# OBSERVATIONS ON THE FORCES OF MORPHOGENESIS IN THE AMPHIBIAN EMBRYO

By C. H. WADDINGTON

Zoological Department and Strangeways Research Laboratory, Cambridge

(*Received* 15 *October* 1942)

(With Two Text-figures)

## INTRODUCTION

Recent years have seen considerable advances in our knowledge of chemical interactions between different parts of developing embryos, and of the metabolic processes by which the stimulating evocators are released. We have also acquired further information, on the biological level, of the correlations between parts which lead to the formation of units organized into definite patterns. On the other hand, the forces which actually bring about the changes in shape which are perhaps the salient feature of early development have remained almost unstudied. Several hypotheses have been put forward. Thus some years ago, Glaser (1914, 1916) suggested that the folding of the neural plate into a groove is due to a weakening of the inner surface of the neural epidermis followed by an imbibition of water; the swelling consequent on this is supposed to extend to weakened inner surface more than the stronger outer one, so that the cells are deformed into truncated cones, and the whole layer folds. This conception has recently been criticized by Brown, Hamburger & Schmitt (1941) on the basis of accurate density determinations, which failed to reveal any evidence of imbibition. They suggest that the shape changes are primarily due to 'an increase in the "attractive" forces between molecules in the adjoining cell surfaces of prospective neural tissue cells so that the area of contact is actively increased'. Without making any hypotheses as to the physico-chemical mechanisms involved, Holtfreter (1939) has also emphasized the importance of attractive or repulsive forces dependent on the surface properties of the cells; in his case he was concerned with interactions between masses of tissue differing from one another in histological type. Finally, several authors (e.g. Harrison, 1936; Needham, 1936; Waddington, 1940) have drawn attention to the possibility that the facts could be explained if we could postulate the formation of submicroscopic fibrils (an orientated cyto-skeleton) within the cytoplasm.

The present communication presents data which bear on these possibilities from a number of angles. It had been hoped to record the observations in a more elaborate and statistical form, but as it seems improbable that the work can be continued in the immediate future, it has seemed best to make an interim report on the material, which, as far as it goes, is fairly straightforward and unlikely to be substantially altered by more quantitative study.

## 1. THE ORIENTATION OF YOLK GRANULES

At first sight the most obvious way of testing the suggestion that there is an orientated cyto-skeleton would be by the use of polarized light. Observations of this kind have been made on the chick embryo by Hobson (1941); and there are of course many studies on the eggs of invertebrates, where, however, the anisotropy has not in general been corre-

lated with form changes. In the amphibian embryo, this simple method cannot be satisfactorily used owing to the presence of large numbers of highly refractive yolk granules. These effectively obscure any double refraction which might be shown by the cytoplasm. The only recorded exception to this is in the long flask-shaped cells lining the early blastopore lip, where Waddington & Picken (cf. Waddington, 1940) observed a weak double refraction in the terminal processes, which are free of yolk, probably because they are too thin to contain the granules.

The presence of yolk granules, although it prevents the use of polarized light, can itself be made the basis of a method of studying a possible cyto-skeleton. The granules in most species of Amphibia are not perfect spheres, but are somewhat elongated ellipsoids. If there is a strongly orientated fibrillar structure in the cytoplasm, it would be expected that the granules would also be orientated with their long axes parallel to the fibrillar direction. This possibility has been investigated in a number of situations in which it might be expected to occur in embryos of *Triton alpestris* and *taeniatus*.

### (*a*) *In the mitotic spindle*

It is becoming generally accepted that the mitotic spindle is a tactoid in which fibrous protein molecules are orientated in parallel (reviews by Darlington, 1939; Waddington, 1939*a*). The conditions are therefore such as should lead to the orientation of yolk granules. The spindle is, however, normally empty of granules, being formed of clear cytoplasm partly derived from the nucleus. Only rare cases of included granules have been found. In all these the orientation of the granules has been very exact, the long axis being parallel to that of the spindle. There seems no doubt therefore that yolk granules do become orientated if they lie in a region of cytoplasm which has a strongly fibrillar structure.

