA), tricarballylic acid, ortho-cresolphthalein complexone (also known as pthalein purple or systematically as 2:6 xylenolphthalein-α:α'-bis(imino) diacetic acid, and hereafter referred to as OCC) and glutamic acid have been used as complexing agents as well as EDTA and sodium citrate.

Embryos were cultured in calcium-free solutions at various pH values in order to discover the pH limits for the disaggregation of the various tissues. The action of EDTA and the citrates was investigated in a similar manner. The results of such experiments provide an indication of the part played by the hydrogen ion concentration on adhesion. These experiments may reveal changes in the response of the cells to such treatments as the embryos develop.

a) Experiments on the disaggregation of Xenopus laevis embryos in calcium-free media at various pH values.

Embryos whose jelly coats and vitelline membranes had been removed by hand in normal Holtfreter solution were washed in calcium-free solutions, at pH 6.9 to 7.0; they were then transferred to the calcium-free solution at a chosen pH. The pH of these solutions was controlled by buffers. M/20 sodium carbonate-sodium bicarbonate buffer was used for the range 9.2 - 10.5; this buffer has a high osmotic pressure, nearly the same as normal Holtfreter solution and in consequence the disaggreg-

ting medium had an osmotic pressure nearly twice that of normal Holtfreter. 0.001M 2-amino-2-methyl-1, 3-propanediol and hydrochloric acid were used for the range pH 8.0-9.6, and various molarities of potassium cyanide for pH 9.5 to 10.0 (0.004 M -0.025M KCN). These buffers overlap in some ranges and the purpose of this was to provide some means of detecting effects due to the chemical nature of a given buffer and not to the pH being studied. Potassium cyanide surprisingly enough does not kill embryos at stages up to early neurula - possibly due to some changes in the cytochrome system of respiration, and it was due to Holtfreter's attempts to impair the respiration of embryos (Holtfreter, 1943 a) that its production of disaggregation due to the alkalinity of its solutions was discovered.

The embryos were placed ten at a time in petri dishes with paraffin wax bottoms (with small depressions to prevent the embryos being moved by accidental shaking of the dishes). The action of the solutions on them was observed for the first five minutes and then at 10, 20, 40, 60 and 120 minutes from being placed in the solution. The medium surrounding them was replaced with normal Holtfreter saline at the end of this period by repeating washing with normal Holtfreter until the pH reached 6.9 - 7.1. The embryos were examined at various intervals over the next three days. The bicarbonate-carbonate and the 2-amino-2-methyl-1,

3-propanediol buffers were used for most of the experiments and the results of treatment at various pH values, listed in Table 1, refer to the action of these buffers alone. The few experiments done on the action of potassium cyanide were found to have similar results.

Results

All cells from stages from early blastula to early neurula disaggregate in these media if the pH is above 9.6. Such cells will reaggregate on their return to normal Holtfreter, and they will then adhere to any other sort of cell. As neurulation begins the tissues fail to disaggregate under this treatment, beginning with the neurectoderm and spreading finally to the endoderm. There is apparently no change in the pH at which disaggregation begins, but merely this development of a resistance to disaggregation. A detailed description of this phenomenon is given in the description of the action of EDTA as a disaggregating agent where the results are identical. The effects of calcium-free solutions on stages earlier than morula (Nieuwkoop stage 6½) are of a somewhat different nature. Treatment with calcium-free solutions tends to destroy the cell surface membrane. If this is done on fertilised but uncleaved eggs the whole egg lyses save for a small portion at the animal pole, which is heavily pigmented. During cleavage stages the resistant area of the cell

TABLE 1.

Distribution of tests on the disaggregation of Xenopus embryos by alkaline media with respect to Nieuwkoop stage and pH of the medium. The left-hand column under each stage gives the total number tested, the right hand one the number of embryos that disaggregated.

Nieuwkoop stage	7		9		10	
pH.						
10·2	-	-	-	-	3	3
9·95	10	10	10	10	10	10
9·90	10	10	-	-	8	8
9·85	-	-	-	-	7	7
9·80	8	8	9	9	3	3
9·75	10	10	10	10	10	10
9·62	-	-	-	-	10	10
9·60	10	10	10	10	10	9
9·58	10	2	10	0	10	3
9·54	-	-	-	-	10	0
9·50	10	0	10	0	10	0
9·20	10	0	10	0	10	0

for stages 11-13 see overleaf.

TABLE 1 cont.

Nieuwkoop stage.

	11		12		13	
pH.						
10·2	9	9	-	-	-	-
9·95	6	6	-	-	-	-
9·90	7	7	8	8	-	-
9·85	10	10	10	10	-	-
9·80	8	8	-	-	-	-
9·75	10	10	10	10	10	10
9·60	10	9	10	10	-	-
9·58	10	0	10	0	10	0
9·54	-	-	5	0	-	-
9·50	7	0	-	-	-	-
9·20	10	10	-	-	-	-

surface increases in size until the cells become entirely resistant to the lysing action of calcium-free Holtfreter on their walls; this condition is reached at about stage 6½. The embryos disaggregate in the same manner as at later stages and this disaggregation and the lysis only occur if the pH is above 9.6. There is no evidence that the pH value at which these processes begin alter during the cleavage period.

The disaggregates obtained by this method contain some material derived from cytolysis of the cells and a fair amount of jelly is visible between the cells as well. The jelly may or may not be produced by cytolysis and its derivation is discussed later. It is very sticky, particularly in the presence of calcium ions, and cells adhere to it firmly. If the pH is raised suddenly to about pH 12 this jelly contracts violently. On addition of calcium ions it sets into a firmer mass to judge from the movements of cells entangled in it when the petri dishes are shaken.

Disaggregation appears to be an identical process with regard to its visible aspects for all the buffers tried for pH values from 9.6 to 10.0. At higher pH values cytolysis occurs extensively and rapidly. Holtfreter (1943a) has described the process in T.torosus, and it appears to be similar to disaggregation in X.laevis. In consequence only the salient features of disaggregation are described here.

This description is derived from observations of many disaggregations. The first sign of disaggregation was a slight loss in the brilliance and reflectivity of the surface coat of the embryo. The surface coat of the Xenopus embryo at stages before nourulation shows a series of colours upon it of the kind due to optical interference. These may be due to some form of diffraction grating structure of the selecting type or to the optical interference set up in thin films. Shortly after the embryo was placed in the disaggregating medium these colours disappeared. This was followed by the loss of small granules from the embryo; they appeared to emerge from the surface coat. These granules are a few microns in diameter and may perhaps be identical with the pigment granules found in the coat. (see Holtfreter, 1943a for extensive discussion of the nature and function of the surface coat.) In the blastula stage these signs first appeared at the animal and vegetal poles of the embryo, but on entering the gastrula stage the dorsal lip of the blastopore became the main site for the initial attack of the disaggregating medium. In the gastrula stage the vegetal pole ceased to be a point of initial attack although the animal pole continued to be so. The action of the disaggregating medium at the animal pole in blastula and gastrula stages produced a round wound in the surface coat extending only a few degrees out from the pole.

This wound did not enlarge itself after a few minutes but remained at the same size until the enlarging vegetal or blastoporal wounds merged with it. These sites of the initial attack of the alkaline media are the same as those observed by Bellamy and Child (1924) in their investigation of metabolic gradients in amphibian embryos.

Soon after the loss of granules the surface coat of the embryo began to tear open together with the underlying ectoderm. The ectoderm from the animal pole region tended to curl up into tight rolls. As the gaps in the surface coat enlarged, cells began to emerge and fall out of the embryo. There did not appear to be any active extrusion of cells, but merely a passive shedding of them through the wound. At the same time the jelly material began to emerge from between the cells. The freshly disaggregated cells frequently were of elongate shape but these soon rounded up into a spherical form. The hyaline protoplasmic margins of the cells (Kuhl, 1937, termed them hyaloplasm margins) showed very little motion in the disaggregated cells other than a slow cydosis. Cyclosis is the rotation of a lobe of the hyaline margin around the cell (see Figure 1). As the surface coat broke up small pustules appeared in it where it was about to break open. The contraction of the coat together with that of the underlying cell layers in the dorsal regions of the embryo appeared to be the cause of the stripping of the ectoderm and neural

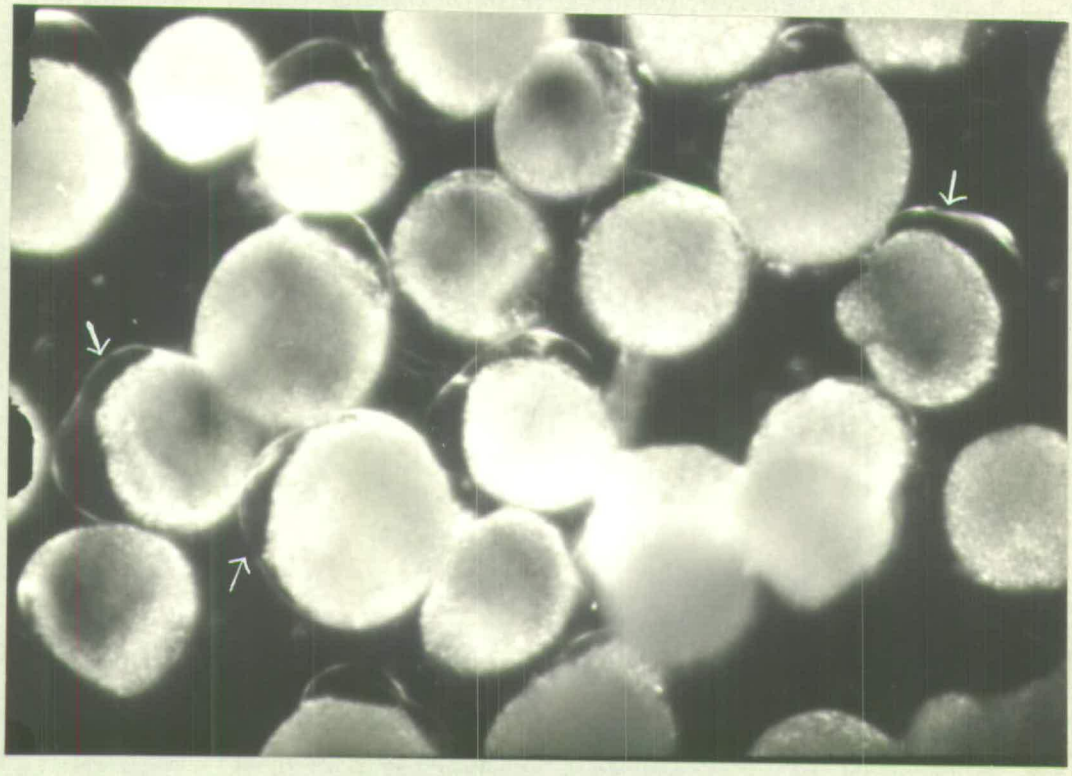


FIGURE 1.

Blastula cells of T.alpestris under dark ground illumination. The hyaline margins of the cell (see arrows) were performing the movement called cyclosis when this photograph was taken. From film 12. Scale, 1 cm = 37 micra.

plate to one side of the disaggregate. The endoderm and mesoderm of gastrula and early neurula stages disaggregated easily but the neural plate became resistant towards the end of gastrulation. Later the mesoderm and finally the endoderm became resistant. As disaggregation continued the embryo lost its mechanical strength, probably as a result of the weakening of the adhesions, and it slumped down in the bottom of the dish as a loose pile of cells. Gentle shaking spread this pile of cells over the dish, though the stickiness of the cells and of the jelly tended to impede their spreading. The breaking open of the embryo revealed a small number of yolk platelets which appeared to lie between the cells. They seemed to be extracellular and not the products of cytolysis during disaggregation. They can be compared with the yolk platelets found in histological sections lying between cells. However there is a certain amount of cytolysis during disaggregation which produces yolk platelets, or a risk of its occurrence. The separation of the cells spread outwards from the blastopore, most rapidly ventrally, most slowly dorsally. The disaggregation which began at the animal pole did not spread far. If the surface coat had been injured a slight disaggregation began at the site of the wound. Fragments of neurectoderm held together by thin and attenuating strands of surface coat were the

last tissues to disaggregate. On no occasion was cell division seen amongst cells of post-cleavage stages in calcium - free media.

The use of such alkaline solutions to disaggregate the embryos may damage the cells by reason of the very great difference in the chemical behaviour of the various molecules forming the outside of the cell (and to a lesser extent internal ones as well) between these pH values and those normally associated with their life. Yamada (1950) and Holtfreter (1944b) have shown that exposure of various amphibian embryonic cells to such alkaline solutions may alter their presumptive fate. Other means of disaggregating the embryos may be less damaging if less alkaline media can be used. It was partly with this object in mind that the various agents which complex calcium have been used as disaggregating agents. Firstly, their action was investigated in order to discover the lowest pH value at which they would bring about disaggregation, which in turn would provide some suggestion as to the nature of the chemical groups on the cell binding calcium to itself. There is a competition between the calcium binding groups of the cells and those of the complexing agent for calcium ions. A measure of the amount of calcium held on the cells, that in solution, and that bound to the complexing agent will permit the calculation of the stability constant for calcium binding by the cell at that pH. If the stability constant can be determined for a number of pH values the results can give

information about the chemical nature of the bonds holding calcium. The occurrence of disaggregation for a given chemical agent can be compared with the stability constants which have been determined and thus some assessment can be made of the question of whether it is calcium binding mainly or only to a lesser degree which is responsible for cell adhesion. The methods by which these calculations have been made are described later, but the stability constants for the calcium complex of EDTA at various pH values are given below in the introduction to the experiments on the determinations of the pH thresholds for disaggregation by various complexing agents.

b) Experiments on the disaggregation of Xenopus Laevis embryos in various media which complex calcium. i EDTA

EDTA 0.0005M - 0.005M in calcium-free Holtfreter buffered either with 0.009M phosphate or 0.001M tris buffers to various pH values in the range of 5.6 - 9.0 or with 0.001M 2-amino-2-methyl-1, 3-propanediol from pH 8.5 - 9.5 was the complexing agent used for the greater part of this work. The stability constant of the calcium complex at various pH values in given in Table II.

TABLE II

Logarithmic stability constants for EDTA calcium complex at various hydrogen ion concentrations

pH	3	4	5	6	7	8	9	10
10% excess of free EDTA	2.0	2.3	3.8	5.5	6.3	7.3	8.3	9.2
100% excess	2.0	2.5	4.3	6.0	7.3	8.3	9.3	10.2

This table is taken from Chaberek, Bersworth and Martell, 1955.

Examination of Table II shows that EDTA only begins to show appreciable complexing power for calcium when the pH is above 6. Therefore the action of EDTA was tried only over the range pH 6 to 9.5. The experiments were arranged in the same manner as those described above. The pH values 6.0, 6.5, 6.8, 7.0, 7.5, 7.8, 8.0, 8.5, 9.0, 9.2, 9.5 were tried.

Results:

Embryos of Xenopus laevis of stages from early blastula to late gastrula were found to disaggregate in this medium

if the pH was at above pH 7.8. A few experiments on T.alpestris material showed that the pH threshold was much lower for this material than for Xenopus embryos; the threshold lay in the range 6.0 - 6.2. At pH 9.5 the processes of disaggregation in Xenopus embryos were similar to those found in disaggregation with alkaline media lacking complexing agents. At successively lower pH values there was less and less cytolysis, and disaggregations done below pH 8.4 showed little or no cytolysis. In those disaggregations done below pH 9.2 no jelly appeared, and the free cells have no stickiness for one another. During disaggregation the surface coat broke up more easily than under the action of alkaline media lacking complexing agents, and there was less rolling up of the ectoderm. The various cell layers of the embryos all disaggregated at about the same rate, which indicated that there was little difference in the methods of coherence of cells in the various cell layers. All the various sorts of cell would reaggregate if the culture medium contained ionic calcium and had a pH near 7. Preblastular and postgastrular stages do not behave in the manner described above on disaggregation with EDTA.

The effect of EDTA on preblastular stages is described first. For these stages the pH threshold for disaggregation has not been so precisely determined as for blastular and gastrular

stages, but it appears to lie between pH 7.8 and 8.0.

EDTA damaged the cell surface of unfertilised Xenopus laevis eggs at pH 8.0 and the outermost membrane of the egg was seen to peel away from the wound and slowly dissolve in the medium. This wound first appeared at the vegetal pole provided that the cell surface was not damaged during the removal of the vitelline membrane. In the course of an hour the egg cytolysed under the action of 0.001M EDTA, and the only solid remains were yolk platelets and a small pigmented nubbin about 50-70 micra in radius derived from the surface layers of the egg at the animal pole. After fertilisation the cell surface became resistant to this lysing action of EDTA; this resistance spreads from the animal to the vegetal pole during the cleavage stages. At the early blastula stage (Nieuwkoop stage 7) EDTA has no visible action on the embryos other than the production of disaggregation. Cell division continued in the presence of EDTA during cleavage stages, but this does not occur during the disaggregation of later stages. The first few cleavages began fairly normally in the presence of EDTA, but the cell surface broke up in the vegetal regions and those portions of the embryo began to lyse. The lysis spread towards the animal pole and disarranged the position of the deepening furrows. In consequence of this lysis inside

the embryo it became possible to remove pieces of the cell surface in the animal portion of the embryo which had not been attacked by the EDTA. Some pieces of the surface were isolated in this manner and given to Dr. G. Selman for examination by electron microscopy. These observations indicate that it is the cell surface which develops a resistance to the lysing action of EDTA. By the 8-cell stage the dorsal half of the embryo was entirely resistant to the lysing action of EDTA, although disaggregation occurred. The cleavage furrows opened out during this disaggregation and were seen to pass into the cytolytic regions ventrally. In these opening furrows the white region described as newly formed cortical material by Selman and Waddington (1955) may be seen. T.alpestris cleavage stages behaved in the same manner, but unfertilised eggs of this species were entirely resistant to lysis by EDTA. This difference from unfertilised eggs of Xenopus might be due to a strong binding of calcium ions to the cell surface before fertilisation. In consequence the EDTA would not remove much of the calcium from the cell surface in one extraction. Evidence for the strong binding of calcium by unfertilised eggs of Arbacia was found by Mazia (1937), and he also found that the degree of binding lessened on fertilisation. Lansing and

Rosenthal (1952) give evidence for the calcium binding shown by this material being located in the cell surface. It is of interest to consider whether or no the cell surface material described here is identical with Holtfreter's surface coat (1943).

Embryos which were placed in calcium-free Holtfreter at the same pH (8.0) at cleavage stages were undamaged in the course of two days, though eventually their development became abnormal and they died at 3 - 4 days. As mentioned earlier if the pH is above 9.6 these stages lyse in the manner described above.

Mid-neurula and later stages were found to become resistant to the disaggregating action of EDTA. When neural closure became complete it was impossible to disaggregate the embryos with EDTA. The neuroectoderm was the first region to become resistant to the action of EDTA, and this was followed by the somites and notochordal tissues. The lateral plate mesoderm next became resistant and finally the endoderm failed to disaggregate under the action of EDTA. These observations are shown in figure 2, which also includes the results of the tests on the ability of the cells to reaggregate. A small number of tests were done on explants of the various separate regions of the embryos with similar results. No

FIGURE 2.

Showing the effects of EDTA on the embryos of Xenopus at various stages, and the reaggregating ability of the cells of the disaggregates thus formed.

evidence was found for the possibility that the pH threshold for disaggregation might rise as the embryo developed. All stages that were disaggregated by EDTA did so if the pH was above 7·8. Later stages did not disaggregate at any pH up to 10·0, and at higher pH values only showed cytolysis.

b. Experiments on the disaggregation of Xenopus laevis embryos in various media which complex calcium. ii. other complexing agents.

Similar but incomplete tests were done with the complexing agents NTA and OCC ; identical results were obtained.

Citrates have a complexing affinity for calcium and the complex is fairly stable. Schubert and Lindenbaum (1952) report that the stability constant at pH 7·0 for the calcium complex with citrate and with tricarballylate have the values (arithmetical) of 1410 and 66 respectively. These values are very much lower than that of the EDTA complex but nevertheless indicate fairly stable complexes with calcium. Oxalates show a similar behaviour. Feldman (1955) used citrates and oxalates for the disaggregation of Triturus embryonic cells with success. His results suggest that these agents brought about disaggregation by removal of calcium from the embryos.

By testing the disaggregating action of citrate, tricarballylate, and glutamate (stability constant at pH 7·0 of

Ca form = 38,Martell and Calvin,1952) it is possible to compare the effects on disaggregation at various points in a range of calcium stability constants. Thus some idea of the calcium binding ability of the cell surface or cement can be got.

Feldman prepared his solutions to be 0·017M in citrate and 0·02M in glycine made up in calcium-free Holtfreter solution. 0·017M solutions of citrate, tricarballylate and glutamate were prepared, and their pH was adjusted to the required value with sodium hydroxide solution.

At pH 7·8 the citrate-glycine medium disaggregated Xenopus gastrulae and early neurulae, and the process was very similar to that described for the action of alkaline media at pH 9·6 or above. Some cytolysis happened and a certain amount of jelly material was formed. Reaggregation occurred subsequently provided that the stage disaggregated was not later than very late gastrula. Mid and late neurulae did not disaggregate in this medium. The action of this medium on cleavage stages was not tried.

The tricarballylate-glycine medium was used at pH 9·0 on stage 11 embryos, and there was partial disaggregation of 19 embryos out of 20, one embryo being almost unaffected. At pH 8·7 there was no disaggregation of a group of 10 embryos

of this stage. At pH 9.6 disaggregation was complete with embryos of this stage. All the disaggregates re-aggregated on return to normal Holtfreter. In appearance the process was identical with the disaggregation produced by the citrate medium.