### (*b*) *In the immediate neighbourhood of an elongating spindle*

The metaphase spindle in amphibian embryonic cells is rather short and broad. During anaphase it elongates considerably, pushing out in two directions through the yolky cytoplasm. If there was any pre-existing orientated skeleton in this cytoplasm, or if the cytoplasm was highly viscous, one would expect the elongating spindle to cause an orientation of the yolk granules in its immediate neighbourhood. This does not seem to occur. At metaphase the ends of the chromosomes protrude in a haphazard way into the cytoplasm, lying between and amongst the yolk granules. In spite of this, no evidence of orientation is visible in the granules immediately against the sides of anaphase spindles.

### (*c*) *In the flask cells of the young blastopore*

In the two situations just mentioned, orientation might have been expected within a cell, but this would not necessarily have had any particular relation to morphogenetic developments. In this and the next two sections, evidence will be presented as to the orientation in cells whose shape is definitely connected with morphogenesis.

As has already been mentioned, the cells lining the early blastopore in the young gastrula become drawn out into long 'flask-shaped' ovals, with a thin neck reaching down to the blastopore from the thicker body which lies farther within the embryo. In the thinnest ends of the necks, no yolk granules are present, and the material has been shown to be anisotropic. In sections, however, the yolk granules within the body of the cells are found not to be orientated to a noticeable extent (Fig. 1*a*). The granules which

lie farthest down in the neck may, indeed, have their long axes parallel to that of the cell but this seems only to be true when they are so narrowly confined that there is no room for them to lie in any other direction. Farther inwards, in the wider regions of the cell, the granules seem to lie completely at random. A full statistical analysis would be necessary to establish this strictly, but at least the appearances make it clear that any orientation which occurs is very much less than could account for the elongation of a more or less spherical cell into the long thin flask shape.