The glutamate-glycine medium was tested on stage 10 - 10½ embryos of Xenopus. At pH 6.7 and 8.35 there was no visible action on groups of 10 embryos. At pH 9.04 there was a slight loss of reflectivity from the surface coat but no other effect, at pH 9.44 there was a slight disaggregation of the embryos just dorsal to the yolk-plug. These latter pair of tests were done on groups of 8 embryos each. The disaggregates reaggregated on return to normal Holtfreter solution.

Discussion of the results of experiments on disaggregation

The compounds that have been studied for their ability to produce disaggregation show a great range of stability constants to their reactions with calcium. These range from that of EDTA which is so high that only a very small amount of ionic calcium is in equilibrium with the free agent to glutamic acid which has a very low stability constant.

The figures quoted for the calcium stability constants mostly refer to pH 7. As the pH of the medium in which they are present rises so the calcium stability constant rises. It is hardly surprising that disaggregation becomes possible at some pH value in any of these media, because previous observation has shown that disaggregation will occur in the absence of calcium ions or of a complexing agent above pH 9.6. The exact pH threshold for a given complexing agent will depend on three variables. Firstly the degree to which the adhesion of cells is controlled by factors which do not involve the action of calcium, and this may vary with pH. Second the change in the stability constant with pH for calcium binding by that portion of the embryo responsible for adhesion, and lastly the rise in value of the stability constant with pH for the disaggregating agents considered.

Qualitatively the results obtained support the idea that the removal of calcium causes disaggregation. The series of agents, citrate, tricarballylate and glutamate require successively higher pH thresholds to cause disaggregation, which would be expected from the values of their stability constants at pH 7. Of course no value is known for the stability constants for the calcium complexes of these agents at their pH thresholds for disaggregation but at any given pH they may be expected to maintain the same relative order as

at pH 7. These experiments do not in themselves indicate the degree to which the three factors outlined above act, but they provide the basis for the later assessment of this problem for they provide the pH thresholds for a number of agents which cause disaggregation. So far no account has been taken of other chemical effects which these agents may have upon the embryos. These other effects of course may act upon cell adhesion and in part determine the pH threshold for disaggregation. A priori the agents used in the manner described are not expected to have any specific action on chemical features of the embryos other than their calcium or magnesium content. Nevertheless some suggestion of such action is provided by the fact that the pH threshold for disaggregation with EDTA is 7.8, whereas for citrate it is of the same value or lower, although EDTA has a stability constant about a thousand times greater than citrate at the same pH. Moreover disaggregation with citrate produces a jelly between the cells, whereas this does not appear in disaggregation with EDTA.

This jelly may result from the cytolysis of the cells but it may perhaps be derived from an intercellular cement, or from the swelling of a layer of material on the cell surface. It has been noticed by Yamada (1930) and Holtfreter (1943b).

The general indication from these experiments that the cell adhesion of these embryos is controlled to a considerable extent by calcium ions was made by Holtfreter (1943b). The present work describes the determination of the pH thresholds for disaggregation for a variety of stages and disaggregating agents. The phenomena observed in the treatment of cleavage stages with EDTA etc. do not appear to have been described previously. At present their significance is obscure.

The intention of the experiments described above was the provision of information on the action of calcium and pH on cell adhesion. In the next section consideration is made of the results of studies on cell adhesion made with enzymes, in order to give some idea of the importance of various organic chemical groupings in cell adhesion.

c. Experiments on the disaggregation of Xenopus laevis embryos with enzymes.

The enzymes trypsin and ribonuclease were used in these experiments. Testicular hyaluronidase was used as well; but on assaying it for possible contamination with ribonuclease by the method of Davidson et al. (1957) much ribonuclease was found. The results of experiments done with hyaluronidase were identical with those done with

ribonuclease. So far it has been impossible to obtain hyaluronidase free of ribonuclease.

Effects of trypsin

Moscona (1952), Trinkhaus and Groves (1955) and other workers have used trypsin digestion for the disaggregation of chick and mammalian material. The trypsin was dissolved in culture media free from calcium and magnesium salts. They found it possible to reaggregate the free cells obtained by this method. Trypsin is thought to attack proteins at their arginyl or lysyl portions, according to Sumner and Myrback (1951). Its action is greatly promoted by the presence of calcium ion and it is thought by some, e.g. Haurowitz et al. (1945) to have little action on native proteins compared with its action on denatured proteins. These facts suggest that it will attack mainly those proteins which are basic and denatured.

Two sets of experiments were arranged. In the first whole embryos or explants from them were placed in trypsin solutions to see whether disaggregation would occur. In the second embryos which had been disaggregated at high pH were washed with trypsin solutions to discover if the jelly material would dissolve. Trypsin obtained from Messrs. Lights was used. Solutions of various strengths were made

up in normal and calcium-free Holtfreter solution, buffered to pH 7.1 with 0.001M tris and hydrochloric acid or pH 7.8 with the phosphate buffer described earlier. The pH optimum of the enzyme is 7.8. All experiments were done on embryos of Nieuwkoop stages 10 - 11.

In the first set of experiments groups of four embryos were treated with 3%, 1%, 0.6% and 0.06% w/v solutions of trypsin in normal Holtfreter at pH 7.1. No disaggregation or extensive cytolysis occurred. There was however a slight damage to the surface coat shown by the loss of fine granules from it, which continued for many hours. The embryos continued to develop fairly normally throughout a lengthy treatment with the enzyme. A few embryos were treated with trypsin dissolved in calcium-free Holtfreter solutions. In these cases a slight cytolysis occurred with 3% trypsin. However since the trypsin was found to contain an appreciable amount of magnesium it is probably that sufficient magnesium is present in the solution to offset the absence of calcium.

The effect of treatment embryos with trypsin prior to disaggregation with EDTA was tried. After a few preliminary trials it was found that exposure of stage 11 embryos to 1% trypsin in normal Holtfreter for one minute was sufficient to prevent subsequent disaggregation by 0.001M EDTA. Groups of

five embryos were exposed for one minute to trypsin solutions in normal Holtfreter solution at the following concentrations 3%, 1%, 0·6% and 0·06% before transfer to EDTA. Immediately after the trypsin treatment the embryos were not damaged visibly. After 40 minutes in EDTA those embryos which had been treated with 3% trypsin showed no sign of disaggregation. Whereas control embryos were fully disaggregated. Those embryos which had been pretreated with trypsin were now in the form of a ball of cytolytic material firmly gummed together by what appeared to be a thick and viscous jelly. Those embryos which had been treated with 1% and 0·6% trypsin showed similar conditions though these were not so fully developed. The embryos treated with 0·06% trypsin showed a moderately complete disaggregation although there was appreciable cytolysis. These embryos showed an easier separation of the ectodermal cells from one another than in the controls, but the mesodermal and endodermal cells were more cohesive. This situation is the reverse of that obtained in the straightforward disaggregation of such stages with EDTA, in which the endoderm is the least cohesive and the ectoderm the most cohesive tissue. Such embryos showed reaggregation on return to normal Holtfreter solution.

In the short while for which the embryos are exposed to trypsin it is unlikely that the concentration of trypsin inside the embryo reaches equilibrium with that outside.

Consequently most of the action of the trypsin will appear in the outer portions of the embryo, where it is present in highest concentration. If trypsin acts by breaking down the protein structure involved in cell cohesion this would account for the lessening of cell cohesion in the ectoderm but would not account for the apparent increase in cohesion in the other tissues.

In the second set of experiments the embryos were first disaggregated with the bicarbonate-carbonate buffer or with the 2-amino-2-methyl-1, 3-propanediol buffer at pH 9.80 for an hour; they were then washed with normal Holtfreter at pH 7.8 (phosphate buffer). Sufficient solid trypsin was added to give a 1% w/v concentration in the medium. Those portions of the ectoderm which had not been disaggregated fully underwent a violent inrolling. The jelly between the cells began to dissolve and after several hours the cells began to cytolyse. Those disaggregates which were returned to normal Holtfreter solution (with several washings) after the trypsin had dissolved the jelly, failed to reaggregate. Cells from such disaggregates maintained movements such as cyclosis and cells frequently came into contact with one another, but no firm adhesions were formed. Such behaviour suggests that trypsin acts on the adhesive

proportion of the cells. If embryos were treated for a shorter while with trypsin, some reaggregation was possible, but the cells were very adherent to the glass of the culture dish, and formed a thin monolayer on its surface, see figure 3. This pair of observations suggests that the jelly may be necessary for reaggregation, but other explanations for the failure of reaggregation after treatment with trypsin are possible.

Although trypsin has a fairly specific site of attack on proteins the multiplicity of places where it may attack the cell make the interpretation of the results difficult. It is remarkable that trypsin has very little effect on embryos if no other treatment is made. This might be due to the occurrence of anti-tryptic agent in the embryos. Evidence for the occurrence of a heparin-like substance in the surface of the eggs of some sea-urchins is described by Heilbrunn (1956). Horwitt (1940) showed that heparin can have an anti-tryptic action. In the interpretation of those experiments in which disaggregation with EDTA was tried after treatment with trypsin one is troubled by an uncertainty as to whether the trypsin acts on the cells prior to the application of EDTA or whether the action is due to the trypsin present in the embryo when the treatment with EDTA is begun. It is

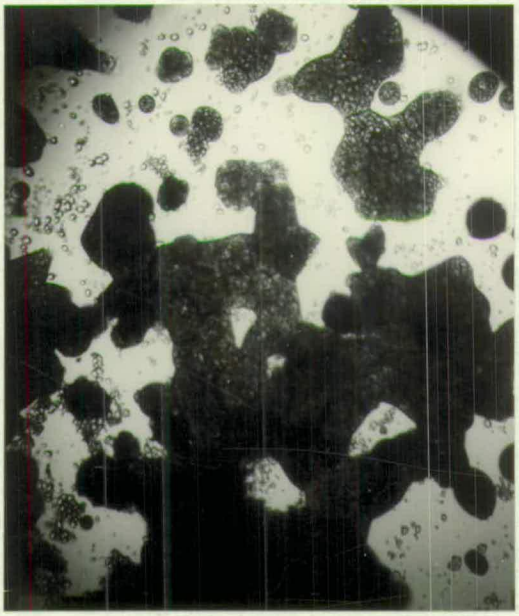


FIGURE 3.

Monolayer of Xenopus gastrula cells reaggregated after slight treatment with trypsin. Microneg.Pan. 8 seconds. 1" objective, x 10 eyepiece. Scale. 1cm = 60 micra.

possible that trypsin acts on the embryos prior to the application of EDTA and makes the cells far more permeable to calcium and magnesium. In consequence the EDTA removes the calcium and magnesium from the interiors of the cells and causes cytolysis. Some evidence for this theory is found in the fact that the enzyme papain appears to increase the permeability of the cell membrane to calcium (see page 86). Such a theory would explain the lack of apparent action of trypsin on embryos in media rich in calcium, for the medium would contain sufficient calcium to maintain the concentration of calcium within the cells at a fairly high level. This theory does not require the existence of anti-tryptic agents; and it suggests that the structures attacked by trypsin are of little importance in cell adhesion. It is unlikely that the other possibility that trypsin only acts when EDTA is present is correct, for trypsin will act on cells which have been isolated by other methods of disaggregation, to dissolve the jelly and destroy the power of reaggregation. It should be possible to test this problem by a series of experiments on calcium loss by embryos after treatment with trypsin. Since trypsin alone does not disaggregate embryos it would seem that protein structures of the kind attacked by trypsin cannot be of great importance in cell adhesion. Yet treatment with trypsin prevents subsequent reaggregation of the embryonic cells.

This problem can be accounted for by the theory that established cell adhesion can continue even though a protein component of the adhesive system is damaged, but that the power of re-establishing adhesion is lost. If the presence or absence of a hydrolytic action of trypsin on the cell surfaces or cementing structure could be demonstrated in the intact embryo this problem could be solved. In the above discussion the assumption has been made that trypsin acts on protein structures to produce materials which are less adhesive than their precursors, but it is possible that the reverse is true. If this were so a very different interpretation of the results would be necessary. The results suggest that the jelly contains a protein component. And that some protein component is important in cell adhesion. Moscona (1952) and other workers who used trypsin for the disaggregation of chick material did not find the subsequent reaggregation was prevented. Whether this difference is due to the nature of the material or to the culture methods used is uncertain.

Effects of ribonuclease

Ribonuclease A is the enzyme which attacks ribonucleic acid, hydrolysing part of it to a mononucleotide state, though a portion is left in a state of comparatively high polymerisation.

A protease-free sample of the enzyme was used. This enzyme is most stable at pH 2.0 - 4.5 but its pH optimum is 7.7, at which pH however it is quite rapidly destroyed. A partial compromise was made in its use between these two choices, and it was used at pH 7.0 in a 0.0005M tris buffer.

Its action on the jelly obtained from disaggregates made at pH 9.6 - 9.8 was tried. Six stage 12 embryos were disaggregated in 0.01M 2-amino-2-methyl-1, 3-propanediol in calcium-free Holtfreter at pH 9.8. Much jelly was formed between the cells. The embryonic disaggregates were washed in calcium-free Holtfreter solution buffered to pH 7.1 with 0.001M tris, and then the freshly prepared ribonuclease was added giving a final concentration of about 12 micrograms per millilitre of ribonuclease in the culture dishes. In the course of 15 minutes the jelly was observed to become much more diffuse and to disappear in many places. There was no other visible action. The embryos were then returned to normal Holtfreter solution and signs of reaggregation were looked for next day. None were found, though controls reaggregated normally.

The results suggest that the jelly contains a ribonucleic acid component. They also suggest that the removal of a ribonucleic acid component removes the ability for

reaggregation from the cells, but this component is not necessarily identical with that contained in the jelly. Since treatment with ribonuclease does not inhibit the movement of the cells such as cyclosis, it seems probable that it may inhibit reaggregation by destroying their ability to readhere even though they come in contact with one another.

Reaggregation of embryonic cells of Xenopus laevis

If the cells which have been disaggregated by some of the methods described previously are returned to fairly normal conditions of culture they may reunite into multicellular bodies. This reunion is called reaggregation. In order for this union to take place two conditions are necessary. Firstly the cells must either be in contact or be able to come into contact. Secondly they must be able to adhere in these contacts. The term 'reaggregation' is usually used to cover the processes involving both of these conditions. Frequently its use is extended to the process of the sorting out of the different types of cells into groups according to their type. Roux (1894) described reaggregation of blastomeres of Rana fusca in terms of the cells coming together and adhering. Reaggregation, including cell sorting, was described by Wilson (1907) for sponges and for coelenterates (1911). More recently Moscona and Moscona (1952) have found that chick embryonic material will reaggregate. The importance of adhesion in reaggregation is clear, and it was for this reason in part that the work to be described was done.

Roux considered that the isolated cells of R.fusca did not show random movements, but that they moved towards one another in a directed manner. He called this movement

'cytotaxis' (1896), earlier in 1894 he had used the term 'cytotropism' for the same phenomenon. He proposed that the movements were directed by the response of the cells to chemical emanations from other cells. A similar mechanism has been suggested to account for the behaviour of the slime moulds, (Acrasiales) by Bonner, (1947). He and others have found much evidence for the idea that isolated single cells of the resting stages move towards others by chemotactic methods and thus unite to form the fruiting bodies. Voigtlander (1932) and Kuhl (1937) reinvestigated the reaggregation of amphibian embryonic cells. They found no evidence for the occurrence of any other than random movement of the cells in the reaggregation process. If adhesion occurs on contact the random movement of the cells will in due course bring about the union of the cells. Roux observed the rate and mode of approach of pairs of cells, but his observations were on a small number of selected pairs of cells and cannot be regarded as being statistically significant. Kuhl used blastomeres from Triturus embryos and made time-lapse films of the movements of the cells. He found that the movements of the cells statistically were random and that cell contacts were as readily made as lost. This latter observation implies that no reaggregation occurred. Likewise Roux's material showed little or no reaggregation.

There was no success in producing reaggregation of embryonic amphibian cells into large groups which would undergo continued differentiation and in which the cells were sorted out into definite tissues until the experiments of Holtfreter (1944a). For sponges and coelenterates extensive reaggregation of this nature had been described from the earliest observations.

The methods of disaggregation used by Holtfreter were chemical, whereas those of Kuhl were mechanical, which resulted in much cell damage. Kuhl used blastomeres whereas Holtfreter used gastrula or late blastula cells. These or other differences may account for the fact that Kuhl failed to obtain reaggregation. But this failure may be no more than a reflection of the fact that in the very dispersed cultures used by Kuhl random movement would have to be continued for a very long while for significant reaggregation to take place, even if adhesion occurred at every contact. Since Kuhl failed to obtain reaggregation, it can be objected that his experiments do not test whether the movement of the cells is random or directed. Holtfreter (1943a) considers that it is unlikely that the movement of reaggregating cells is directed, but he offers no more evidence than that provided by direct observation. Later (1947a) he repeats the suggestion, however, pointing out that cells in close proximity may exhibit reciprocal attraction. In consequence it seemed desirable to

reinvestigate the question of whether the movement of the cells is random or directed.

Evidence for the chemotaxis of cells in embryonic material has been given by Holtfreter (1947a) who studied the movements of the melanophores of R.pipiens tadpoles when lecithin implants were made in the body wall. Likewise Twitty (1945) considers that the reaggregation of the melanophores in the black form of the axolotl is brought about by chemotaxis. Twitty and Niu (1948) obtained evidence that chemical factors affect dispersion of chromatophore cells from amphibian embryos. Galtsoff, (1925) studying the reaggregation of sponge cells, did not find any evidence directed movement of the cells.

The movements of the cells have been studied by making time lapse films of reaggregations. These films also provide much evidence on the way in which adhesions are formed between the cells and on the part played by cell adhesion in the formation of the reaggregate. The results of the experiments described in the previous section provide suggestions as to the nature of cell adhesion. Further experiments on the effect of various chemical and physical factors on cell adhesion in reaggregation were made and are reported in this section of the thesis.

The sorting out of the different cell types from one another

which may occur in reaggregation, has been investigated by Holtfreter (1943a) and others. He found that there is no sorting out of the cells of gastrulae in their reaggregation during the formation of the aggregate. In his paper with Townes (1955) they report there is no sorting of the cells till some while after the reaggregate is formed. It is fairly clear that there can be no great degree of sorting of the cells while the cells are moving together and forming their first adhesions, for if it were otherwise the separation of the various sorts of cells from one another could be seen. Nevertheless it is possible that there may be some degree of sorting, which though not obvious to brief examination, would be revealed by statistical enquiry. Cell sorting could be brought about by directed movement. It could also occur if the only cells to form adhesions were of like kind or if like pairs of cells formed stronger adhesions than unlike. In the latter case it would be necessary for the weaker adhesions to sever more frequently than the stronger, if sorting were to be brought about. Chiakulas (1952) has described such differential cell adhesions in wound healing. The results of the analysis of the films have been used to examine this problem of cell sorting.

However it seems probable that cell sorting occurs after the reaggregate has formed. In consequence of the

opacity of the cells it is extremely difficult to detect cell movements within the reaggregate. Histological preparations have been made of a number of reaggregates in order to obtain evidence on the occurrence of cell sorting.

All studies on cell sorting may be liable to the objection that it has not been shown that the various cell types survive disaggregation and reaggregation. Trinkhaus (1956) suggests that cells may dedifferentiate and then redifferentiate into another type of cell. In order to settle this objection it would be necessary to mark individual cells of a known type in some harmless manner so that they could be traced in the reaggregate. Technically this procedure is very difficult to perform. A less satisfactory method of investigation is to reaggregate tissues of a previously known presumptive fate and interaction, and to test the reaggregate for the appearance of cells of unexpected types. However the isolation of only a few cell types at a time may obscure changes which would take place when many other cell types are isolated with them, as in reaggregates of whole embryos. An example of such a change is that described by Lopashov (1955), in which changes in cell type occur when the total number of cells of one presumptive

fate in a mass is large. Another is found in the work of Yamada (1940), who found that chorda cells would induce mesoderm tissue of one sort to give rise to a number of organs which were not amongst the organs usually formed by that tissue. Despite these objections experiments on the isolation, disaggregation and reaggregation of tissues of known presumptive fate have been done to test the possibility that changes in cell type occur. But by reason of these objections the results and interpretations of such experiments should be regarded with great caution.

Analysis of films of reaggregation. a, filming methods.

Embryos were disaggregated in the carbonate-bicarbonate buffer at pH 9.8 or in EDTA at pH 8.0. When disaggregation was complete, which is after a period of from forty to sixty minutes, the disaggregating medium was replaced with normal Holtfreter solution at pH 6.9-7.1. The process of reaggregation was observed thereafter.

A special type of culture dish was used to hold the reaggregations which were being filmed. The construction of this is indicated in figure 4. The dish is constructed so that good optical conditions for filming are had. The reaggregating embryonic cells are inside a glass ring, which is cemented with polythene onto a slide of 1mm. thickness. This slide lies in a petri dish, and is surmounted by an inverted watch glass with a coverslip set in its centre. The medium inside the ring adheres to the coverslip above by capillary action. Thus the medium forms a column whose ends are the plane surfaces of the coverslip and the slide. This column contains the optical path used in filming. The petri dish has an optical flat set in its centre so that all the boundaries traversed by the optical path are plane surfaces. This condition prevents distortion of the image of the reaggregate. The petri dish is flooded with Holtfreter solution in order that evaporation from the medium inside the glass ring might be slight. An air space is present inside the inverted watch glass, which stands over the slide, in consequence the medium inside the glass ring is not in connection with that in the petri dish, but adheres to the coverslip set in the watch glass. This air space provides oxygen for the

VIEW OF CULTURE DISH USED FOR FILMING.

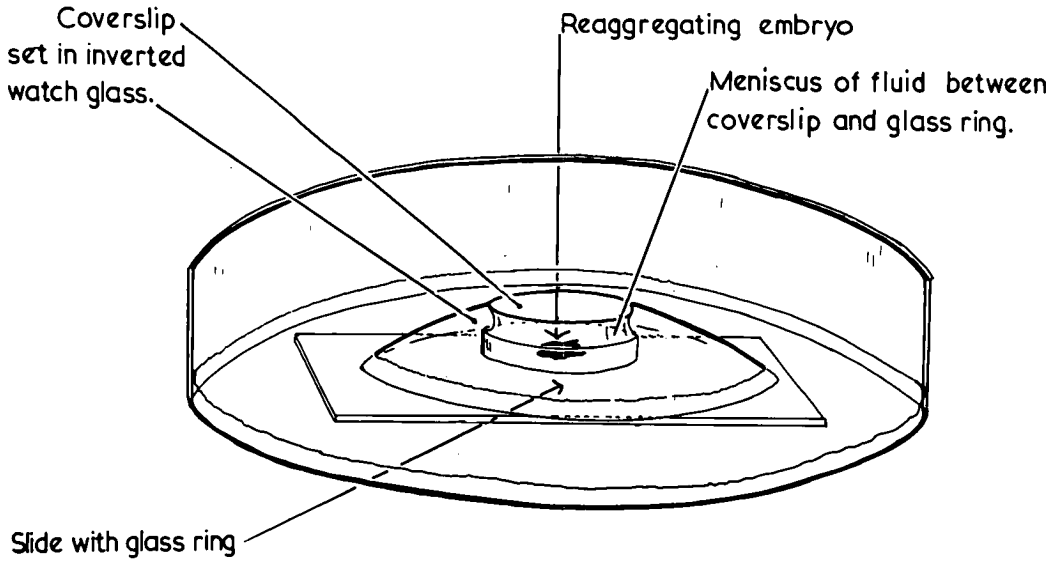


FIGURE 4.

reaggregate. The medium inside the glass ring is held to the coverslip above by capillary attraction so that there is a narrow meniscus between the top of the ring and the coverslip, through which gases may diffuse in and out of the medium.

This dish was mounted on the stage of a microscope and time-lapse films were taken using low and high power objectives. Disaggregation was done in the dish provided by the glass ring and slide and the whole culture dish was put together on the stage of the microscope just before filming was to begin. The filming was done by Mr. E. Lucey of the film unit, Institute of Animal Genetics, University of Edinburgh. Films of the reaggregation of eight Xenopus gastrulae were made, six of these were filmed using the 1" objective of the microscope, two with higher power. Nine films of the reaggregation of Triturus blastulae were made. Six of these films were taken using a 16mm. phase contrast objective, two with dark ground illumination at a similar magnification and one with normal illumination with a 16mm. objective. A short technical description of the films is given in appendix 1. For those films taken at higher powers or under phase contrast illumination, the petri dish was removed from the composite dish in order to provide the small working distance required by the optical system.

The negatives of these films are the property of the Institute of Animal Genetics, University of Edinburgh. The Institute and I possess positive copies of these films. An edited film with a spoken commentary has been made: it is hoped to submit this film together with the thesis.

The observations made from the films have been added to by direct

examination of reaggregation. For such examination the material was cultured in the dishes formed by the glass rings and thin slides, and these dishes were stored inside pairs of petri dishes in between observations.

b. General observations on reaggregation.

This description of reaggregation of the cells of the gastrula of Xenopus includes the results of direct observations with those derived from the films.

At the end of disaggregation the cells lay on the bottom of the culture dish. The cells were frequently packed so thickly that it was impossible to tell whether they were properly separate from one another. Furthermore in such a situation it was impossible to examine the nature of cell movements, in part by reason of the degree to which cells obscured one another. Moreover it was unlikely that cells in such proximity would show movement for they were in close contact. Finally the arrangement of the cells was unlikely to be particularly random for they tended to lie close to their original neighbours. In consequence of this tendency any behaviour of the cells resulting in cell sorting would tend to be obscured, because the cells would already be partly arranged in groups according to their kind. Even if there was no actual sorting out of the cells reaggregates would still show an apparent cell sorting. For these reasons the cells were mixed up and spread more openly on the dish by shaking it gently. Such a technique cannot result in a very effective mixing of the cells but appears to give a moderately well mixed population. This mixing of the cells was

done before the culture medium was replaced with normal Holtfreter culture medium. The reaggregates from such cell populations showed clear sorting out of the various types of cell, as has been reported by Townes and Holtfreter (1955). The degree of mixing of the cells was much reduced after their reaggregation and this fact indicated cell sorting.

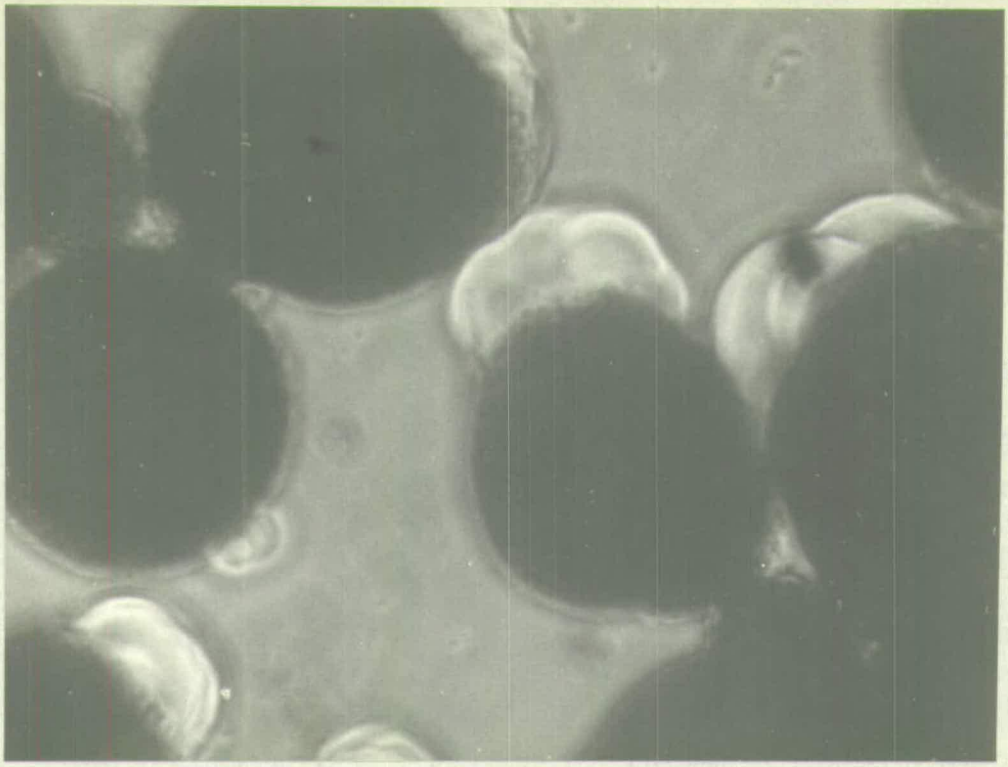
The first reaction of the cells disaggregated at pH 9.8 to the return of normal Holtfreter medium appeared to be a slight clotting of the cells and jelly. This reaction did not occur with cells which had been disaggregated with EDTA. The onset of this phenomenon was exceedingly rapid if it occured, and I am not certain of its existence. The jelly between the cells seemed to become much more resistant to the movement of the cells in it when it was shaken. The cells appeared to become more adhesive almost immediately. It would be interesting to investigate this phenomenon more completely.

For an hour or so little change was observed in the population of cells. The cells lay still save for the movements of the clear hyaline margins. Cells often lay in contact, but no firm adhesion seemed to form between them. The hyaline margins moved in the circular movement round and round the cells known as cyclosis. This movement proceeded slowly (say one revolution per five minutes) in the absence of calcium, and more rapidly in the presence of calcium. Soon after the return of ionic calcium to the medium the rate of cyclosis was much increased. Examination of the films appears to show that cyclosis was most rapid early in the process of reaggregation and that the rate declined thereafter, even in cells which failed to make connections. It would be of interest to know

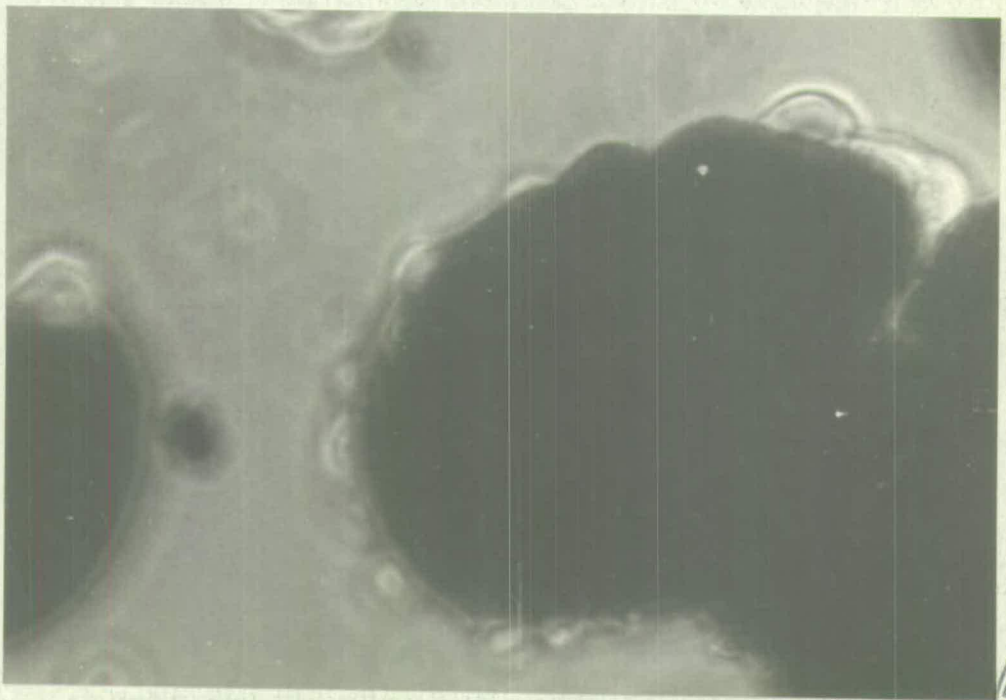
whether this decline is connected with changes in the culture medium or in the cells themselves.

In the more densely packed portions of the population, the hyaloplasm margins came into contact whenever they happened to be pointed towards one another. These were the first contacts made by the cells. Generally these contacts lasted a very short time. The bulges moved away from one another and the connection broke without any sign of mechanical strain between the cells. This would seem to indicate that there is no true adhesion between the cells but that they are merely pressing close on one another. Yet later on these cells could often be seen to adhere to one another. Frequently the bulges appeared to push hard against one another and to become deformed without any movement of the main mass of the cells happening. From this it may be concluded that the bulges have little structural rigidity. The transitory contacts that were formed did not appear to affect the motion or position of the bulges. On occasion the direction of circulation of a bulge was seen to reverse after collision with another, but there was no inhibition or restriction of movement. In these respects the cells do not behave like the fibroblast populations studied by Abercrombie and Heaysman (1954) which show an inhibition of movement in certain directions on the formation of contact, this phenomenon being called 'contact inhibition' by these authors.

After a while the bulges tended to become blebby and of complex form, and their regular circulation around the cell ceased. These changes may be seen in films 16 and 17. Figure 5 shows the blebby form of hyaline bulge. The blebs were protruded in various



(a)



(b)

FIGURE 5.

Phase contrast views of cells from T.alpestris blastulae. Compare with figure 1 and notice the development of a blebbiness in the bulges, present in a slight degree in (a) and to an extreme degree in (b). From film 13. Scale 1cm = 17·3 micra.

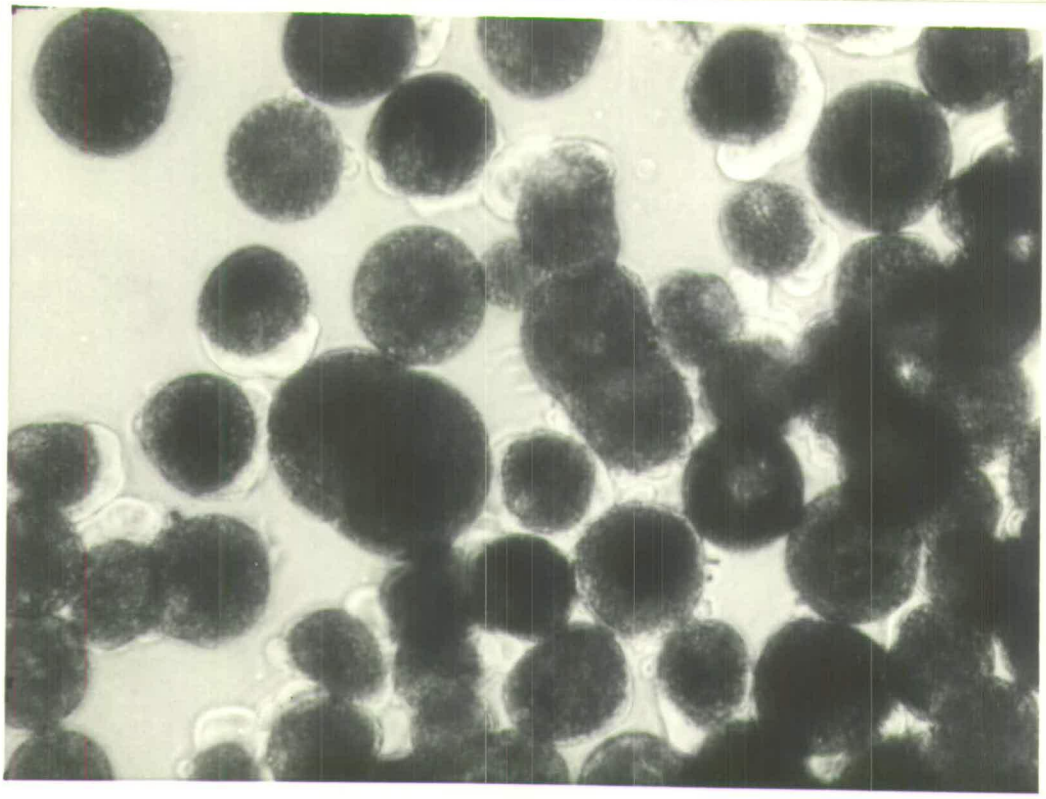


FIGURE 5 cont.

Phase contrast view of T.alpestris cells showing the development of a moderate degree of blebbiness in the hyaline bulges. From film 17. Scale 1cm=35•6 micra.

positions around the cell. These changes appeared at the time that firm adhesions of the cells began to occur, both in adherent cells and in isolated ones.

The formation of these adhesions may be seen from time to time in the films, in particular in films 15 and 16. The hyaline margins of two cells were seen to come into contact. Instead of parting or remaining in the same state the opaque endoplasms of the cells appeared to dart towards one another. Figure 6 shows the stages of this phenomenon. The endoplasms moved towards one very rapidly over a distance of about 10 micra. This distance is too great for the phenomenon to be due to the inability of the optical system to resolve between the two objects. Such an inability would give the impression of apparent fusion of the two objects when they lay closer together than the resolving distance of the system. In this case the system has a resolving power of 2 microns.

After this rapid fusion of the two cells a series of slower adjustments were seen to take place. The area of contact of the dark endoplasms with one another increased and the clear hyaline bulges became confined to the free surfaces of the cell. This process increased the area of mutual contact between the cells. Holtfreter (1947a) describes this phenomenon and attributes it to the action of the forces of surface tension. It may be seen in film 13. After two cells had joined their hyaline bulges were less active, and as more cells joined together the bulges disappeared.

The number of cells adhering to one another is increased by another factor. Cell division was seen to occur during reaggregation,

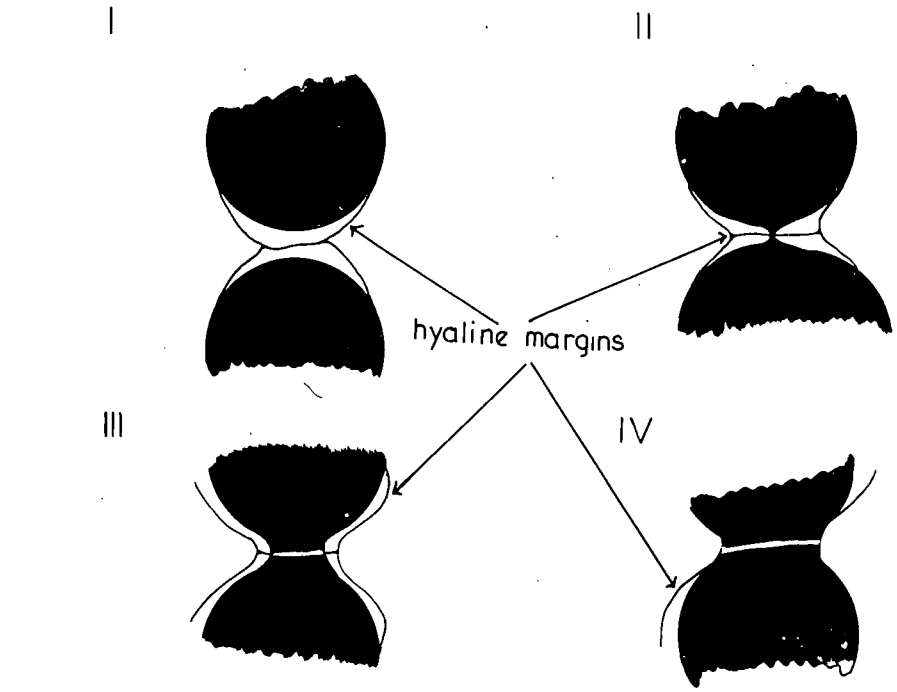


FIGURE 6.

The endoplasms are shown in black. In stage I they lie opposite but no adhesion has formed. The rapid darting out of the endoplasms takes place in stage II; followed by the gradual increase in the area of contact between the endoplasms, stages III and IV. The cell membranes are shown black on white for the hyaline margins, white on black between the endoplasms.

the daughter cells remain adherent. Division results in an increase in the total surface area, and if less than 36·5% of the surface of each cell is concerned in contact with the next, there is an actual increase in the area of surface onto which other cells may join. Furthermore division results in a lateral movement of the daughter cells,in consequence of which they come closer to other cells,thus promoting aggregation. It was noticed that all the hyaline material disappeared somewhile before the furrow formed. This observation may bear relation to that of Mitchison and Swann (1955),who observed that the cortex of the sea-urchin egg became much stiffer shortly before cell division. The newly formed daughter cells frequently showed very active hyaline margins. The cells formed their adhesions with little more movement than that shown by the hyaline bulges. Amoeboid movement of individual cells was not seen (compare with Holtfreter,1946,1947b). Cell division and the small movements caused by the irregular motions of the hyaline margins appeared to account for the slight movements shown by whole cells. The cells on the periphery of the population, which were often far from one another, showed active hyaline margins shortly after they were returned to normal Holtfreter solution, but this activity soon died away. These peripheral cells remained in the same place and continued to survive for several days.

As a consequence of this lack of motion of the cells,adhesions only occur in those portions of the population, where the cells lie so close that the movements of the hyaline margins and the slight movements of the cells have a fair chance of bringing them in contact. Thus at a certain separation of the cells reaggregation

will not occur. Since the dimensions of the hyaloplasm margins on cells are of much the same value from one cell to another it follows that at a certain density of population of the cells they will be just unable to touch one another and reaggregate. This density thus will be reached quite abruptly if one considers successively less and less dense populations. The result of these expectations was found to occur. The less dense parts of the population of cells failed to reaggregate. Since the formed reaggregate contracted in the horizontal dimensions a zone clear of cells was left between it and those portions of the field which had failed to reaggregate. Outside this zone unaggregated cells were found. Figure 7 shows this clear zone.