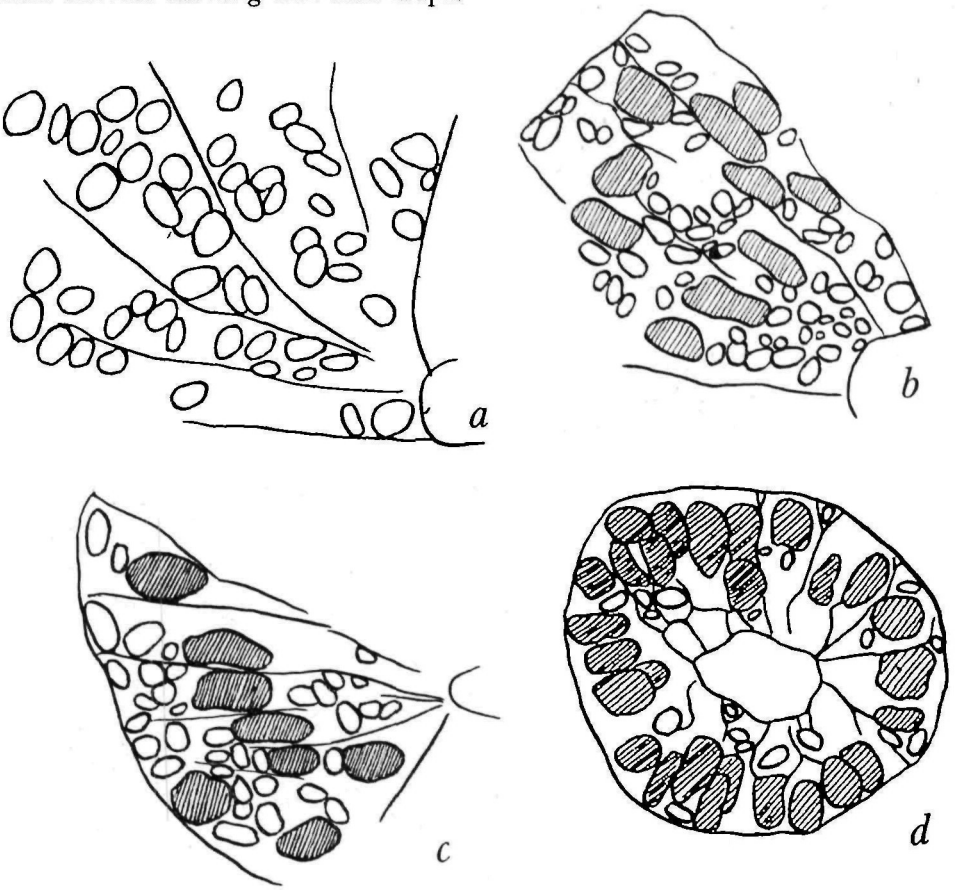


Fig. 1. *a*, Camera lucida drawing of the terminal ends of some 'flask-cells' extending down to the early blastoporal groove at the bottom right. Yolk granules indicated by hollow ovals, nuclei lie further towards the centre of the egg and are not visible. *b* and *c*, Similar drawings of groups of cells from the just closed neural tube; nuclei hatched. *d*, Lens vesicle shortly after becoming free from the corneal epithelium.

### (*d*) *In the neural groove*

The changes of shape in the cells forming the neural plate, groove and tube have not yet been adequately described. Many authors have drawn attention to the elongation of the originally cuboidal cells of the ectodermal epithelium into a columnar shape, which then narrows on the outer surface to form a thin truncated cone. These changes do not, however, take place simultaneously over the whole area of the neural plate. In particular, the first change from a column towards a cone, which initiates the folding of the plate, occurs towards the edges of the neural area. The externally visible effect correlated with

this is the elevation of the neural ridges, outlining the still unfolded neural plate. It appears probable that a detailed study of the cell changes proceeding during these early stages in the folding would limit the types of hypothesis which could be advanced to account for the phenomena.

At present, however, we are concerned only with the orientation of yolk granules within the elongated columnar or conical cells. The granules in these cells appear to be in process of fairly rapid utilization, and they are not always easy to distinguish; the well-formed granules are accompanied by masses or more or less amorphous yolky material which is partially digested. The granules whose shape remains definite have been drawn in the figures, the other material being omitted (Fig. 1 *b*, *c*).

It is apparent that there is no noticeable orientation even in the very elongated cells of the early closed neural tube. In these cells the nucleus is usually long and oval in shape. Its long axis always lies parallel to that of the cell as a whole. It might be suggested that this is evidence that the cytoplasm in which the nucleus lies has an orientated structure. It is clear, however, that the cells are often so narrow that the nucleus could not be fitted in in any other way. The orientation of the nucleus can probably be more plausibly considered as a direct consequence of constriction due to the narrowing of the cell.

### (*e*) *In the lens*

During the first stage of the formation of the lens in *Triton* the cells of the inner layer of the ectoderm elongate, forming a single-layered columnar epithelium. This then folds inwards, and becomes cut off as a small vesicle. Both in the columnar and the vesicle stages, the individual cells are considerably longer and narrower than in the original epithelium. No trace of orientation of yolk granules can be found (Fig. 1 *d*), except for such as are confined in very narrow spaces between cell surfaces; in such cases the orientation can be attributed to constriction by these surfaces rather than to a cytoplasmic micro-structure.

By the time the lens fibres are formed, the yolk granules have disappeared.

## 2. THE SHAPE OF CELLS IN MOVING CELL STREAMS

The investigation of early development by means of marks made with vital dyes has revealed the frequent occurrence of considerable translocations of cells by means of streaming movements. Very little is known about the physical forces involved in these movements, although preliminary measurements of their order of magnitude have been made by Waddington (1939*b*). Hypotheses as to the nature of the forces have to take account of the fact, recorded by many authors (e.g. Spemann, 1931; Waddington, 1942), that small packets of cells, transplanted into other regions, retain specific capacities to move in definite directions. This argues for the existence of some fixed polarity in the cells.

A conceivable basis for such a polarity would be a fibrillar organization of the cytoplasm, with orientation of the fibrils. A cyto-skeleton of this kind might have visible effects either on the orientation of the yolk granules or of the cell shape. Fixed and stained preparations of streaming regions of amphibian embryos have therefore been examined in an attempt to detect such effects; the material used was the roof of the primitive gut of a mid-gastrula and the overlying presumptive neural tissue (Fig. 2*a*, *b*). No evidence of any orientation of yolk granules could be detected. Moreover, as the

figures show, the cells are sensibly iso-dimensional, with no marked elongation in the direction of movement. Even the mitoses are not regularly orientated, and this is true not only in the tissue some distance away from the lip of the blastopore, but in the cells in its immediate neighbourhood, where the movement is most vigorous.