After the cells had joined with one another, they became closely packed so that the body they formed tended to have a small surface area. The stages of this process may be seen in figure 8. This diagram shows stages in the reaggregation of the cells of a Xenopus gastrula taken from film 9. Cells were seen to develop many contacts with one another. These groups of cells then began to show movements of elongation and contraction. Often these movements were of a vigourous nature such as may be seen in films 9 and 17. Frequently large groups of cells moved actively in one direction. The motion of such groups of cells appeared to be due to their contraction towards a certain fixed point. No movements were seen in those groups of cells which had no point of adhesion to the culture dish. During such movements the hyaline margins of the outer cells of such groups appeared to show a co-ordinated amoeboid movement, but at present it cannot be resolved whether this is an active

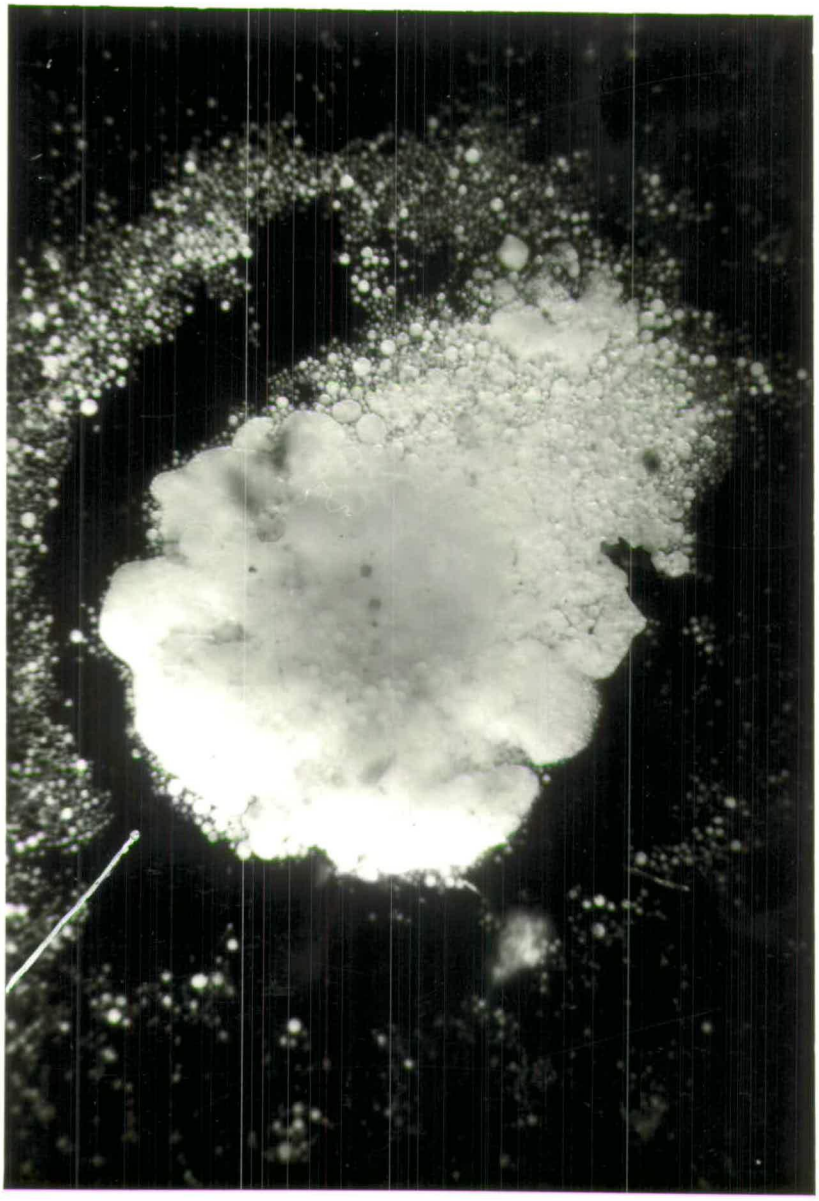


FIGURE 7.

4a). A dark ground view of the reaggregate of a Xenopus gastrula. This view shows the zone free of cells which the contraction of the reaggregate forms between itself and those regions where the cell density is too low for reaggregation. Scale. 1cm = 442 micra.

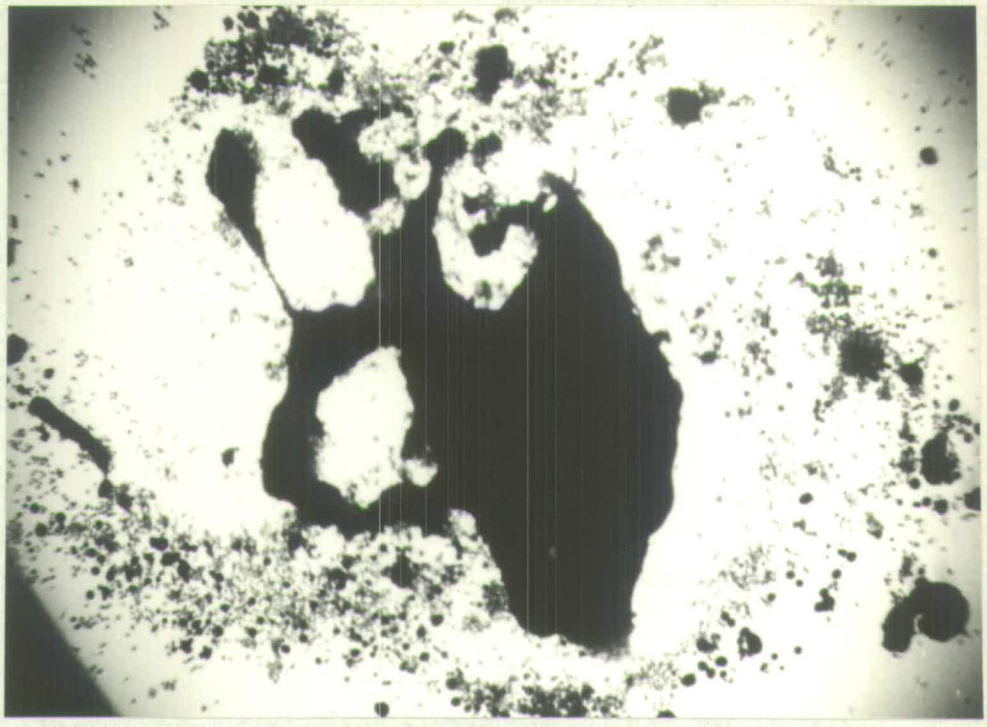
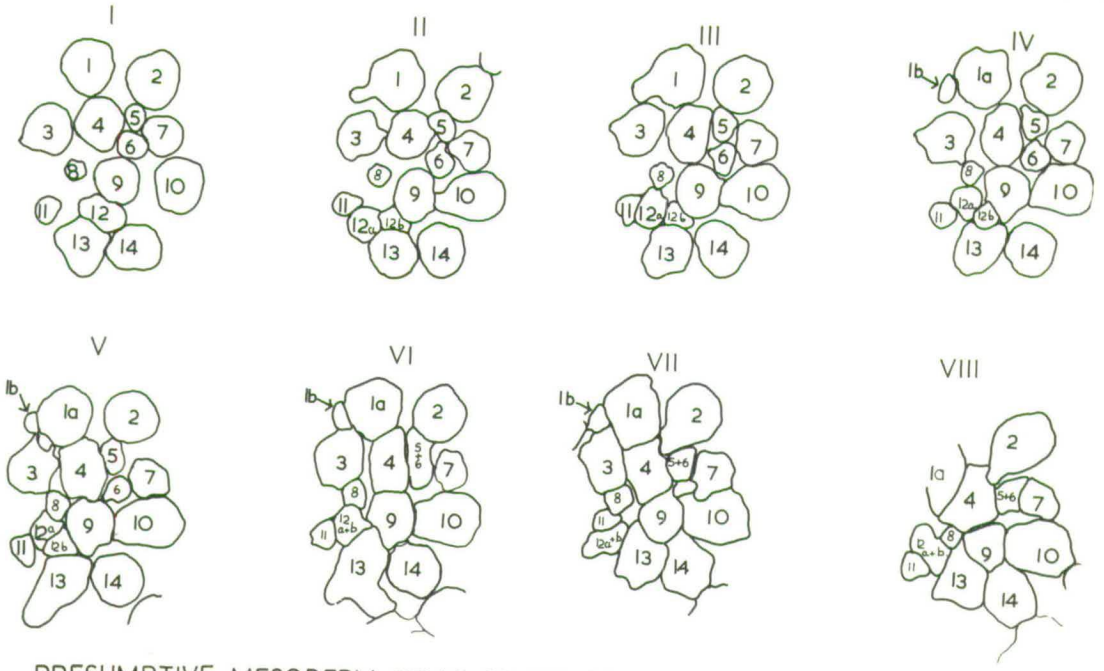


FIGURE 7 cont.

(b). Another view of a Xenopus gastrula reaggregate showing the clear zone between main reaggregate and the unreaggregated cells. Here the unreaggregated cells have formed small peripheral reaggregates in places. Same scale as (a). From film 6.



PRESUMPTIVE MESODERM CELLS OF XENOPUS GASTRULA SHOWING THE CHANGE IN CELL SHAPE AS REAGGREGATION PROCEEDS; I-VIII. From a time lapse film.

FIGURE 8.

Showing the successive stages I-VIII in the close packing of cells which have readhered. Cells marked with subscripts a or b are the daughter cells of one cell present in stage I. From film 9.

promotion of the movements or a passive response of the cells to being dragged over the surface of the dish.

On occasion a group of cells was seen to be attempting to move towards two or more different points. Later such groups split into portions each of which centred on one of these points. Cells lying between these points often became very stretched during the early stages of separation, as though they were under considerable tension. Such cells may be seen in figure 9. These cells maintained their attachments with both the separating portions and often had one or both of their regions of attachment to other cells drawn out into fine thread-like processes as the separation of the two portions increased. In general the main body of such a cell lay in the margin of one portion of the group and a process reached from it to find attachment in the other portion. More rarely a cell lay between the two portions and was attached to them by processes reaching out from either end. As the two portions separated the cell and its processes became more extended, and they appeared to be under considerable tension. Ultimately the processes snapped; and on the few occasions when this was seen to occur it was noted that the pieces of the processes slowly withdrew, one into the cell of its probable origin and the other onto the cell of its attachment. But this interpretation raises the question of the nature and origin of these threads. Are they elongated filopodia or threads of surface coat of the type described by Holtfreter (1943a) ? And if they are filopodia are they formed from a single cell or from both the cells concerned in an adhesion? Unfortunately no evidence on

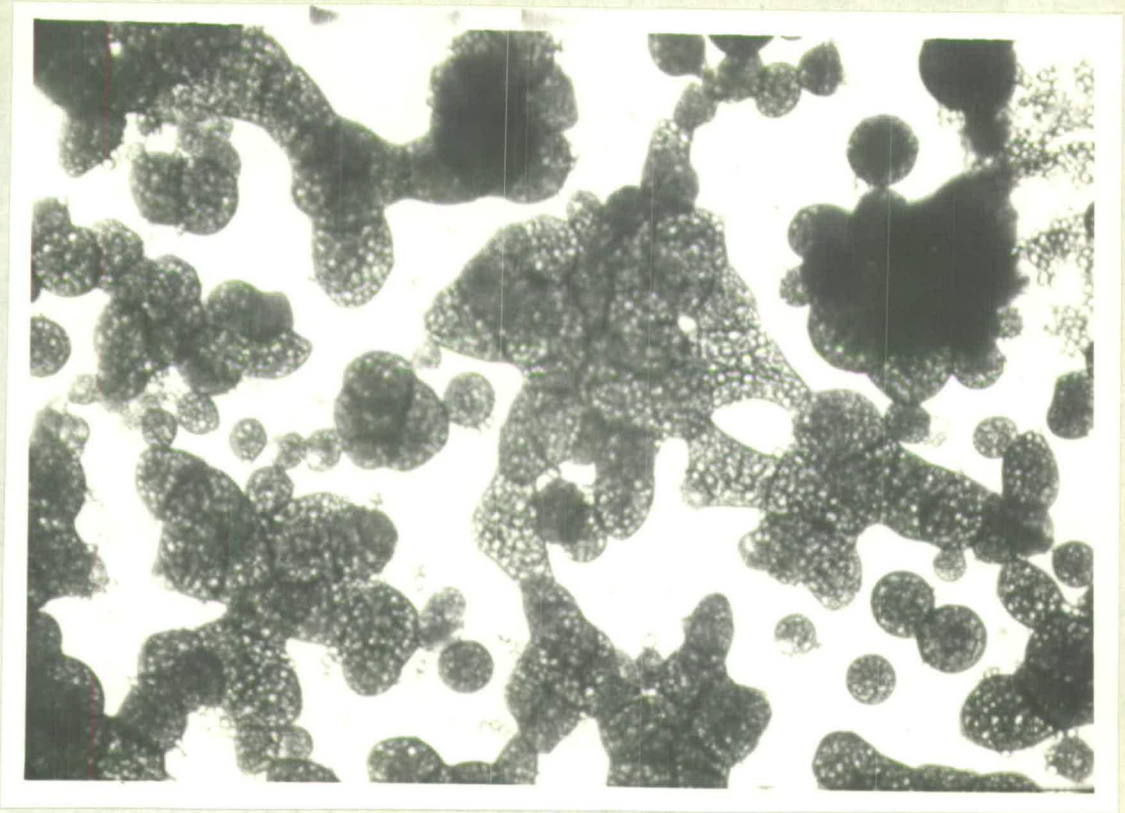


FIGURE 9.

(a). A still from film 9 showing the elongated form of the reaggregating cells of Xenopus at mid-gastrula stage : this form is presumed to indicate considerable tensions in the cells. Scale 1cm =57micra.

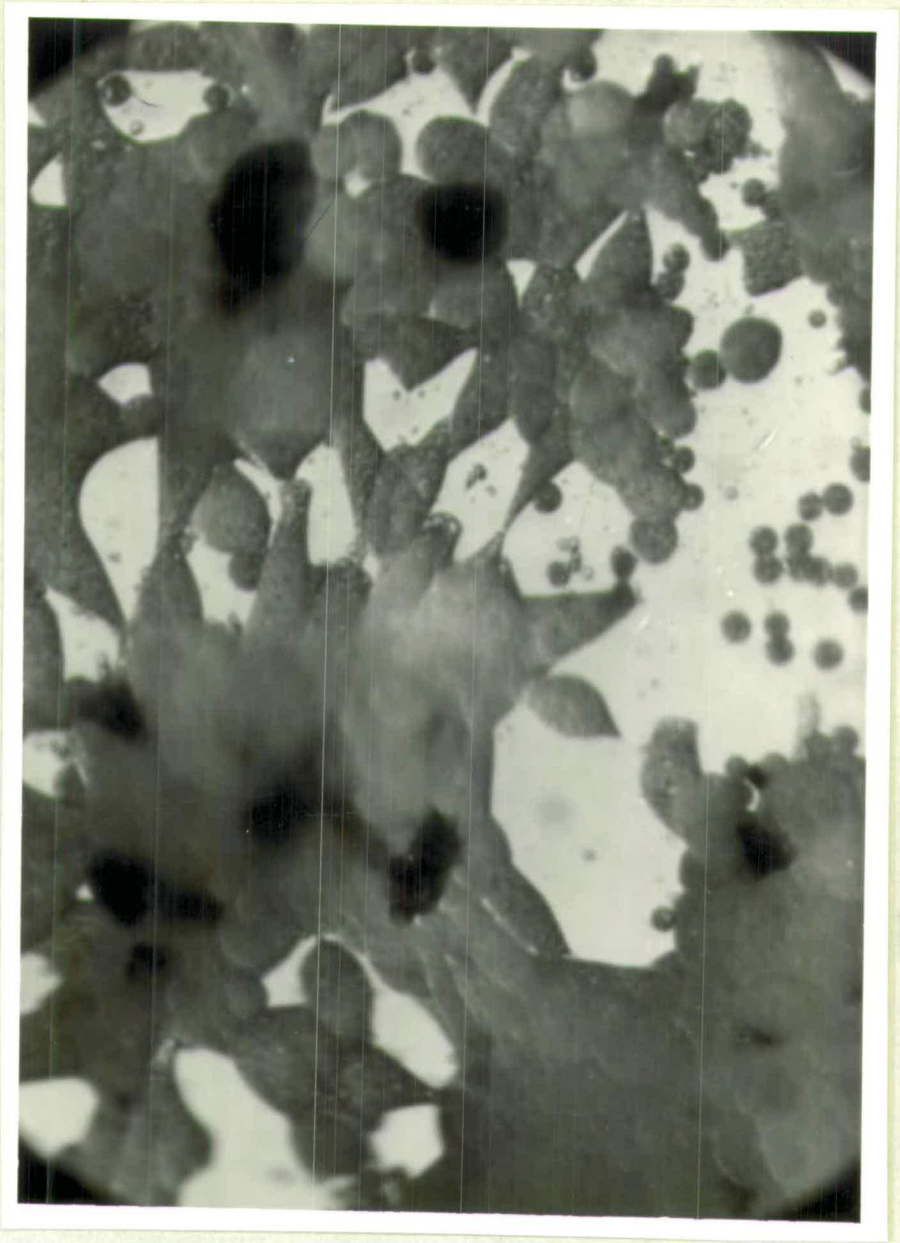


FIGURE 9 cont.

(b).A view similar to 9a, but showing an even greater extension of the cells stretched between the several close-packed reaggregates. Scale 1cm = 45 micra. 10 sec, exposure on Microneg Pan film,through binocular microscope x 5 objective, x 10 eyepiece.

these points has yet been obtained. These filaments may reach 400 microns in length. They were found to be negatively birefringent when in normal Holtfreter solution. Figure 10 shows two of these filaments.

There is no evidence that this violent separation of groups of adherent cells into several portions represents any extensive degree of cell sorting, for the separated groups usually contain the same composite selection of cells. However this observation does not provide a critical test of this question. It seems that the cells tend to contract together and that any point of firm attachment of the cells to the surface provides a centre towards which the cells will necessarily contract. In reaggregates formed from originally very dense populations of cells the number of cells will be such that these separations will be obscured. That there are such regions of particularly firm adhesion of the cells to the culture dish may be due to the nature of the surface of the culture dish, e.g. patches of dirt. Such a fact would explain why this phenomenon of groups separating from one another is only seen in a few of the reaggregations. Little investigation has been made of these features of reaggregation, but they would appear to offer a useful field for future research.

The cells contracted into tightly packed groups. Thereafter few changes which can be seen occur in the reaggregate. The surface of the newly formed aggregate was in nature like that of a new layed cobble street with portions of individual cells standing out in relief on the surface. Soon however it took on the appearance of

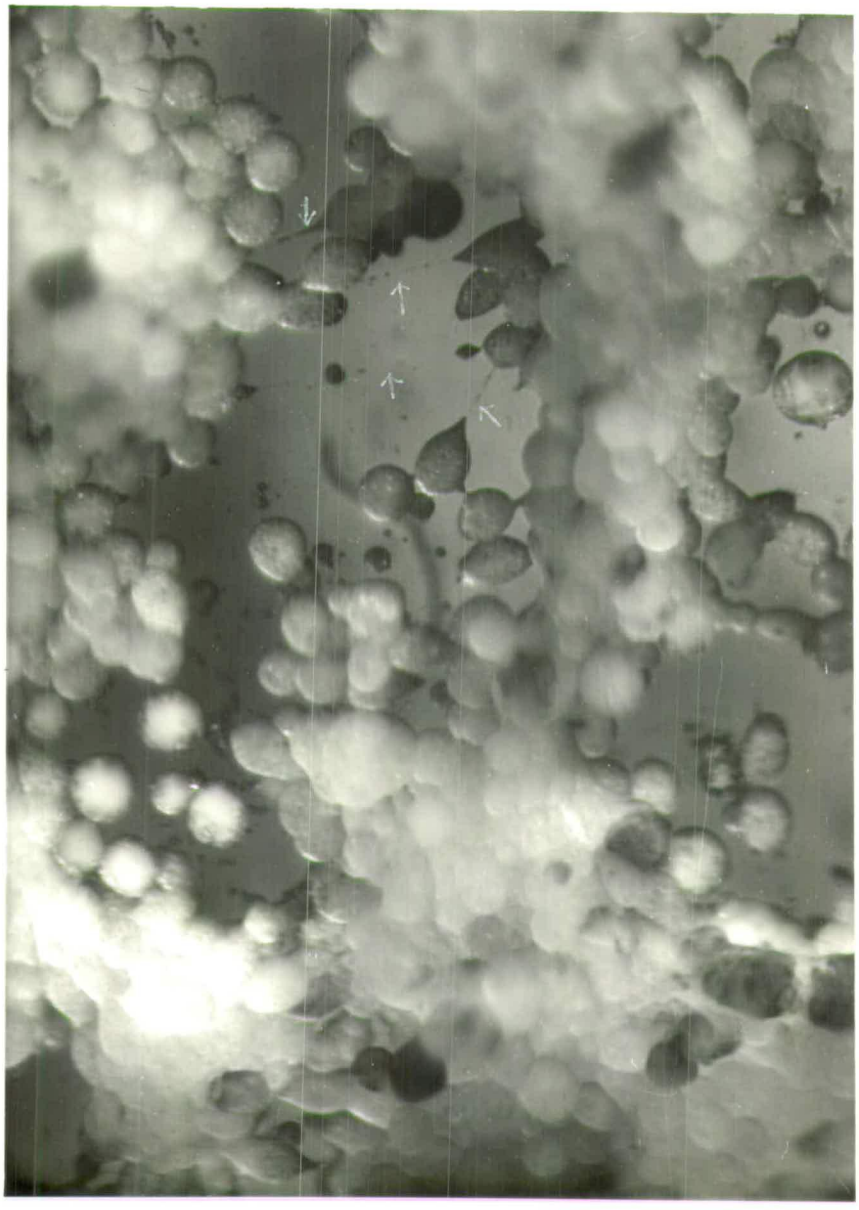


FIGURE 10.

Showing the filaments (indicated by arrows) formed between cells formerly in close contact, which are moving into different aggregates. Microneg Pan. 60 sec. exposure. x 5 objective and x 10 eyepiece. Direct and reflected illumination. Scale 1 cm = 85 micra.

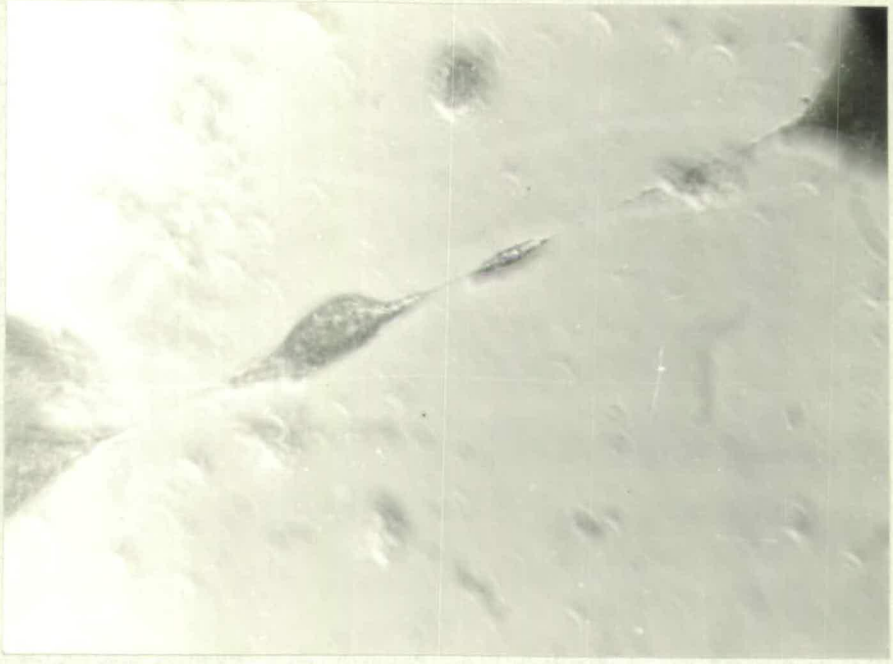


FIGURE 10 continued.