### 3. THE TENSILE STRENGTH OF THE CELL SURFACES

It is clear that alterations in the surface forces of the cells of different regions of the embryo, or of different parts of single cells, might produce changes in the shape of tissues or organ rudiments. No systematic attempts to detect such alterations seem to have been made. Almost our only source of information as to the surface forces in embryonic amphibian cells is the investigation of Harvey & Fankhauser (1933). They measured the tension at the surface of the newly fertilized egg of *Triturus* (*Diemyctylus*) *viridescens* by observing the flattening of the egg under the influence of gravity. The value

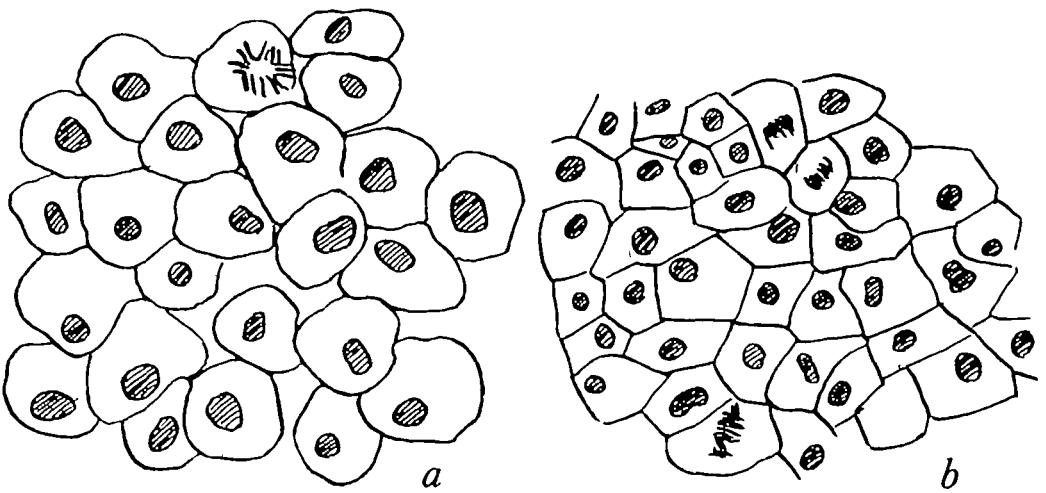


Fig. 2. *a*, A group of cells from the roof of the primitive gut of a mid-gastrula; they are moving in the direction of the top of the page. *b*, A group of cells of the presumptive neural plate overlying the last; the outline of the intercepts of the cells with the surface of the gastrula is indicated; the cells are moving towards the bottom of the page.

obtained was extremely low, less than 1 dyne/cm. Similarly, low values are typical of 'naked protoplasm' in general (cf. Newton Harvey, 1937). It cannot, however, be assumed that the surfaces of embryonic cells can always be considered to fall under the heading of 'naked protoplasm'. For instance, the newly fertilized eggs were studied by Harvey & Fankhauser immediately after they had secreted from the surface a fertilization (or vitelline) membrane whose strength is of quite a different order of magnitude. There is no reason why the cells should not in later development produce similar surfaces, whose strength might thus be considerably greater than that measured on the stripped egg.

Casual observations made during the normal operations of experimental embryology suggest that something of the kind may be true. Small fragments of tissue being transferred from place to place in pipettes sometimes come in contact with the water-air interface. Fragments of young embryos are immediately torn to pieces by the surface forces, but the tissues of much older embryos (late tail-buds and swimming stages) are

obviously tougher, and may be quite uninjured. This suggests a possible method of investigating the tensile strength of the cell surfaces.

Two methods have been used for testing the reactions of cells to applied surface forces. In the first, fragments of tissue were lifted by needles into the air-liquid interface of solutions of saponin, whose surface tension had been measured. In the second, the tissues were brought into the interface between water and various organic liquids. The embryos used are *T. taeniatus*.