A filament drawn out of either end of a cell; note the secondary thickening in the filament near the cell. From 17 hour old reaggregate of a Xenopus early gastrula. Photographed on FP3 film, 5 sec. exposure. Phase illumination, x 20 water immersion objective, x 10 eyepiece.

worn cobbles for the surface became smooth. The surface was at first formed from all types of cell, but after a while as found by Townes and Holtfreter (1955) it became formed mainly of pigmented ectodermal cells. This change is the main outwardly visible sign of the extensive cell sorting which was found to occur. The smoothing of the surface appeared to be accompanied by the reformation of the surface coat, as described by Holtfreter (1943a). However the actual detection of this coat is a matter of some difficulty, and proof of its actual existence by dissecting it off the cells was tried in only three cases.

As the reaggregates contracted into closely-knit groups a certain number of spherical and apparently moribund cells were rejected from the mass. These cells appear to be unable to adhere to the cells of the aggregate, and this may account for their separation from it.

Reaggregates from cells separated with alkali were found to live for 9-10 days, at the end of which period their yolk platelets had vanished and considerable differentiation had taken place. Presumably death occured because their supplies of food from the yolk platelets were exhausted. The reaggregates from cells disaggregated with EDTA lived for 3-4 days when a sudden and violent disaggregation and cytolysis happened. The reason for this earlier death is suggested in the section of this thesis on the existence of cementing material.

<u>The analysis of the films to decide whether the movements of the cells are random or directed.</u>

In the reaggregations, which have been studied there has been very

little movement of the cells before the first adhesions are formed. This fact may be seen clearly in the films. Furthermore reaggregation only occurs at such a density of cell population that the cells are quite or very nearly in contact to begin with. That there is such a critical density of the population, for reaggregation to be possible, suggests that any mechanism producing directed movements of the cells can only act over very short ranges. The main movements of the cells responsible for the production of adhesion are the protrusions of the hyaline bulges, for these are the chief movements which bring the cells into contact with one another. Thus the problem which has been analysed is whether the directions of protrusion of these bulges are random or directed.

These bulges might be directed, not with respect to individual cells but with respect to some more general feature of the cell population, e.g. density of population or to some precise feature, e.g. the location of a certain type of cell. Such a direction of the bulges would not aid reaggregation, for the bulges would tend to be on one side of all the cells in a given area. Consequently contacts would be made between cells only in those situations where the bulge of one cell touched the endoplasm of another; whereas if the direction of protrusion of the bulges was random contacts can be made between two bulges. Thus with a random direction of protrusion contacts will be formed between cells that are further apart than is possible if the direction of protrusion were directed. Nevertheless it is possible that such a mechanism exists; in consequence statistical tests have been used to investigate this possibility.

Another possibility is that the bulges are directed between pairs of cells so that they either tend to face one another or to hold some other relation to one another. This effect might be due to either of the two following possibilities. Firstly it might be a general influence of the cells on one another, which however would be obscured in closely packed populations of cells, for any cell acts and is acted upon equally by the surrounding cells. Such a phenomenon would not aid the reaggregation of cell populations of uniform density, but would assist the reaggregation of cells on the periphery of a population. This possibility has not been tested statistically because of the lack of films showing reaggregation at the periphery of a population. Moreover any such action would have little effect on reaggregation of the whole population. Secondly there might be an orientation of bulges between specific types of cell. Such a mechanism would assist cell sorting. Statistical methods have been used to test this possibility.

Before describing these tests it is well to mention that the studies of Voigtlander (1932) and Kuhl (1937) were done with cells which showed extensive amoeboid movement across the culture dishes.

The statistical tests.

The directions of protrusion of the hyaline bulges may be seen in the phase contrast films of the aggregation of T. alpestris blastulae. When the films were projected frame by frame the directions of protrusion of the hyaline margins could be read off on an octant grid. Such grids are shown in figure 11, where they have been superimposed on the cells A and B. The octants were

DIAGRAM TO ILLUSTRATE METHOD OF INVESTIGATING POSSIBLE ORIENTATIONS OF HYALOPLASM BULGES.

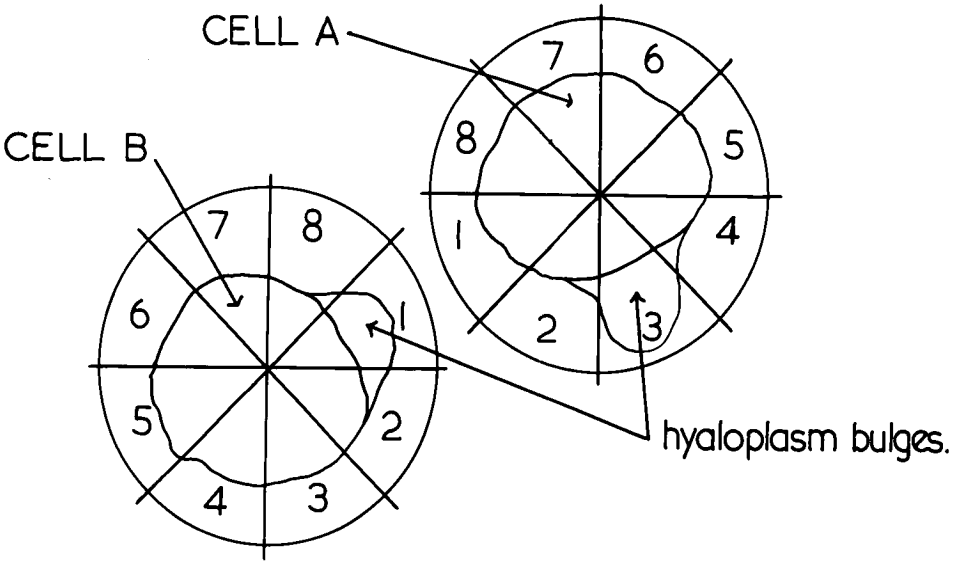


FIGURE 11.

arbitrarily numbered in the manner shown. The type of octant shown over cell A was used in the portion of the work on the orientation of the bulges in single individual cells, which is now described. The octants were placed over the images of the cells projected onto a screen from the film, with an arbitrary but constant orientation of the octant for all the cells in a film. Whenever a protrusion appeared in a certain octant the number of that octant was scored. The nature of the movements of the bulges were analysed by scoring the positions of the bulges at intervals throughout the film. If the direction of protrusion of the bulges is random the distribution of observations in the classes 1 - 8 will be random. The randomness of the scores made were tested by the χ^2 test, and the values of χ^2 are shown in Table 3.1.

In order that the cells chosen for examination might be similar and that there was no selection which might bias the results, the cells were chosen according to the following conditions. The cells examined reaggregated subsequently, but formed no adhesions (and thus directed orientations of the bulges) during the period analysed. This precaution precludes the selection of cells which failed to reaggregate. This condition was further narrowed to those cells which reaggregate solely by the contacts formed by the protrusion of their hyaline margins and not by any other slight movements of the cells. So only those cells which were just able to come into contact with other cells by means of their hyaline bulges were chosen. The film was examined at intervals of about 12 frames apart, during which interval the bulges would be expected to make

TABLE 3.

Scores in the classes 1-8 of the octants occupied by the hyaline bulge in the cells of reaggregating embryos of T.alpestris.

1st set of 4 cells.

16	13	15	5
9	6	14	3
21	7	12	8
24	5	3	7
22	6	5	9
19	5	4	15
16	11	8	13
13	13	8	7
8·31	10·33	17·03	13·09

Values of χ^2

For f= 7, the 5% value of χ^2 is 14·1

2nd set of 7 cells.

14	23	20	16	16	21	21
15	13	22	7	14	8	17
24	19	9	27	15	13	22
21	10	19	23	13	18	14
18	10	16	15	23	18	16
10	12	11	11	18	12	11
13	17	19	19	17	17	8
16	24	15	11	12	21	19
8·9	13·72	9·02	19·33	5·23	9·21	9·15

Values of χ^2

between one and two revolutions of the cell. Individual cells were followed for about 64 or 128 observations.

Owing to the small amount of film which was suitable for analysis by this method only two sets of cells have been analysed by this method. The results are given in Table 3. The first set of cells lay on the periphery of a population, the second set towards the centre of another population.

The second possibility that the cells of certain types might show a mutual interaction was tested by the following method of investigation. A group of cells obeying the same conditions as described above was chosen. A pair of cells such as A and B in figure 11 were considered. The octant grids of the two types shown were placed over the images of a pair of cells: one sort of grid on each cell. The octants numbered 1 were set opposite so that they represented the closest approach of the bulges. The positions of the bulges in adjacent pairs of cells were examined at intervals of about 12 frames. The number of the octant that the bulge of one cell occupied was read off on each occasion and added to the number obtained from the adjacent cell. Thus scores in the classes 2 - 16 were obtained. If the bulges show random orientation with respect to one another, the distribution of the scores will be a triangular one with a mean in the class 9. The results of the examination together with the theoretical distribution of results if the positions held by the bulges are random are shown in Table 4. Nine pairs of cells were examined, unfortunately this number is too small for results which are satisfactory in terms of either the

TABLE 4

Scores of the pairs of octants occupied by the hyaline bulges of adjacent cells arranged in descending order in the classes 2-16. The random expectation for 64 observations is included.

Random expectation.					
1	1	1	4	7	
2	3	5	6	6	
3	3	12	9	6	
4	10	9	17	9	
5	11	10	5	7	
6	7	13	13	9	
7	10	14	15	14	
8	14	14	17	20	
7	16	11	15	8	
6	20	11	4	14	
5	12	9	9	7	
4	2	7	3	6	
3	8	3	7	8	
2	7	4	0	5	
1	3	2	1	2	
	20·24	8·54	30·59	21·45	Values of χ^2

For $f = 14$, the 5% value of χ^2 is 23·7.

See overleaf for data on the remaining five pairs of cells.

TABLE 4 cont.

6	5	0	0	2	
3	10	1	4	2	
8	9	10	6	2	
5	9	12	5	2	
13	7	7	2	4	
11	20	4	5	4	
9	11	14	7	9	
20	14	27	12	11	
15	14	11	10	7	
12	7	15	12	7	
9	5	5	4	5	
10	6	2	3	4	
3	5	5	5	6	
3	2	12	4	1	
0	3	3	0	1	
17·38	26·8	47·26	15·5	8·07	Values of χ^2

number or number of types of cells examined.

The system of numbering the octants is not ideal for it provides no clear test of a slight tendency towards orientation, for some of the values, such as 8 + 1, are lost in the largest class of results. The results of the scoring were analysed by the χ^2 test, and these values of χ^2 are included in Table 4.

The values of χ^2 obtained for the distribution of orientations of the bulges of cells considered singly are given in Table 3. The majority of these values are not significant at the 5% level, but two are significant at this level. The results thus tend to support the theory that their directions of protrusion are random, but the presence of the two anomalous results suggests that it would be very desirable to examine more cells. If it is assumed that the cells examined in section ii of Table 3 are all identical the values for each class may be summed for all the cells, in which case $\chi^2 = 15.27$, which gives a result not significant at 2.5% level.

Table IV gives the values of χ^2 obtained for the distribution of mutual orientations between cells. Six of the results are not significant at the 5% level, but three are. Again the only definite conclusion that can be had from the results is that a larger sample is desirable. Since the first set of observations suggest that the orientation of the bulges is random it would be expected that the second set would show the same effect, for any mutual orientation shown by the cells would also appear in the orientation of the bulges when this was considered singly. The observations for each portion of the analysis were done simultaneously

when the nature of the orientation of the bulges of cells considered singly had not been analysed.

Miscellaneous experiments on the physical and chemical conditions necessary for the reaggregation of cells.

These experiments were done in order to reveal the physico-chemical nature of the processes involved in adhesion. Experiments were done on the pH limits for reaggregation and on the effects of replacing the calcium in the Holtfreter solution by magnesium or strontium. Some observations on the effect of the nature of the surface of the dish used for reaggregation upon the reunion of the cells are given.

The experiments on the pH limits for reaggregation are described first. Midgastrulae (stage $10\frac{1}{2}$-$11\frac{1}{2}$) of X. laevis were disaggregated in EDTA at pH 8.0. When disaggregation was complete the medium was replaced by normal Holtfreter solution buffered at the following pH values with a 0.001M tris buffer, 7.0, 7.5, 8.0, and 8.5, and with a 0.0084 M phosphate buffer at pH 5.3, 5.9, 6.3 and 6.8. Groups of ten embryos were tried at each pH value. Reaggregation was complete in those embryos cultured at pH 6.3, 6.8, 7.0 and 7.5, and partially complete at pH 5.9 and 8.0. No reaggregation occured at the other pH values tried.

These observations are closely related to some made from attempts to reaggregate embryos on 3% agar-agar or 3% gelatin surfaces. At first trial it was found to be impossible to obtain reaggregation on such surfaces. Since both the agar-agar and the gelatin were ash-free and had been made up in distilled water it seemed possible

that the lack of reaggregation might be due to a lack of calcium in the surface that these substances presented to the cells. Nevertheless one would expect diffusion from the normal Holtfreter solution to remedy this lack rapidly. When the agar-agar or gelatin were made up in normal Holtfreter solution the cells placed on such surfaces still failed to reaggregate. It was then decided to measure the pH of the gelatin and of the medium above it. The pH of the Holtfreter solution was measured electrometrically and found to vary very little from pH 7.0 even if it had been left over the gelatin for a long while. The pH of the gelatin was measured with indicators. Daniolli (1941) pointed out that owing to the adsorption of charged indicator molecules onto the surface of the protein molecules in the gel the measured pH was not the same as the true pH of the bulk of the gel. (It may be wondered whether the term pH can be applied to the complex conditions obtaining in these systems). In consequence of this adsorption the bulk pH of such systems should be measured with indicators which are not adsorbed onto the molecules by reason of their having the same sign of charge as the molecules at that pH. Preliminary measurements suggested that the bulk pH of the gelatin was above its isoelectric point (pH 4.7 as reported in Kruyt, 1949). Consequently the anionic indicators bromocresol green and chlorophenol red were used. A pH of 5.1 was found by matching the colour formed by these indicators against those they formed with various pH standards. It was then decided to buffer the gelatin to pH 6.6 with phosphate buffer. When this was done reaggregation succeeded, provided that calcium were included in the gelatin. These observations suggest the

necessity of having calcium ions and a suitable pH on the actual surface on which the cells reaggregate, though it is possible that failures were due to the lowered calcium concentration and pH in the immediate vicinity of the gelatin surface. Either of these interpretations is supported by the fact that if the heaps of disaggregated cells lay several layers deep on the surface of plain gelatin, then all the cells reaggregated except those of the layer in contact with the gelatin. More accurate measurement of such matters as the pH gradient on either side of the gelatin surface might decide between these two possibilities.

It was found to be possible to reaggregate cells of X. laevis gastrulae on surfaces of glass, both plain and coated with the silicone whose proprietary title is H.S.1107, polythene, 'perspex' and paraffin wax. The hydrophobe surfaces such as polythene or paraffin wax gave reaggregates which showed little adhesion to the surface. These cells reaggregated on such hydrophilic surfaces as glass or polythene which has been flamed adhered to these surfaces more strongly. These observations suggest that the cells are more adhesive to hydrophilic surfaces than to hydrophobic, an observation which is not in agreement with those of Holtfreter (1947a), who found that oil droplets were very adhesive to these cells. The reason for this disagreement may have no other source than that he used sorts of hydrophobic surfaces different from those used in this work.

The experiments in which the calcium of the culture medium was replaced by magnesium or strontium were done in this manner. Five

embryos each from stages 10 and 11 were disaggregated in EDTA, and in due course this was replaced by calcium-free Holtfreter solution containing either 0.005M magnesium sulphate or chloride or 0.0005M strontium chloride. In the next section of the thesis it will be shown that the concentration of calcium required for reaggregation has a lower limit of about 0.0005M.

Those cells in solutions containing magnesium were slow to develop the first adhesions of reaggregation; taking about 20 hours to reach the condition shown by normal control reaggregates in about 3 hours. The cells were very adherent to the base of the dish and no contractions of the reaggregate or sorting of the cells appeared to occur. The hyaline margins of the cells were of a pseudopodial form; see figure 12. An extensive monolayer of epithelial cells developed on the periphery of the group.

With strontium replacing calcium the cells reaggregated to an even slighter degree, and those adhesions which were formed were very weak.

The general chemical similarity of magnesium and strontium to calcium suggest that they would replace calcium in the physiology of the cell to some degree, and the observations support this idea. A desirable extension of this work would be the examination of the effects of various concentrations of magnesium and strontium upon reaggregation. For this would enable one to obtain some idea of the nature of the colloid molecules which are affected in their adhesive properties by replacing calcium with other divalent ions, see Kruyt (1949).

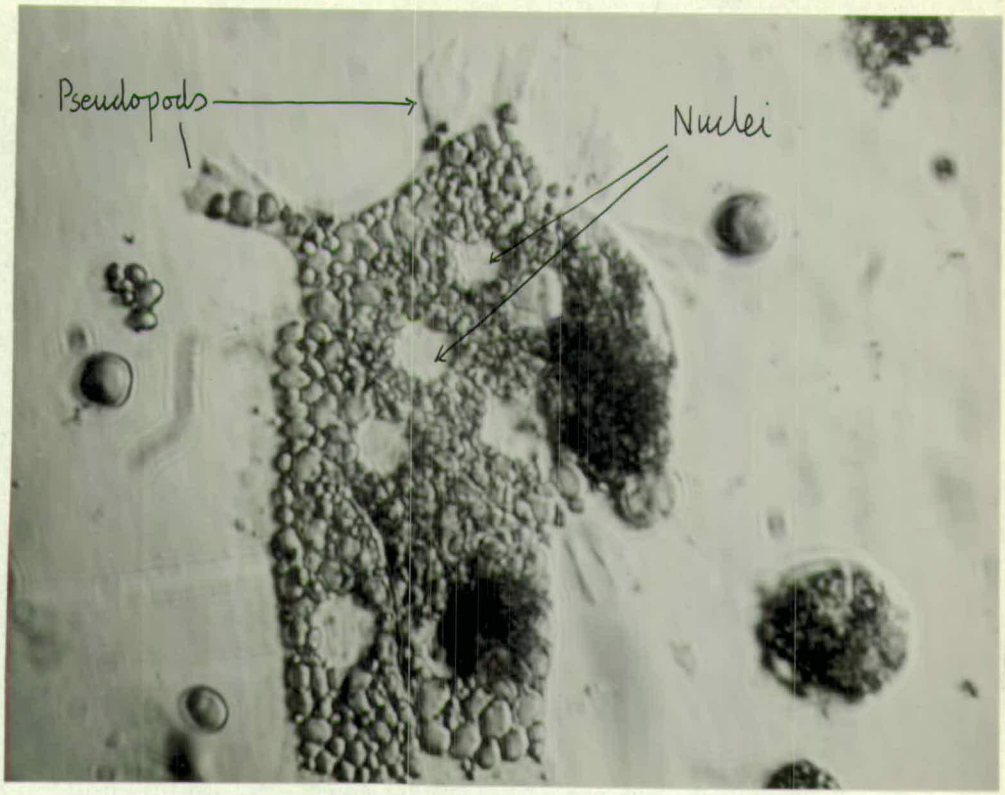


FIGURE 12.

Phase contrast view of small reaggregate of 7 cells from mid-gastrula of Xenopus. Cultured two days. Note the extreme flattening of the cells on the dish, and the pseudopodia, typical of the cells in the presence of magnesium. Photographed on Microneg Pan, 30 sec. exposure. x 20 water-immersion objective, x 10 eyepiece. Scale 1 cm = 25 micra. The axis of the phase system was not central with that of the objectives etc. This accounts for the apparent N.E. illumination of the cells.

Observations on the maintenance of cell type and on cell sorting during reaggregation.

The possibility that cells may change their type when they have been treated with unusual chemical or physical agents has been mentioned earlier. Before undertaking studies on the sorting out of the different cell types from one another which seems to occur in the reaggregate it was desirable to investigate this possibility.

Two sets of experiments were set up. In the first conditions were similar to those with which Holtfreter (1944b) obtained neuralization of prospective epidermis from early gastrulae of Amblystoma punctatum. Explants were made of the median prospective ventral epidermis of early gastrulae of X. laevis. Twenty such explants were disaggregated in calcium-free Holtfreter solution at pH 9.8, and eighteen with EDTA. The cells were allowed to re-aggregate in normal Holtfreter solution and the reaggregates were cultured for three days. At the end of this period they were fixed in Smith's solution, embedded in paraffin wax, cut at 7 micra thickness and stained with celestin blue and eosin. In no case was there any sign of the formation of neuroid or neural structures in these groups of cells. Since it has not yet been possible to obtain the neuralization of such material by any means it is impossible to have controls for this experiment; nevertheless the results suggest that such cells are unlikely to be affected by the treatments used in their disaggregation.

The second set of experiments was to test that function of

embryonic cells such as induction were not effected by disaggregation. Boucher (1953) investigated this possibility. Her method of disaggregation was to use calcium-free Holtfreter solution at pH 10.0. She showed, as had been suggested by Holtfreter (1944b), that the inductive capacities of disaggregated organiser cells were unaffected by disaggregation, though there was perhaps a disarrangement of the specific regional properties found in the intact organiser from late gastrulae. Her experiments were repeated; however EDTA solution was used for disaggregation. Embryos of T. alpestris were used because the abnormal nature of gastrulation of X. laevis, described by Nieuwkoop and Florschutz (1950), prevents exact localisation of the presumptive areas of this embryo. The reaggregated organisers of nearly 100 embryos of various stages were implanted under the ventral ectoderm of early gastrulae. (Mangold's 'Einsteckung' technique). Dr. S. Brahma gave great assistance with these operations. After these implanted embryos had been cultured for four days they were fixed, sectioned and stained in the manner described above. The results are unfortunately somewhat incomplete for many of the embryos died owing to fungal infection. Inductions occured both in a control and in the experimental series; though there was a high percentage, about 20%, of embryos in which no secondary induction developed in both series. These results are unsatisfactory but suggest that disaggregation with EDTA does not greatly affect the inducing action of the organiser cells.

Since the two series of experiments give rise to the conclusion

that both cell types are stable to the methods of disaggregation used, it seems reasonable to investigate the occurence of cell sorting.

No extensive study has been made of cell sorting, for the subject lies somewhat outside that covered by this thesis. Consequently investigations have been confined to examination of histological sections of the reaggregates. These sections were prepared from reaggregates from embryos disaggregated by EDTA or normal Holtfreter solution at pH 9.8. They were fixed, sectioned and stained in the manner described above.

The cells of reaggregates which had been cultured for two or more days showed appreciable differentiation. Cells of all the organ types present in the tissues disaggregated were present, and these cells formed well-knit organs, appearing to be completely separated from cells of other types. In reaggregates of one or two days age the cell sorting was less complete. Groups of cells of very drawn-out form were seen in the sections of such reaggregates; these appeared to be similar in form to the 'bottle cells' described by Holtfreter (1943b), as occuring at the blastopore during the onset of gastrulation. Perhaps these cells indicate the occurence of active morphogenetic movements in the reaggregate as they appear to do in the normal gastrula. The jelly, which is formed in the disaggregation of embryos at pH 9.8, survived in places in the reaggregates forming patches which stained deeply with celestin blue. The finding that these cells sort out or segregate according to their type in the reaggregates is in agreement with the results of Townes

and Holtfreter (1955).

Discussion of the results of the investigations on the nature of reaggregation.

In the introduction to this analysis of the processes involved in the reaggregation of embryonic amphibian cells the question of the mode by which the cells make contact was posed. The results of the analysis of the films suggest that the cells make contact with one another by random protrusion of their hyaline bulges. Townes and Holtfreter (1955) come to a similar conclusion. The movements of the cells were practically confined to the production of these protrusions. The 'cytotaxis' described by Roux (1896) does not appear to occur.

The work of Weiss (1945) describes the orientation of cell migration in media containing fine fibrous structures. The jelly which formed between the cells when they were disaggregated at pH 9.8 might contain such fibres, although attempts to stain such fibres by Lillie's reticulum method did not reveal them. De Laubenfels (1934) considered that the reaggregation of the sponge Iotrochota birotulata was controlled by the fibres of jelly material that formed between the cells. However the lack of such a jelly in those disaggregates which had been made with EDTA makes the possibility of such a mechanism in the reaggregation of X. laevis embryos most unlikely. The results of the analysis of the films also argue against such a mechanism.

It was observed that the cells from blastulae and gastrulae adhere equally well to one another whatever their type. The

results of investigation of post-gastrular stages are expressed in figure 2. It shows that the cells become unable to develop adhesions both between their own and different kinds. This change begins with the neural cells and finally reaches the endoderm: this order parallels the order in which the tissues lose their ability to be disaggregated with EDTA. These changes in the capability of the cells to reaggregate and in their reaction to EDTA suggest that the mechanism by which the cells adhere changes at this stage of development. Such a change in the mechanism of cell adhesion would provide a means whereby the sorting out of cells could occur, as was suggested by Townes and Holtfreter (1955). The results of the present work indicate that the change appears to be from a mechanism in which the calcium ion is important, to some other kind or kinds of adhesion. If some means of moving the cells around inside the reaggregate exists, then the cells will tend to sort into groups which show their greatest adhesiveness towards one another. If each type of cell adheres most strongly to its own type, and less strongly to cells of other types, the cells will segregate into groups of definite composition. Such selective adhesiveness might result from the cells developing forms of adhesion specific to their type, and this is the hypothesis which has been put forward by Townes and Holtfreter (1955), Weiss (1950), and Tyler (1946). A simpler explanation can be conceived to explain the segregation. It is that one form of cell adhesion is replaced by another, equally general, and that this replacement does not occur simultaneously for all cell types but in succession

from one cell type to another. In consequence cells of one type are always capable of adhering to one another, but cells of different type will only adhere to one another when their methods of adhesion happen to be similar. As the change begins in the first type of cell to be affected it will sort out from all others. The change will then occur in the second type of cell to be affected and it in turn will separate out from the remainder. And so on. The successive changes in the method of adhesion suggested by the evidence given in figure 2 would support such a theory. However it is possible that there is a successive and not simultaneous development of the type-specific form of adhesion.

In considering the various manners in which cell adhesion might affect cell sorting the important part played by the movements of the cells has been neglected. In the present study evidence for the occurence of such movements has been obtained from the films, in which contractions and extensions of groups of cells have been seen. The histological slides have also revealed evidence for cell movements, e.g. 'bottle cells'. Unfortunately no evidence has been obtained as to how these movements are brought about.

Evidence on the mechanism of cell sorting has been obtained from studies on the reaggregation of other embryonic material such as chick. Moscona (1956) found that the cells of dissaggregated chick embryos assorted according to type. The degree of differentiation of the assorted cells, which is the main feature by which their type may be recognised, was found to depend upon the relative pro-

portions of the different cell types mixed. In reaggregates of mixed generic origin (mouse and chick) he obtained the somewhat surprising result that the cells assorted according to their organ and not according to their generic type (1957a). He found no evidence for possible changes of cell type contrary to the results of Trinkhaus and Groves (1955), who found that there was some evidence for such changes. Until it becomes possible to trace individual cells throughout reaggregation it will be impossible to resolve such points of difference. The results of such experiments should be born in mind when considering the evidence which has been obtained that cell type is unaffected by disaggregation and reaggregation.

In the consideration of the effect which the development of selective adhesiveness might have on cell sorting no suggestion was made as to the possible cause of such differences. The question of the cause of these differences leads onto the consideration of what causes the cells to adhere. Weiss (1950) discussed the nature of the cell surface in general terms. He considered that the development of selective adhesiveness results from the production of a suitable configuration of molecules on the surface of the cell: he called this theoretical phenomenon 'ecaptation'. In itself this term provides no further explanation of the causes for adhesion and its probably specific nature.

Holtfreter (1943b) explained the adhesion of cells in terms of the action of surface forces. If the different types of cells have different surface tensions, selective adhesions will tend to form.

The remarkable specificity of certain types of cell in their adhesion was revealed by the experiments of Wilson (1907), Galtsoff (1925a, 1929) and de Laubenfels (1927) and Spiegel (1955) who found that mixtures of sponge cells separated out into groups according to their specific type. The experiments of Moscona, quoted above, run somewhat counter to this. A few experiments done in the course of this work show that the same specific separation occurs in amphibia. Gastrulae of T. cristatus and T. vulgaris were disaggregated together in EDTA and the cells well mixed together. On reaggregation the cells of each species separated out, the large colourless cells of T. cristatus being clearly separate from the small pigmented cells of T. vulgaris. Tyler (1946) suggested that this specificity of adhesion, which of course is manifested in the segregation of the cells, was due to some type of of antigen-antibody reaction. This suggestion had previously been made by Loeb (1945). Spiegel (1954, 1956) has demonstrated that the reaggregation of sponge or amphibian embryonic cells may be prevented by supplying suitable antibodies to the cells. However this phenomenon cannot be interpreted as being clear evidence for the presence of an antigen-antibody reaction in cell adhesion, for any impairment of the cell surface will probably greatly alter its adhesive properties. And Rothstein (1954) points out that the cell surface carries a great number of possible antigens, not all of which will be concerned with adhesion, with which the antibodies will react. Thus treatment with the general assortment of antibodies that Spiegel used will probably damage the cell surface in a

manner so unspecific that it will be impossible to make any definite conclusions. (Incidentally Townes and Holtfreter (1955) point out that the mechanism of adhesion proposed by Tyler is more properly called an auto-antibody reaction). Nevertheless Tyler's theory could be tested experimentally; though the technical difficulties would be great as Clayton's work on the distribution of antigens in newt embryos shows (1953).

Unfortunately neither immunological methods nor surface tension investigations would provide much evidence as to the nature of the chemical groupings responsible for cell adhesion, although they might reveal its very specific nature in certain types of cells more clearly than the present experiments on cell sorting do. Moreover the technical difficulties of such experimental methods are considerable (see Newton Harvey, 1954, on surface tension). Thus it seemed that the problem of cell adhesion and of the nature of differences in this respect between cells could be better treated by another approach.

The cell surface can be considered as bearing an electrical double layer upon it. This layer is born by all charged surfaces which are charged by preferential adsorption or exclusion of ions from the surrounding medium upon the surface. This preferential adsorption or exclusion will tend to set up a charge on the surface which will be balanced by a corresponding charge of opposite sign in the layer of the continuous phase closest to the surface. This surface charge will be at a certain potential, and the potential is related to the surface tension. The values of such potentials are of course much

affected by the number of ions in the medium around the surface.

If two electrical double layers are close they will interact in the following manner. They will tend to repel each other because of the like sign of charge and to attract each other due to the London-Van der Waals attraction. The repulsive force acts only in the thickness of the double layer and has the features of an exponential function. The force of attraction falls off as an inverse sixth power law with the distance from the coll. In consequence the force of attraction tends to predominate close to the surface. Kallman and Willstatter (1932) have shown that these forces of attraction may extend to 10-100 Angstrom units from the surface. Overbeek (1949) has summed these two effects for a variety of theoretical situations. He showed that the potential energy against the separation of the two surfaces may reach the order of ergs per square centimetre of their surface when the approach is of the order of 5 Angstrom units. In all cases attractive forces predominate over repulsive forces if a sufficiently close approach of the two surfaces is made. If the force of repulsion is large the approach of the two surfaces has to be very close before attraction predominates. If however the force of repulsion is lowered, which may be done by lowering the potential of the surface charge, attraction predominates over repulsion for greater separations of two surfaces. Whether or no two surfaces succeed in cohering depends upon their chance of making a sufficiently close approach for attraction to occur.

The surface potential depends upon the chemical nature of the sur-

face, for this determines which ions it will preferentially adsorb or exclude, and upon the ions present in the surrounding medium. This theory of the interaction of charged surfaces accounts for their adhesion. It should be pointed out that adhesion can also be brought about by the formation of non-polar chemical bonds between two surfaces. Cell surfaces are like other surfaces and will develop electrical charges upon themselves. Thus cell adhesion is presumably partly or entirely brought about in the manner suggested above. Owing to the nature of the forces involved adhesions will thus be formed very rapidly once a sufficiently close approach is made by the two surfaces. Non-polar chemical bonds will probably form more slowly between the two surfaces. The rapid formation of the adhesions described from the film, is thus probably an example of the adhesion resulting from the interaction of the two electrical double layers.

These properties of the electrical double layer also find expression in such phenomena as surface tension and the antigen-antibody reaction. The antigen-antibody reaction is merely a special case of the mechanism of adhesion described.

The effect of the ionic nature of the medium on the surface potential is considerable. Bungenberg de Jong (1949), in discussing the effect of specific ions upon colloids, points out that the effect of reversal of the charge of the surface, which the adsorption of specific ions upon a charged surface has, increases with the valency of the ion. The concentration of divalent ion necessary to bring about the same reversal of charge as that produced by a certain concentration of

univalent ion is of one tenth to one hundredth the value. If the colloid is negatively charged, as it will be above its isoelectric point, these ions that affect the charge will be cations. Such ions as calcium will alter the charge, and consequently the surface potential, far more effectively than univalent ions such as sodium.

The reduction of charge that addition of calcium or magnesium to the medium brings about, results in a fall of the surface potential and consequently the force of repulsion between like surfaces diminishes. At a sufficient reduction of potential the forces of attraction will be strong enough to maintain adhesion. The pH of the medium would also affect the charge of the surfaces and thus pH limits to adhesion would occur. The results of the work have shown that calcium is of great importance in the maintenance and reforming of adhesions. pH limits for cell adhesion have been found. The sudden formation of adhesions suggests that they are due to the interaction of two surfaces whose London-Van der Waals forces are able to hold one another together. This theory does not provide a simple explanation of how cells may show specificity of adhesion, but the cells of the early embryonic stages do not show such specificity. If there existed patterns of different surface potential upon the various parts of the cell surface the specific adhesion of later stages might be explained as well. The next section of this thesis is concerned with attempts to show that cellular adhesiveness is dependent on the surface charge.

Originally I thought that the theory could best be tested by measuring the surface charge of the cells in various electrolytes, which were experimentally known to affect cell adhesion. This was tried but proved technically very troublesome. In consequence another approach was taken. Surface potential is affected by ionic adsorption, and if the surface adsorption of an ion can be demonstrated and correlated with changes in the adhesiveness of the cells then evidence will have been obtained suggesting that the theory is correct. The ion chosen for investigation was calcium, of whose effect on adhesion much evidence has already been given. A certain amount of work was done on magnesium as well. This method of investigation may lead to more information being obtained about the chemical nature of the cell surface, for as Bungenberg de Jong (see above) has shown the particular reversal of charge effects shown by a group of ions are related to the chemical nature of the surface.

The distribution of calcium and magnesium ions in normal, disaggregating, and reaggregating embryos of Xenopus laevis.

The introduction to this work has been given immediately previous. The methods, results and conclusions, as well as the introduction have been published (Curtis, 1957).

In order to determine whether the calcium is adsorbed onto the surface of the cells which can adhere to one another the general distribution of calcium and magnesium (with which calcium may easily

be confused) in the embryo was examined. The possibility of the adsorption of calcium by cells which then become able to adhere was investigated by measuring the uptake of calcium by cells placed in a medium in which they subsequently reaggregated. This uptake was measured by the decrease in calcium concentration of the medium surrounding the cells. In order to show that this process had the characteristics of adsorption the kinetics of the uptake were examined. Consequently it was necessary to know the permeability of these cells to calcium and their total normal holding of these ions. The total amounts of calcium and magnesium in the embryos were measured on groups of embryos of various stages which had been ashed. If the adsorption of calcium permits readhesion of the cells the removal of calcium from the surface should permit dis-aggregation, and thus the loss of calcium by the cells during dis-aggregation was measured. EDTA was used for disaggregation or OCC, and the amount of calcium they complex can be measured.

Analytical methods:

The methods developed by Headridge (1956, 1957) and Chalmers (1954) were used for the determination of calcium and magnesium ions. These methods allow the distinction of the two ions. They depend on the titration of the ions with EDTA using another complexing agent as an indicator (e.g. eriochrome black or murexide). By doing these titrations at pH 10 and then again at pH 12 the concentration of magnesium ion may be separated from that of the calcium ion. A slight modification of the methods that these authors used was made, for titrations were done using a micrometer mounted

microburette of the Scholander type (that is a displacement burette) instead of an ordinary burette. In consequence estimations of the calcium content of samples down to 0.1 ml could be done. The end-points of the titrations were determined spectrophotometrically, using a Unicam S.P.500 spectrophotometer. The results of Headridge (1957) indicate that calcium and magnesium may be determined to within 10% for quantities down to 0.02 micrograms. A series of control estimations was run during the course of the present work and gave similar results.

The precautions against contamination of the titrations by ferric iron which Headridge (1956) introduced were used. The EDTA was standardised against calcium chloride solutions, which in their turn had been standardised with silver nitrate. All solutions were made up in glass distilled water, which had been previously analysed for calcium and magnesium by the methods described above. The titrations were done on samples whose volume had been measured by weighing to the nearest 0.0001 gm. It was found that the formula for the 'metal buffer' whose use Headridge (1956) recommends in the titration, tended to give free magnesium ions, though it should not in theory. In consequence this buffer was adjusted by additions of EDTA until it gave neither free magnesium ions or EDTA.

The calcium and magnesium ions obtained by ashing groups of embryos were estimated by these methods. Owing to the large amount of phosphate in the ash, solution of the ash with dilute acid did not extract these ions efficiently. In consequence the phosphate ions were removed by passing the ash, suspended in distilled water,

through a column of the anionic exchange resin Amberlite IRA-401 (chloride form). This treatment appeared to give a satisfactory removal of phosphate from the sample and in consequence the calcium and magnesium ions came into solution. The ashing was done in platinum crucibles and the dry weight (dried at 110°C) of the embryos was measured.

Groups of from 20 to 30 embryos were used for the estimation of total calcium and magnesium in them. After the removal of their vitelline membranes they were transferred to calcium-free Holtfreter solution buffered at pH 7.0. The blastocoel or archenteron cavities were opened with needles and the fluid washed out with a pipette, so that the calcium or magnesium in this fluid would not be included in the total estimation. This precaution prevents the inclusion of calcium from the Holtfreter solution in the estimates. It tends to confine the estimation of the ions to those which are inside or on the cells, which is the distribution of interest in the present enquiry. It may be thought that the washing in calcium-free Holtfreter solution allows a loss of these ions from the cells by permeation, but evidence, which will be described, suggests that such a loss if any must be very small. The embryos were then given a second washing in calcium-free Holtfreter solution. A sample from this washing was set aside for the analysis of calcium, so that it could be shown that no calcium was transferred in the drop of medium in which the embryos were carried to the next stage. In the next stage the embryos were either placed in platinum crucibles for ashing, or into EDTA or OCC solutions for disaggregation.

Groups of from 20 to 40 embryos, prepared in the manner described above, were used for the estimation of the amounts of calcium removed from them by disaggregation in EDTA or OCC solutions. When EDTA solutions were used for disaggregation, in the manner described earlier, the amount of calcium and magnesium that was removed from the embryos by the EDTA could be estimated by back titration of the remaining free EDTA with standard calcium solutions. Owing to the fact that samples of about 1 millilitre volume must be used for such estimations the loss of calcium in the course of a disaggregation can only be followed from a few determinations. This is somewhat unsatisfactory. Consequently another method of estimating the amount of calcium which had been removed from the embryos was used. Orthocresol phthalein complexone (OCC) complexes calcium, with a sharp increase in the light adsorption at 572 millimicra at pH 8.0. This increase was found to obey the Beer-Lambert law over the range 0.00001M to 0.0002M calcium, provided that the pH is kept constant. Pollard (1956) found that the OCC complex with calcium did not obey the Beer-Lambert law exactly. This slight difference between his and the present findings is probably to be explained by the fact that he used OCC at pH 10.5, at which pH the rate of change of colour density with pH is very great, whereas at pH 8.0 this rate of change is slight. In consequence very slight changes in pH would affect his results appreciably, while in the present work such changes would have a slight effect. Nevertheless it was necessary to maintain the pH most carefully and in consequence the OCC was buffered with tris at 0.006M concentration, a higher concentration than used normally.

Embryos disaggregated without damage in CCC and reaggregated normally thereafter. By using these optical density methods of estimation the increase in complexed calcium in the course of a disaggregation could be followed from a larger number of measurements than when EDTA was used for disaggregation. The optical density of small samples taken from the disaggregation was measured in the spectrophotometer. The samples were then returned to the disaggregation; in consequence the disaggregation could be sampled at frequent intervals. Both methods of following the loss of calcium from the embryos during disaggregation were used. Both these methods do not allow the distinction of complexed calcium from complexed magnesium and the measurements made are of the two ions together.

The uptake of calcium ion by the reaggregating cells was examined by following the loss of calcium ion from the medium in which the cells were. Preliminary work showed that the uptake of calcium at any given concentration in the medium was complete in less than two minutes. Such a time is too short for more than one or two samples of the medium to be taken, and in consequence it would only be possible to obtain two or so measurements on which to base an uptake curve. Obviously this is not a satisfactory method of investigation.

The method used, was to allow the uptake of calcium at a low external calcium concentration to come to equilibrium; samples were taken from which the uptake could be measured. The external calcium concentration was then raised to a known value and the uptake at equilibrium measured again. This process was repeated with nine

successively doubled initial calcium concentrations. By this means the uptake of calcium with increasing external calcium concentration was followed. The embryos, in groups of from 55 to 70, were dis-aggregated in EDTA at pH 8.0 in the usual manner and then washed repeatedly with calcium-free Holtfreter solution at pH 7.0. It was found that four washings were sufficient to lower the concentration of EDTA remaining with the cells to a very low value. A sample of the final washing was taken and analysed in the manner described above for its content of EDTA; the value obtained was used in correcting the calcium ion concentration of the first solution of calcium with which the cells were mixed.

The cells were treated with each concentration of calcium for 15 minutes. The concentration of calcium in the medium at the beginning of a period was obtained by calculation from amount of calcium in the medium at the end of the previous period and the amount of calcium in the stronger medium which had just been added to the cells. These two values were obtained by the methods of analysis described above. Mixing of the solutions and the cells was assisted by placing the culture dish on a mechanical rocker. In calculating the uptake of calcium corrections were made for the volume of the cells and the concentration of the solutions due to evaporation.

Results.

First the results of the analysis of whole embryos is considered. The total amount of calcium in the embryos appeared to vary considerably from batch to batch of eggs. The figures given in Table 5.

TABLE 5.

Total calcium and magnesium content of Xenopus embryos after ashing : expressed in micrograms per embryo. Accurate to ± 0·02 micrograms.