After preliminary tests of a number of different saponin concentrations, solutions in tap water were made up of the dilutions $2 \times 10^{-5}$, $10^{-5}$ and $5 \times 10^{-6}$ by weight. The surface tensions of these were measured by pulling a clean platinum wire through the surface. The values obtained (three measurements on each solution) were: solution 1, 50·4 dynes/cm.; solution 2, 55·8 dynes/cm.; solution 3, 60·6 dynes/cm. The tap water, measured at the same time, gave a value of 73·6 dynes/cm.

At the surface of all three solutions, naked newly fertilized eggs broke immediately and completely, as might be expected. In unfertilized eggs which had been kept at room temperature for 3 days, the surface had become extremely solid, and was not disrupted at the surface of any of the solutions or even of tap water.

Tissues of embryos ranging from young gastrulae to early tail-buds were studied. Tissues from all regions, in all stages, were broken on the surface of solution 3, with the highest surface tension. Differential behaviour was, however, shown with respect to the other two solutions with lower tensions. The ectoderm of the mid-gastrula was broken, though rather slowly, on solution 1, and rapidly on solution 2. By the early neural plate stage, the neural plate itself survived on solution 1, though the epidermis broke slowly. In the neural groove stage and later, both neural and epidermal tissues survived on this solution. Beginning in the open neural plate stage, a most interesting differential reaction of the neural tissue to solution 2 began to be manifested. If transverse sections of the neural tissue were carefully lifted into the surface, it was apparent that the inner surface of the neural tissue broke more readily than the outer. The difference was particularly marked in the neural groove stage, at which the inner convex surface broke down slowly while the outer concave surface survived uninjured. By the early tail-bud stage, neither surface of the neural tube was broken on solution 2, although the tension was sufficient to tear open the just-closing neural ridges, and flatten the tube into a shallow groove.

The epidermis was throughout slightly weaker than the neural tissue. The mesoderm was weaker still, but survived on solution 1 by the early tail-bud stage. The endoderm was the weakest tissue of all, failing on solution 1 even in the tail-bud stage. These observations are summarized in Table 1.

In using the second method, the interfacial tensions between tap water and the various liquids was not directly measured, but reference was made to physical tables (Landolt-Börnstein, 1912) where values of sufficient accuracy could be obtained. It was clear that the tissues used in these experiments were very rapidly injured by the quantities of the substances which dissolved in the water, and all tests were therefore made as quickly as possible; even so, probably no great reliance should be placed upon them, except as confirming the general picture emerging from the experiments with saponin solutions.

In general, tissues raised into the water-organic solvent interface broke at lower surface

tensions than those recorded for the saponin solutions. Thus all tissues tested broke or the water-carbon tetrachloride interface, with a tension of about 45 dynes/cm., although the neural tube of tail-bud embryos was only slowly disrupted. This neural tissue survived on the water-benzene interface, with a tension of about 35 dynes/cm., but other tissues (epidermis and endoderm) broke. Rather surprisingly, the epidermis was broken even on the water-chloroform interface (about 25 dynes/cm.), but this had no effect on the neural tissue or notochord of mid-tail-bud stages. The epidermis survived the lowest

Table 1. *Behaviour of tissues in solutions* 1 (*surface tension* 50·4 *dynes/cm.*) *and* 2 (55·8 *dynes/cm.*)

| | Solution 1 | Solution 2 |
|---|---|---|
| Unfertilized egg fresh | Broke immediately | — |
| Mid-gastrula: | | |
| Ectoderm | Broke slowly | Broke quickly |
| Mesoderm | Broke, surface of primitive gut going slowly | Broke quickly |
| Endoderm | Broke quickly | Broke immediately |
| Early neural plate: | | |
| Neural plate | Survived | Lower surface broke more quickly than upper |
| Mesoderm | Broke slowly | |
| Endoderm | Broke quickly | |
| Neural groove: | | |
| Neural tissue | Survived | Difference between surfaces very marked |
| Epidermis | Survived | Torn off mesoderm, but broke only slowly |
| Early tail bud: | | |
| Neural tube | Survived | Torn open but cells not disrupted |
| Epidermis | Survived | Survived, but cut edges torn away from mesoderm |
| Mesoderm | Survived | Broke slowly |

tension applied, that of the water-ether interface (about 11 dynes/cm.). At the tail-bud stage, the endoderm was the only tissue which could not support this tension, and it was only slowly disrupted. In the earlier stages, however (open neural plate and younger), all tissues were broken on this interface.