Stage.	Calcium	Magnesium
	6·01	
	5·66	0·11
Late blastula	5·22	0·12
	4·25	
	3·36	
	2·54	
	5·37	
	4·68	0·07
Early and mid-gastrula.	4·53	0·14
	3·27	
	2·12	
	4·00	
	3·62	0·12
Early neurula	2·55	
	2·28	
	2·01	0·09

suggest that the value fell from blastula to neurula stages during the development of any one batch.

Secondly the results of the measurements of the amount of calcium and magnesium removed by the EDTA show that the amount of these ions removed during disaggregation fell slightly from blastula to neurula. These values are shown in Table 16. Since the total amount of magnesium in the embryo is lower than the amount of calcium and magnesium removed in disaggregation it seems probable that little magnesium is removed in disaggregation. Figure 13 shows the amount of calcium and magnesium lost by the embryos during disaggregation as a plot of cumulative loss against time. Curves A and B refer to measurements made on the disaggregation of gastrulae with EDTA. Curves C and D were obtained by following the course of the loss of these ions with CCC; curve C refers to the disaggregation of a gastrula, curve D to a neurula. Disaggregation in all cases was visibly complete at 45-70 minutes. These curves indicate that once disaggregation was visibly complete the rate of loss of calcium and magnesium greatly diminished and fell to a value too low to be estimated by the methods used. However it was found that if the embryos had been decapsulated by the method of Spiegel (1951), in which papain is used, the loss of calcium and magnesium from disaggregated embryos in EDTA solution continued until almost the whole amount of these ions must have been removed, after a few hours embryos treated in this manner cytolysed. Whereas the disaggregated cells of embryos decapsulated by hand survived for several days in EDTA. These observations suggest that the calcium and magnesium content of the cells is divided into two portions. The smaller of

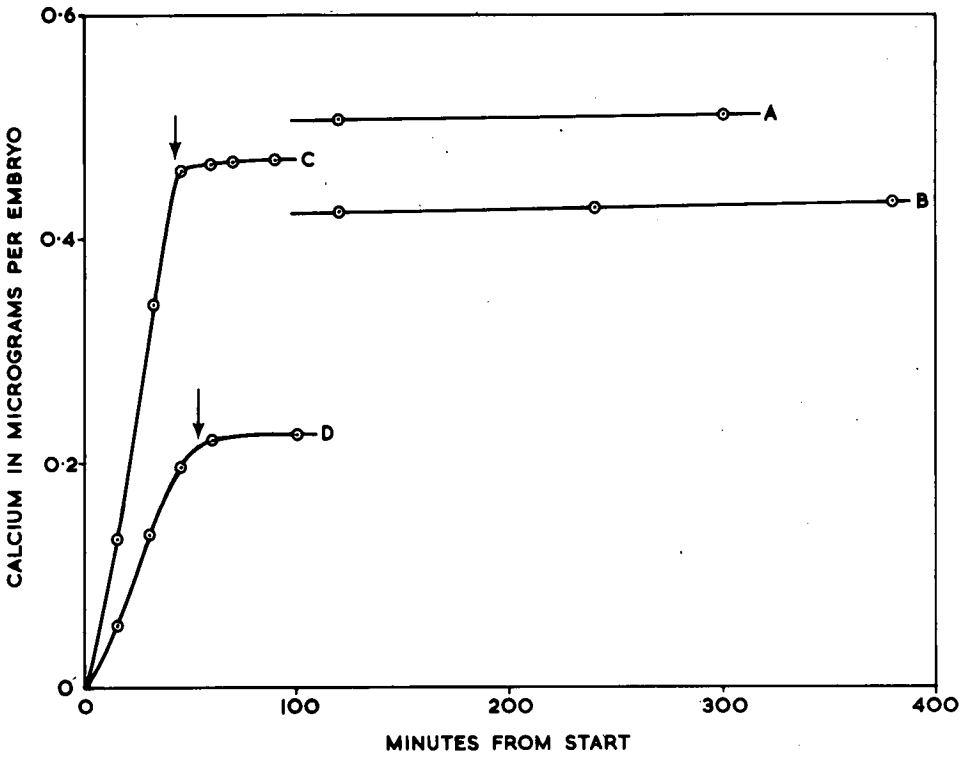


FIGURE 13.

Curves showing the cumulative removal of calcium and magnesium from Xenopus embryos during disaggregation. The arrows indicate that disaggregation was visibly complete at this time.

TABLE 6.

Amounts of calcium and magnesium (measured together) removed from Xenopus embryos by disaggregation with EDTA. Expressed in micrograms per embryo : accurate to ± 0·05 micrograms.

Late blastula.	0·25	0·53	0·38
	0·32	0·47	
Gastrula.	0·26	0·33	
	0·28	0·48	
Neurula.	0·32	0·25	0·36
	0·27	0·28	

these portions is easily removed by EDTA treatment, and its removal is accompanied by the disaggregation of the embryos. The larger portion is not affected by EDTA treatment and does not appear to be concerned primarily in cell adhesion.

The results of the measurements of the uptake of calcium ions by embryos prior to reaggregation are shown in figure 14. The cells took up calcium until the external concentration reached 0·0005M in calcium, above which concentration there was no further uptake. Figure 14 shows the results obtained for blastulae, mid-gastrulae, late gastrulae and early neurulae. Six other uptake curves have been obtained; two each for blastulae, early gastrulae and late gastrulae. These values give curves of similar form to those shown in Figure 14. The data from which these other curves may be plotted are given in Table 7. The cells on which these measurements were made reaggregated subsequently, with the exception of the neurula stages. The form of the reaggregates which developed in the higher calcium concentrations was somewhat abnormal, for cell sorting appeared to be partially inhibited.

The curves shown in Figure 14 are of a form which suggests that the uptake may be an adsorption process. For these curves, which are adsorption isotherms, are fitted by the Freundlich adsorption isotherm, which states that :

$$\text{the mass adsorbed} = (\text{constant} \times \text{external concentration})^{\frac{1}{n}},$$

where n is a number greater than 1.

Halsey and Taylor (1947) showed that a simplification of this isotherm may be achieved if a limit to the adsorption is set.

TABLE 7.

Uptake of calcium in micrograms per embryo by Xenopus embryos prior to reaggregation in media of various strengths of calcium (expressed in millimoles per litre).

	Medium	Uptake	Medium	Uptake
Late Blastula.	0•012	0•05	0•015	0•03
	0•048	0•08	0•053	0•09
	0•17	0•8	0•14	0•6
	0•42	2•8	0•38	1•7
	0•80	4•4	0•77	4•1
	1•51	4•5	1•46	4•3
Early Gastrula	0•010	0•02	0•017	0•04
	0•051	0•05	0•053	0•07
	0•16	0•6	0•15	0•7
	0•39	0•8	0•42	0•9
	0•81	0•9	0•91	1•1
	1•61	0•9	1•20	1•1
Late Gastrula	0•012	0•03	0•013	0•02
	0•062	0•06	0•049	0•08
	0•15	0•1	0•13	0•1
	0•35	0•2	0•37	0•2
	0•79	0•3	0•82	0•2
	1•62	0•3	1•69	0•2

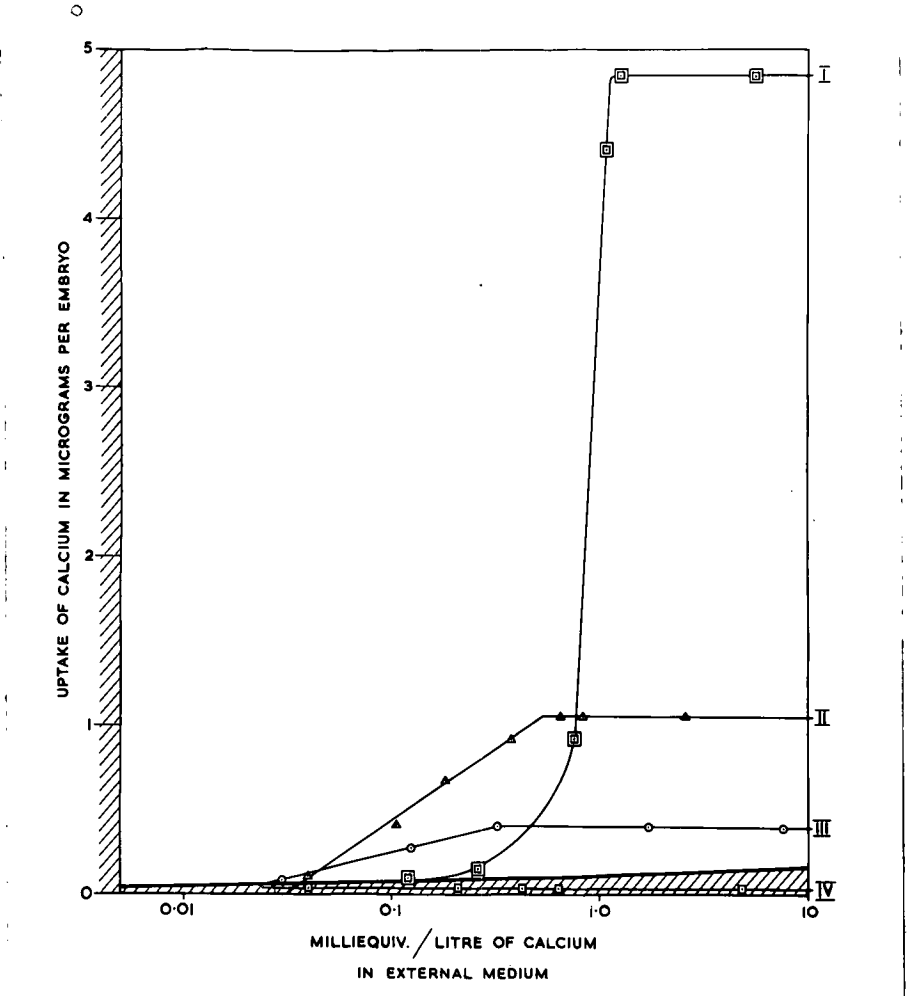


FIGURE 14

Curves showing the uptake of calcium by dissaggregated cells of Xenopus in media of various molarities of calcium. The shaded borders indicate the regions where the analytical methods are not sufficiently accurate for measurements to be made. Curve I relates to a late blastula, II to a mid-gastrula, III to a late gastrula, and IV to an early neurula.

By this means the Freundlich isotherm can be fitted to such curves as these which show an adsorption limit.

Discussion.

The uptake curves for calcium which have been obtained suggest the action of an adsorption process. The kinetics of this uptake do not necessarily prove the occurence of adsorption for certain permeation phenomena have similar kinetics. The extreme rapidity of the uptake and the low values for the outward permeation of calcium and magnesium revealed by the experiments on disaggregation suggest that this process is an adsorption one. More properly the process is probably one of chemisorption, for the adsorption appears to be fairly specifically confined to divalent cations.

The calcium and magnesium removed from the embryos during disaggregation may be located in an intercellular cement. But the calcium taken up in reaggregation is not likely to be held by an intercellular cement because disaggregation will have removed this. Moreover it seems unlikely that the cells can give rise to a new cement in the very short period which calcium uptake occurs. In the next part of this work some evidence for the existence of a cement between the cells will be given and for its removal during disaggregation.

The results of the investigation of calcium uptake agree with those on the reaggregability of the embryonic cells which are expressed in figure 2. For the cells become unable to readhere as their ability to adsorb calcium fails. In figure 2 it is shown that one can disaggregate stages of slightly greater age than can be reaggregated.

This difference may reflect slight changes in the mechanism of adhesion brought about by disaggregation, such as a slightly earlier loss of the ability to hold calcium to the surface than would occur in the intact embryo. But the development of the cells during the three hours or so that disaggregation and reaggregation require may bring the cells to the same stage of development as that at which disaggregation becomes impossible. It is for the same reason that it is impossible to say that the uptake curves relate to a particular stage. If the measurements were repeated at a lower temperature, when differentiation would be greatly slowed, but adsorption processes little reduced, it would be possible to remedy this matter. However it is uncertain that isolated cells develop at the same rate as those in whole embryos. The differentiation of the disaggregated cells would explain other observations such as that about 0.2 micrograms of calcium and magnesium can be removed from neurulae during disaggregation, yet cells of such stages will not readsorb calcium.

The uptake of calcium found in the blastulae and early gastrulae is greater in amount than that released on disaggregation of such stages. This may indicate that there are more calcium binding groups on the cell surfaces than ions to fill them in the normal environment of the embryo. Normally the embryo develops in its own environment fairly well isolated from the surrounding medium, which may contain very little calcium, by the jelly coat and vitelline membrane. The short while during which the embryos are in normal Holtfreter solution prior to disaggregation may not be sufficient

for appreciable adsorption of calcium.

It has been argued from the results of the measurements of calcium and magnesium loss by disaggregated embryos that the permeability of the cells to these ions is low. However this does not necessarily argue that permeation in the opposite direction is as slight. Thus the uptake might be due to permeation. Moreover it has been suggested by Lansing and Rosenthal (1952) that the calcium content of membranes determines their permeability; and thus presumably their permeability to calcium. It may be that the complexing agent removes calcium from the surface of the cells faster than it can be replaced by permeation, and in consequence the permeability to calcium falls. If a weaker complexing agent were used the loss would continue longer for permeation could continue to supply calcium from the interior of the cell for a longer while. This possibility should be tested experimentally. However the rapidity of uptake of calcium suggests that permeation processes are not involved to any considerable extent.

In the first section of this thesis, which was on the subject of disaggregation, the question of the stability of calcium binding by the cell surface was raised. Although the binding appears to be due to chemisorption the concept of a stability constant for this binding still has a useful meaning in deciding about the action of complexing agents on the embryos. It was suggested that if the pH threshold for disaggregation lay at a value at which the stability constants for calcium binding by the surface (probably falling with pH rise) and for the complexing agent (rising with pH) were of similar value,

then one might conclude that disaggregation was mainly due to the complexing action of the agent. If it is assumed that each binding group can hold one calcium atom then the stability constant may be written:

$$k_1 = \frac{(\text{conc. of bound calcium})}{(\text{conc. free calcium in medium})(\text{conc. of free binding sites})}.$$

At half-saturation of the surface, figure 13 shows that a stage II embryo has bound about 0.5 micrograms of calcium, the external calcium concentration being 0.00005M. The embryos of this stage were found to contain of the order of five thousand cells, whose mean radius is of the order of ten micra. These figures were obtained from photographs of disaggregates but are not highly accurate. Such values allow the calculation of the concentration of bound calcium in the surface layer, which is assumed to be of the order of ten Angstrom units thick (Overbeek, 1949). At half-saturation there will be an equivalent concentration of free binding sites. The stability constant that is calculated from these results has a value of 20,000 at pH 7. This value is sufficiently high to make disaggregation by calcium-free media a very slow process. The pH threshold for disaggregation by EDTA is 7.8, at which the EDTA has a logarithmic stability constant of 7.3. At this pH the stability constant for the calcium binding will probably be lower than the logarithmic value of 4.3 measured at pH 7. This thousand or more fold difference of the stability constants at the pH threshold for disaggregation suggests that calcium binding is not the only factor that controls cell adhesion. Nevertheless it is realised that the evidence on which this value for the stability constant has been

calculated is uncertain in many respects. Moreover it is not known how the stability constant for the calcium binding altors with pH. For if it increases with pH, though not so rapidly as that of the EDTA, then the interpretation that disaggregation is mainly due to the removal of calcium is more likely.

The results indicate that calcium is chemisorbed onto the cell surface. They give support to the theory proposed earlier that the adsorbtion of calcium would lower the surface potential of the cells so much that attraction would occur between closely apposed cell surfaces, thus producing cell adhesion. Since this mechanism of adhesion appears to exist only in the blastula and gastrula stages it does not show specificities with regard to cell type for all types of cell adhere to one another. The failure of neurula stages to reaggregate with calcium suggests that another mechanism of adhesion develops at this stage, and the evidences of cell sorting suggest that this is specific with regard to cell type. In figure 2 it was shown that the ability to reaggregate in the presence of calcium failed at different stages for the various tissues. Future experiments might be done to see whether this failure is correlated with the loss of the ability to take up calcium by the respective tissues.

<u>The investigation of the existence of an intercellular cement.</u>

It has been pointed out that cell adhesion may be brought about by intercellular cements. Such cements occur in large quantities in many adult tissues, where they are frequently of mucopolysaccharide

nature, see Stacey (1946). In the earlier sections of this thesis it was suggested that the jelly formed between the cells during disaggregation in alkali might represent such a cementing material.

The distinction between intercellular cement and cell surface becomes very much a matter of definition when the amount of cement is small. In such circumstances the cement may be so closely apposed to the cell surface that in most respects it may be regarded as forming part of the cell surface. Disaggregation with EDTA does not produce a jelly material. It is possible that the jelly is due to a cytolytic reaction of the cells to the action of the alkali, which is not produced by EDTA. Otherwise it may represent a cement which is not affected by the treatment with EDTA and remains on the cell surface, or is dissolved by the EDTA. If the material remains on the cell surface the process of calcium uptake, which has been described as chemisorption, may be an absorption of calcium by a layer of cement. In such a case the cement might be regarded as being part of the cell surface. The possibility that the cement is dissolved by the EDTA has been investigated. It should be pointed out that if a material is found to be removed from the embryos by EDTA, it may only be the result of some cytolytic reaction.

The results of the investigations of the nature of the jelly, mentioned earlier, showed that it formed in alkaline disaggregation, and dissolved below pH 9·2 in the absence of calcium ion (see p 21). It was found to stain strongly with celestin blue. Such staining suggests but does not prove the presence of nucleic acids (Clark and

Powers, 1953). Originally the results of the experiments with hyaluronidase were interpreted to indicate that the jelly was of a mucopolysaccharide nature. In consequence disaggregates made in alkali were fixed and prepared for histological staining. The sections were stained by the periodic acid-Schiff method, which stains the aldehyde groups of sugars. The jelly material did not stain, which suggests that the jelly does not contain any polysaccharide material. It was later found that the hyaluronidase was contaminated with ribonuclease. Pure ribonuclease also attacked the jelly, as did trypsin. These results suggested that the jelly contained ribonucleic acid and protein components.

In view of the above evidence it was decided to examine the EDTA which had been used for disaggregation to discover if it contained ribonucleic acid and protein components.

Methods of analysis.

Late gastrulae of X. laevis were disaggregated in 0.001M EDTA at pH 8.03 in calcium-free Holtfreter solution buffered with trihydrochloric acid (0.001M in tris). Groups of from 20 to 30 embryos were used. The embryos were gently agitated on a rocker during the disaggregation for 40 minutes. At the end of this period the embryos were completely disaggregated into separate cells. These cells were allowed to settle to the bottom of the dish and the supernatant removed. No sign of cytolysis of the cells could be found. The supernatant was centrifuged at 300-400 gravities for 10 minutes. A small quantity of intact cells and a few yolk-platelets formed the sediment at the bottom of the centrifuge tubes. These

yolk-platelets are probably of extracellular origin, see earlier, and if this is so do not indicate cytolysis. The clear supernatant from this centrifugation was examined spectrophotometrically. A typical absorption curve is shown in figure 15. The form of this absorption curve suggests the presence of a nucleic acid component, see Chargaff and Davidson (1955).

It was then decided to attempt a more exact characterisation of this component. Four batches of from 250-260 late gastrulae of X. laevis were disaggregated by the method described above. The supernatant medium was removed and treated in the same manner. It was dried under reduced pressure at -4°C. The samples prepared in this manner contained the salts of the medium, the EDTA and tris and the component or components being studied.

These samples were analysed in the manner described below. The amounts of EDTA and tris in the material could be calculated from their concentration in the weight of solution evaporated. Thus the amount of nitrogen contained in the EDTA and tris could be separated from that due to the unknown material when the total nitrogen content was estimated by the method of Lubochinsky and Zalta (1954) after the material had been given a micro-Kjeldahl digestion. The phosphate content was measured by the method of Briggs (1924) after the material had been digested in concentrated sulphuric acid. Ribose was estimated by the orcinol reaction and deoxyribose by the method of Dische, (as given by Chargaff and Davidson, 1955). The α-amino nitrogen content was measured by the method given by Colowick and Kaplan (1957), which uses the reaction with ninhydrin for estimation.