## DISCUSSION

In the first two parts of this paper, phenomena were examined which might have been expected to reveal evidence of a fibrillar micro-structure of the cytoplasm. No such evidence appeared. The fact that yolk granules can be orientated in the mitotic spindle shows that such an orientation is possible in fibrillar surroundings. Its absence in the cytoplasm of cells undergoing changes in shape is therefore probably significant. At least it may be concluded that if any fibrillar cyto-skeleton is present, the forces exerted by the fibrils are much smaller than those in the spindle tactoid. It then becomes difficult to suppose that they can be strong enough to play any important part in bringing about the shape changes of morphogenesis.

The third part of the paper describes changes in surface phenomena which may be of more importance in this connexion. It should be said at the outset that the quantitative data are of an extremely rough nature. The surface of saponin solutions is known to be peculiar in many respects (Gaddum, 1932), since its tension depends on the length of

time it has been in existence and the rapidity with which it is stretched. The values for the surface tension given in the table were measured by a method which is not directly comparable with the phenomena which may be expected to occur when material is suddenly introduced into the surface, and therefore do not accurately represent the forces to which the embryonic cells were subjected. Moreover, it is possible that the saponin solutions had noticeable effects on the surfaces of the cells immersed in them; and this was certainly the case with the organic solvents used.

It can hardly be supposed, however, that the uncertainties in the measurements of the surface tensions of the saponin solutions affected the order of magnitude estimated, or the relative tensions of the different dilutions. The order of magnitude was almost a hundred times greater than the tensions measured in the naked surfaces of similar eggs by Harvey & Fankhauser. The greater strength of the cells of the later embryonic stages cannot be attributed to the effects of the saponin or the organic solvents, since these would be expected to diminish surface strength rather than increase it. It therefore seems justifiable to draw, from the present experiments, the first conclusion that the surface strength of cells in the gastrula and later stages is of an altogether higher order than that of 'naked protoplasm', being in the region of some tens of dynes per centimetre.

The second conclusion which seems justifiable is perhaps even more important. It is that the surface strength is not the same in all cells at all times. The evidence consistently indicates a gradual increase in strength throughout the gastrula to tail-bud stages. This affects all tissues, but not all equally. During these stages, the ectodermal tissues are always the strongest and the endodermal the weakest (in later stages the notochord seems rapidly to acquire considerable strength). Of the ectodermal tissues, the neural material is the strongest, and it shows behaviour which is perhaps the most interesting of all. The fact that the upper, concave, surface of the neural groove is stronger than the lower, convex, surface is exactly what would be expected if the surface tensions of the cells were responsible for the folding of the neural plate into the neural tube.

Although the evidence is thus in agreement with such a hypothesis, it cannot by any means be taken to prove it. In the first place, what has been measured is the passive strength of the cell surfaces against breakage by an applied force. In so far as the surface has any of the properties of a solid, this passive strength is not the same thing as the active force which the surface could exert to change the shape of the cell. In the second place, the greater strength of the concave surface might be a consequence rather than a cause of the folding. The folding, however it is brought about, is bound to involve a diminution in area of the cell surfaces abutting on the concave side, and this, for instance, by concentrating an ectoplasmic surface layer, might produce an increase in strength of these surfaces.

In the absence of any other well-established hypothesis, however, the suggestion that the folding of the neural tube is caused by changes of the surface properties of the cells remains an attractive one. The present investigation has been concerned only with the cell-water surface. In the embryo itself, forces arising in the cell-cell surfaces would also have to be considered. The relations between the forces in the two types of surfaces require much further study. Brown *et al.* (1941), who also tend to attribute neurulation to surface changes, have stressed the possibility that the primary force may be due to 'increases (in) the intercellular cohesion or "attraction", resulting in an increased area of contact between cells' They show that the area of contact does increase. This would

also be expected as a result of effective contraction of the cell-water surfaces on the upper side of the neural plate, if this was brought about by an increase in their active surface tension. And there is, of course, no reason why cell-water tensions and cell-cell tensions should not both be concerned; in fact, one might suppose a priori that both types of surfaces would undergo simultaneous, though not necessarily similar, changes.