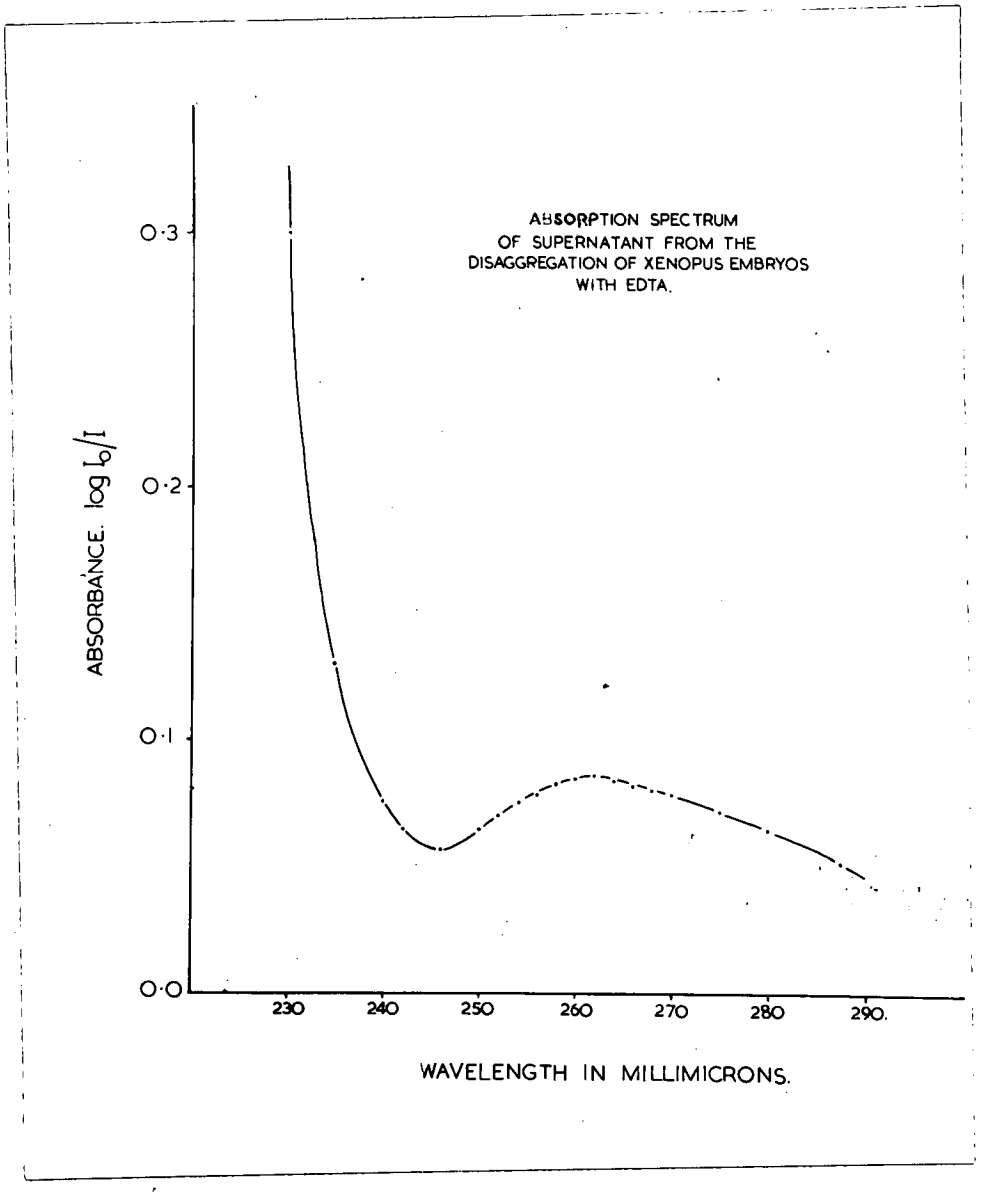

ABSORPTION SPECTRUM
OF SUPERNATANT FROM THE
DISAGGREGATION OF XENOPUS EMBRYOS
WITH EDTA.
ABSORBANCE. log Io/I
0·3
0·2
0·1
0·0
230
240
250
260
270
280
290.
WAVELENGTH IN MILLIMICRONS.

FIGURE 15.

The results of these analyses revealed that all the components sought with the exception of deoxyribose were present. Their quantities were such that by calculation the material appeared to be a ribonucleoprotein containing about 25% nucleic acid. The ribose-phosphate ratio was found to be 0.97. In calculating the percentage of nucleic acid in the material it was assumed that the amino-acids forming the protein chain had an average molecular weight of 140 (see Block and Weiss, 1956) and that the proportion of the four nucleic acid bases were identical so that they had an average molecular weight of 278.

The nucleic acid bases were separated and identified by chromatographic methods. A sample of the material was hydrolysed with 70% perchloric acid for one hour at 100°C. The hydrolysate was spotted onto Whatman No. 1 paper. A descending chromatogram was run in isopropanol- hydrochloric acid -water (170:41:to 250 v/v). Adenine, guanine, cytosine and uracil were recognised from their positions with respect to markers, and from the ultra-violet absorption curves of eluates of the spots from the chromatogram. The extinction values of the various bases account for sufficient nucleic acid to form 25% of the nucleoprotein. This value is in agreement with that obtained from the 260/280 millimicron absorption ratio for the untreated material, which indicates that there is more than 20% nucleic acid present in the nucleoprotein; this method was introduced by Warburg and Christian (1941). Figure 16 shows the curve from which this value was obtained.

A sample of the material was examined for its amino-acid content.

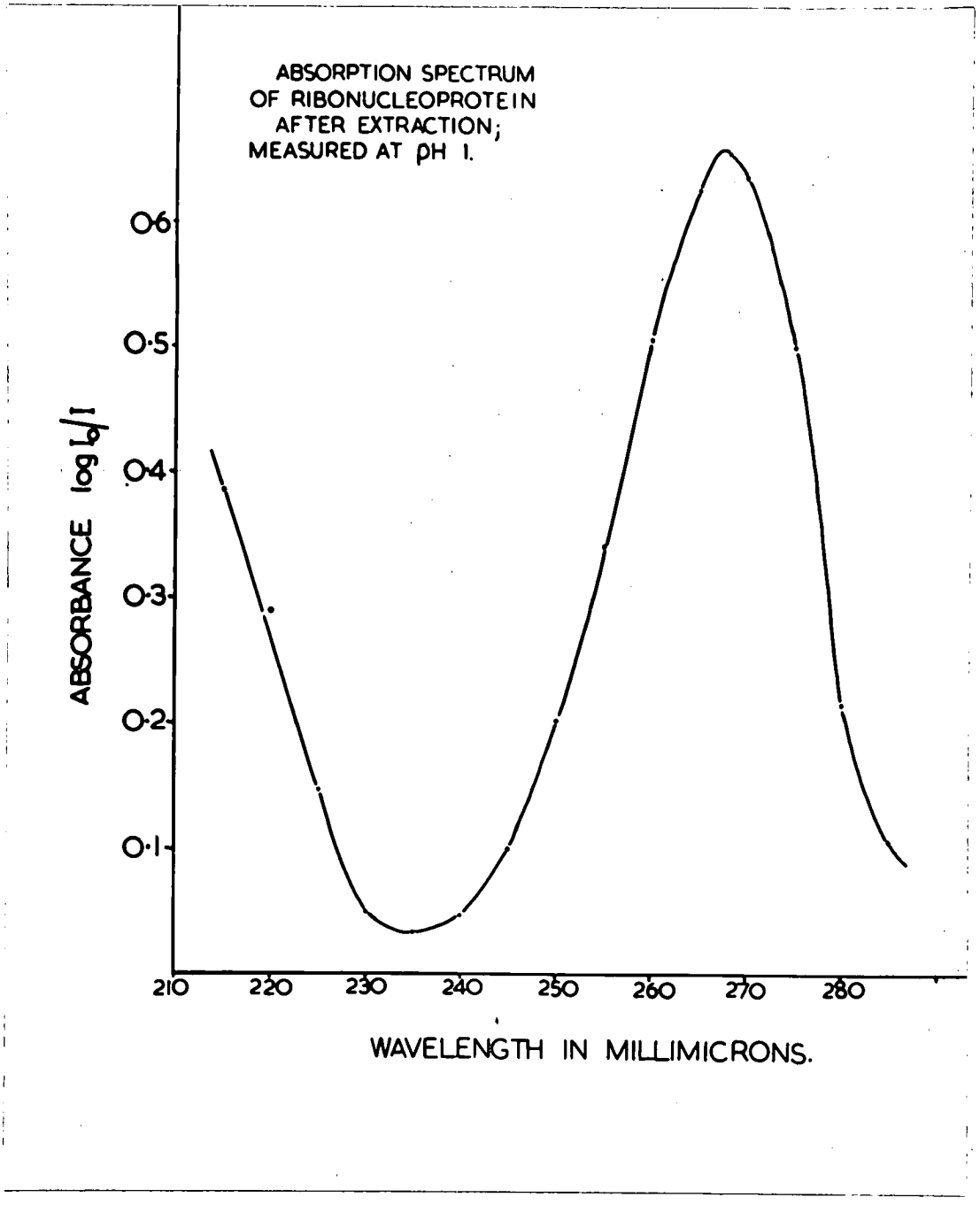

ABSORPTION SPECTRUM
OF RIBONUCLEOPROTEIN
AFTER EXTRACTION;
MEASURED AT pH 1.
ABSORBANCE log I0/I
0·6
0·5
0·4
0·3
0·2
0·1
210
220
230
240
250
260
270
280
WAVELENGTH IN MILLIMICRONS.

FIGURE 16

It was hydrolysed in 6N hydrochloric acid for six hours. A chromatogram of the hydrolysate was run on Whatman No. 1 paper with phenol saturated with water. Qualitatively arginine, histidine and tyrosine have been recognised from their R_f values and from specific reactions. Exact identification of the remaining amino-acids is yet to be done.

A yield of 4 micrograms per embryo of the nucleoprotein was found.

Discussion.

The results of the analyses indicate that ribonucleic acid and protein are present in this material in a ratio of 1:3. The fairly constant proportion of ribonucleic acid to protein which has been found in the four batches examined suggests that they form a true ribonucleoprotein in the embryo. But it should be realised that they may not be in combination on the living material. Until the matter has been further investigated it is probably most convenient to refer to the material as containing a ribonucleoprotein.

Since EDTA appears to remove this ribonucleoprotein from the embryos without damage to the cells it seems probable that it is located on the cell surface or inbetween the cells. It seems unlikely that such a large molecule would permeate out from the cell interior. The precautions taken against contamination by cytolysed cells in the preparation makes a derivation of the nucleoprotein from such cells unlikely. Brachet (1950) points out that yolk-platelets contain some ribonucleoprotein. It is possible that a yolk-lysis reaction of the kind described by Gross (1954) occurs

during disaggregation, but this seems unlikely for intact platelets are found in the medium.

That the ribonucleoprotein is located in the cell surface or in-between the cells is supported by the observations of Brachet (1957), who has observed that gastrula stages stained with methyl-green pyronin show staining of a ribonucleic acid component on the extreme periphery of the cells. Similar observations have been made on embryos stained by this method in the course of this work. If the ribonucleoprotein is located in this position it may be identical with the jelly material. It has already been shown that the jelly behaves in its response to the enzymes trypsin and ribonuclease as though it were a ribonucleoprotein. The insolubility of the jelly in the presence of calcium ions suggests that it may be a nucleoprotein, see Ambrose and Butler (1953).

The results of the analysis of the material suggest that all the nitrogen found is accounted for by the ribonucleoprotein. In making this conclusion it is assumed that the protein has a fairly average composition of amino-acids for this class of compound (see Block and Weiss, 1956). In consequence it seems probable that if any other organic material is extracted by the EDTA treatment it cannot contain much nitrogen. This premise restricts the search for other compounds mainly to the saccharides. Until the analysis of the amino-acids is carried out quantitatively it will not be known whether this assumption is true or false.

General discussion of the relations of calcium uptake and the ribonucleoprotein to cell adhesion.

The problem remains of the function of this ribonucleoprotein or ribonucleoproteins, for it has not been shown that it is one molecular species, in the embryo. Brachet (1950) has drawn attention to the function of the microsomes, which contain much ribonucleoprotein. From a number of lines of evidence he claims that they play an essential part in the mechanism of induction. Brachet, Ruusi, and Gothie (1952) have produced evidence that these microsomes may be transferred from the cells of the inducing mesoderm to the overlying ectoderm. Such an occurence would explain the discovery of ribonucleoprotein on the cell surface or between the cells. Waddington and Sirlin (1955) could not find evidence for such a transfer of microsomes.

Yamada (1950) noticed that the presumptive mesoderm of amphibian gastrulae was neuralised by the action of alkalies, with the formation of a jelly material during the action of the alkali. If this jelly is of similar nature to that described in this work it might be a ribonucleoprotein; in which case one might expect it to have the observed inductive action. Niu (1956) has shown that the inductor material of T. torosus gastrulae releases a substance having many of the properties of ribonucleoprotein when it is cultivated in hanging drop cultures. Induction occurred in such cultures in the absence of contact between the tissues. He obtained evidence for the existence of two or more classes of inductor. Consequently it

seems likely that the ribonucleoprotein described in the present work is of similar origin to those reported by Brachet, Niu, etc.

Since the ribonucleoprotein appears to be similar to those claimed to have an inducing action by the authors mentioned above, it is planned to investigate its inducing action. It has been pointed out that it may represent several ribonucleoproteins. If this is so they might represent the various regionally specific evocators proposed by Toivonen (1950), Nieuwkoop (1952) and others. Resolution of the various possible component ribonucleoproteins and testing of their evocating action might assist the solution of the problem of whether there are or are not specific evocators. However Waddington (1952c) has pointed out that the effects of regional specificity may be due to the tendency for the various portions of the embryos to individuate in their own specific manner.

Without disclaiming the possibility of an evocating action for the ribonucleoprotein other functions which it might have can be suggested. Lansing and Rosenthal (1952) and Mazia (1940) have put forward the idea that the presence of ribonucleic acid on the cell surface determines its power to bind calcium. If the ribonucleoprotein that has been found is regarded as forming a part of the cell surface, which can be removed in certain circumstances without cytolysis, the adhesion of cells might be attributed to the calcium binding by this material. If it is to be regarded as an intercellular cement its stability and adhesiveness might also be attributed to the binding of calcium. However the conclusions of Lansing and Rosenthal and of Mazia are based on the association of these substances in the cell

surface,which is not a necessary proof of their combination. Rothstein and Meier (1951) showed that the cell surface of yeast bound uranium very strongly and calcium less strongly. This binding was attributed to the presence of polyphosphate groups. Nucleoprotein provides a rather similar structure.

Earlier it was suggested that the stability constant of the calcium complex with the cell surface probably declined with pH. This interpretation was advanced to explain the spontaneous disaggregation in calcium-free media above pH 9.6. Thus the value for the stability constant for the calcium binding measured at pH 7.0 was regarded as indicating that other factors as well as calcium binding were required to maintain the adhesion of the cells up to pH 7.8 in the presence of EDTA. If however the stability constant rose with pH, the threshold for disaggregation with EDTA might be solely due to competition between the cell surface and EDTA for calcium. Katz and Klotz (1953) have shown on theoretical grounds that the affinity of proteins and other substances of amphoteric nature for cations generally increases with pH,and demonstrated this experimentally for the calcium binding of serum albumin,as has Nanninga (1957) for that of meromyosin. Schneider (1946) and Gjessing (1951) found that ribonucleoprotein binds calcium and magnesium and gave evidence which suggested that these complexes are most stable at pH 6 to pH 7.

In view of this evidence it seems that the assumption made earlier about the effect of pH on calcium binding was wrong. Thus it seems

possible that the pH threshold for disaggregation with EDTA may be explained in terms of the competition between the EDTA and the cell surface for the calcium ions. Until measurements of the calcium stability constant are made at a number of different pH values it will be impossible to decide whether other mechanisms of cell adhesion occur in this material. Katz and Klotz point out that calcium binding by proteins increases with pH up to pH 10 to 11. Yet the embryos are disaggregated in calcium-free media above pH 9·6. If the calcium is bound by protein this fact would suggest that other mechanisms beside calcium binding are important in cell adhesion. However the results of Schneider and Gjessing suggest that the ribonucleoprotein complex with calcium is most stable in the range pH 6 to 7. This range is that in which reaggregation occurs and is the one in which embryos are very resistant to disaggregation. That the stability constant of such complexes falls on either side of pH 6 to 7 accounts for the spontaneous disaggregation above pH 9·6 and the failure of reaggregation below pH 6. The results of Schneider and Gjessing are incomplete and refer to a different ribonucleoprotein from that isolated in the present work. Nevertheless such results suggest that the binding of calcium by a ribonucleoprotein would account for the observed pH limits for disaggregation and reaggregation. Moreover a ribonucleo-protein material has been identified in those embryos in a

suitable location for action in cell adhesion. The action of ribonuclease and trypsin, which prevent readhesion of the cells, supports this hypothesis. McQuillen (1950) has given evidence that some bacterial surfaces bear a nucleoprotein material upon themselves. Further investigation of the calcium binding by the cell surfaces and by the isolated ribonucleoprotein at various pH values is planned. The results of such work may allow the testing of the hypothesis that cell adhesion is due to calcium binding by a ribonucleoprotein during these blastula and gastrula stages.

There remain two objections to this hypothesis. Firstly how is it that embryonic cells continue to adhere to one another in media as acid as pH 2? Does another mechanism of cell adhesion act at these pH values, even if it is not one normally used in the life of the cells? Secondly it was found that cells which had been disaggregated in EDTA showed an immediate and rapid uptake of calcium. Yet disaggregation has removed a considerable amount of ribonucleoprotein. Is sufficient left on the cell surface for the readsorption of calcium? Or is it resecreted sufficiently rapidly for the observed uptake to occur, though this possibility seems unlikely? Or is it possible that calcium binding and the ribonucleoprotein are unrelated save in that they are both distinctly and separately necessary for cell adhesion? If the ribonucleoprotein is slowly restored to the

cell surface after disaggregation it would account for the fact that, although calcium uptake is rapid, the development of readhesions between the cells is delayed for an hour or so. Such objections form a ground for future work. However considerable evidence has been given for the hypothesis that cell adhesion is due to the surface adsorption of calcium ions. A similar mechanism was proposed on theoretical grounds by Schmitt (1941).

Another matter for further enquiry is raised by the sudden death of reaggregates made from cells separated with EDTA at three to four days of age. Does this represent a delayed effect due to the EDTA complexing metals within the cells which are necessary for the functioning of their enzyme systems?

CONCLUSIONS

The nature of the mechanism of cell adhesion has been examined in amphibian embryos. It has been investigated in terms of the phenomena of disaggregation and reaggregation which represent the loss and regaining of adhesion. The main conclusions of the work described in this thesis are :

(i) The loss of adhesion between cells is connected with the removal of calcium from the cells, and only occurs in certain pH ranges.

(ii) Cells of blastula and gastrula stages form their first adhesions in reaggregation without regard to the types of cell they are in contact with.

(iii) The readhesion of cells of these stages is preceded by an uptake of calcium, whose kinetics suggest surface adsorption.

(iv) A ribonucleoprotein material is concerned with the readhesion of the cells of these stages. It is suggested that it may be concerned with the binding of calcium to the cell surface, and that it may be identical with the jelly material seen in certain disaggregates.

(vi) The cell surface of preblastular stages is not stable in the absence of calcium but becomes stable to such conditions during blastular stages. In neurular stages the ability of the cell surface to adsorb calcium ceases. This failure of calcium adsorption occurs at the same time as a general change in the mechanism of adhesion of the cells. Subsequently the mechanisms of cell adhesion become selective. There thus appear to be at least two different and successive mechanisms of cell adhesion during the development of the embryo. The first mechanism occurs during blastular and gastrular stages and is much concerned with the presence of the calcium ion, the second occurs in later stages and is of a different nature. The first mechanism does not appear to show any differentiation from one cell type to another. The second mechanism appears to be concerned with the selective adhesion of the various cell types.

(vii) In order to account for the phenomena of cell adhesion occurring in the blastular and gastrular stages it has been suggested that adhesion may be due to the attraction which exists between surface when they carry little electrical charge. The nature of the uptake of calcium, the pH thresholds for disaggregation and reaggregation and other phenomena have been shown to fit the expectations of such a theory.

APPENDIX I.

Technical description of the films.

Film No.

1. Xenopus gastrula disaggregated at pH 9·8. 3hrs. filming with 2 sec. exposure and 10 sec. interval. Field 540 by 760 micra. No reaggregation.

2. Xenopus gastrula disaggregated with EDTA. 4 hrs. filming with 2 sec. exposure and 10 sec. interval. Field 570 by 803 micra.

3. Xenopus gastrula disaggregated with EDTA. 2¾ hrs. filming with 2 sec. exposure and 10 sec interval. Field 2000 by 2820 micra.

4. Xenopus gastrula disaggregated with EDTA. 2 sec. exposure for 10 sec. intervals for 4 hrs. and 132 sec. intervals for another 13 hrs.

5. Xenopus gastrula disaggregated with EDTA. 2 sec. exposure at 30 sec. intervals for 6½ hrs. Field 4500 by 6350 micra.

6. Xenopus gastrula disaggregated with EDTA. 2 sec. exposure at 30 sec. intervals for 14 hrs. Field 4500 by 6350 micra.

7. T.alpestris late blastula disaggregated with EDTA. 2 second exposure at 10 second intervals. Timer failed several times for periods of about 5 minutes. Field 570 by 803 micra.

8. T.alpestris blastula disaggregated with EDTA. 18 hrs. filming at 15 second intervals. Exposure 2 sec. Field 570 by 803 micra.

9. Xenopus gastrula disaggregated with EDTA. Filmed for 20 hrs. at 15 second intervals with 2 second exposures. Field 570 by 803 micra.

10. T.alpestris blastula disaggregated with EDTA. Filmed at 15 second intervals and 2 second exposure for 2 hours, when timer failed. Field 570 by 803 micra.

11. T.alpestris blastula disaggregated with EDTA. Filmed for 15 hours at 15 sec intervals and 2 sec. exposures. Field 570 by 803 micra. Little reaggregation.

12. T.alpestris blastula disaggregated with EDTA. Filmed under dark ground illumination with 2/3" objective and x 10 eyepiece with 1 sec. exposures and 5 sec. intervals for 5 hrs. Field 360 by 507 micra.

13. T.alpestris blastula disaggregated with EDTA. Filmed with phase contrast illumination, x 10 objective and x 10 eyepiece, at 5 sec. intervals and 1 sec. exposures. Field 180 by 253 micra. Four separate shots in this film.

14. T.alpestris blastula disaggregated with EDTA. Filmed in the same conditions as film 12.

15. T.alpestris early gastrula disaggregated with EDTA. Filmed with phase contrast illumination, x 10 objective and x 6 eyepiece, for 2 hrs at 5 sec. intervals and 1 sec. exposures. Timer failed after 2 hrs. Field 360 by 507 micra.

16 and 17. T.alpestris blastulae disaggregated with EDTA. Filmed with phase contrast illumination, x 10 objective and x 6 eyepiece, at 5 sec. intervals with 1 sec. exposures. Field 360 by 507 micra.

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