The discussion has so far tended to minimize the importance of fibrization as a factor in morphogenesis. This may be premature. Although the evidence strongly suggests that no importance can be attributed to fibrization within the body of the cytoplasm, the alterations which have just been discussed in the cell surfaces may well depend on the state of fibrillar aggregation of their protein constituents. Something of the kind, in fact, would seem to be the simplest way of accounting for the remarkable polar properties of cells such as those undergoing the invagination movements. Fibrization taking place in the cell surface might cause an increase in strength; and a similar orientation of the fibres in contiguous surfaces might account for the polarity. The positive evidence for such a suggestion is still meagre, but perhaps some support may be found in the observations of anisotropy in the thin terminal processes of the flask cells of the blastopore (Waddington & Picken, see Waddington, 1940). These thin ends consist mainly of the superficial ectoplasmic layer, and the fact that they show double refraction probably indicates that this layer contains orientated fibrils.

## SUMMARY

1. The cells of early amphibian embryos contain ellipsoidal yolk granules. When a granule lies within the mitotic spindle, it is orientated with its long axis parallel to the direction of the spindle fibres. Yolk granules lying in the cytoplasm of cells are not specially orientated unless they lie in narrow spaces between cell surfaces; this is true in cells of the moving invagination streams, in the flask cells of the young blastopore, and in elongated cells of the neural groove and lens.

2. The breaking strain of cell surfaces was tested by bringing the cells into surfaces of known strength and observing whether they withstood the applied tension. The evidence indicates:

(*a*) That the breaking strain of cells from gastrula and tail-bud embryos is some tens of dynes per centimetre.

(*b*) That the breaking strain is not the same in all cells, being highest in neural tissue and lowest in endoderm during the stages mentioned.

(*c*) That the breaking strain gradually increases with age.

(*d*) That in the neural groove stage the strength of the outer concave surface of the groove is greater than that of the inner convex surface.

3. It is suggested that changes in surface tensions may be of importance in bringing about morphogenesis. The possibility is envisaged that the changes in tension may be connected with orientated fibrizations of the surface proteins.

REFERENCES

BROWN, M. G., HAMBURGER, V. & SCHMITT, F. O. (1941). *J. Exp. Zool.* **88**, 353.
DARLINGTON, C. D. (1939). *The Evolution of Genetic Systems.* Cambridge.
GADDUM (1932). *Proc. Roy. Soc.* B, **109**, 114.
GLASER, O. (1914). *Anat. Rec.* **8**, 525.
GLASER, O. (1916). *Science*, N.S. **44**, 505.
HARRISON, R. G. (1936). *Coll. Net.* **11**, 217.
HARVEY, E. N. (1937). *Trans. Faraday Soc.* **33**, 943.
HARVEY, E. N. & FANKHAUSER, G. (1933). *J. Cell. Comp. Physiol.* **3**, 463.
HOBSON, L. B. (1941). *J. Exp. Zool.* **88**, 107.
HOLTFRETER, J. (1939). *Arch. exp. Zellforsch.* **23**, 169.
LANDOLT-BÖRNSTEIN (1912). *Physikalisch-Chemische Tabellen.* Berlin.
NEEDHAM, J. (1936). *Order and Life.* Yale.
SPEMANN, H. (1931). *Arch. EntwMech. Org.* **123**, 389.
WADDINGTON, C. H. (1939*a*). *Introduction to Modern Genetics.* London.
WADDINGTON, C. H. (1939*b*). *Nature, Lond.*, **144**, 637.
WADDINGTON, C. H. (1940). *Organisers and Genes.* Cambridge.
WADDINGTON, C. H. (1942). *Proc. Zool. Soc. Lond.* **111**, 189.

## *Note added in proof*

Since the MS. of this paper was sent to press, I have had an opportunity of seeing the interesting discussion by F. O. Schmitt (*Growth Symposium*, 1942) of the importance of cell surfaces in morphogenesis. Many of the suggestions made there are similar to those advanced in this paper, and a stimulating discussion is given of the role of protein aggregates in cell-surfaces.