Experimental Production of Double Embryos

––––––––

Thesis by

Albert Titlebaum

In Partial Fulfilment

of the Requirements for the

Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1929

Experimental Production of Double-Embryos

by Albert Titlebaum

The egg of the polychaet, Chaetopterus, has never been observed normally to produce double-embryos. It has been found, however, by experimental treatment, such as pressure and low temperature, that double-embryos can be produced.

The egg that develops into a double embryo as a result of the treatment can be detected as early as the first cleavage. The first cleavage, as well as the succeeding ones, of such an egg has been found to be modified in a typical manner from the normal cleavage.

The development of the egg of Chaetopterus has been studied by Wilson ('82), Mead ('97; '98), Lillie ('06). The unfertilized egg contains a large germinal vesicle which immediately starts to break down when set free in the sea waters, the egg proceeding to the metaphase of the first maturation division, at which stage it remains until fertilized. The egg may be fertilized in the germinal vesicle stage, in which case the time for the first cleavage is twenty to thirty minutes longer than for those fertilized at the later stage.

At room temperature (about 22° C.) the first polar body is given off thirteen minutes after fertilization, and the second polar body nine minutes later. At the time of extrusion of the first polar body the egg becomes flattened

(Fig. 1a), its polar axis being the shortest. It then rounds up (Fig. 1b) and becomes flattened again when the second polar body is given off (Fig. 1c). About eight minutes later the egg becomes pear-shaped (Fig. 1e). At this stage the polar axis is lengthened and the animal hemisphere is narrower than the vegetal. The egg then rounds up once more (Fig. 1f) and after about four minutes begins to bulge at the anti-pole (Fig. 1g). As the first cleavage plane cuts through the egg this bulge becomes somewhat constricted off forming the so called polar lobe (Fig. 1h). These changes in shape are more marked in eggs taken from fresh females than from females that have been kept in the laboratory.

The first cleavage occurs forty minutes after fertilization, and divides the egg into two unequal cells, the larger being the CD-blastomere and the smaller the AB-blastomere. The polar lobe is attached by a narrow stalk to the CD-blastomere, and after the first cleavage it passes entirely into that cell. A small polar lobe again appears at the next cleavage and passes into the D-blastomere, which is the largest of the blastomeres of the four-cell stage.

The cleavage of this egg follows the spiral type characteristic of the annelids. Thus the third cleavage is dexiotropic, giving rise to the first quartet of micromeres. These are only slightly smaller than the corresponding macromeres. The succeeding divisions are alternately leiotropic and dexiotropic. A typical young trochophore is formed after about

twenty hours, which soom develops a mesotrochal band of cilia.
This larva gradually metamorphoses into the late trochophore
(Fig. 5a).   The mesotrochal band of cilia is replaced by two
lateral flagella, and a second ciliary girdle develops poster-
iorly.   The eye spots appear on the upper lip of the mouth.
The mouth becomes enlarged transversely, and leads by a cil-
iated oesophagus into a large stomach.   This is separated
by a double fold of endoderm from a smaller intestine.   The
anus opens on the dorsal side, and posterior to this the "ter-
minal papilla" or holdfast is formed.

Pressure Experiments

As has been previously reported (Titlebaum '28) double
embryos can be produced by compressing the uncleaved egg.   In
most of the experiments pressure was applied by means of a
slide and a number one square coverslip.   Large rectangular
coverslips sag somewhat and thus compress the eggs in the
middle of the slide more than those nearer the supports.   The
coverslip was supported by means of two strips of paper whose
thickness was approximately two-thirds the diameter of the
egg.   The strips of paper were soaked in sea-water before be-
ing used.   A small drop containing eggs in the desired stage
is placed between the paper strips on the slide, and the cover-
slip is dropped gently on it.   The excess water is then removed

by means of filter paper placed at opposite edges of the cover-
slip, this latter part of the process being done under the micro-
scope.   The slides are then kept in a moist chamber in order to
prevent hypertonicity of the sea-water due to evaporation.

A compressorium of the type devised by Ziegler was used
in several experiments, mainly as a control for the effects of
lack of oxygen, and hypertonicity of the sea-water.   By means
of this apparatus it is possible to keep fresh sea-water flow-
ing past the eggs while they are under pressure.   However, the
use of the compressorium for small and rapidly developing eggs
is rather difficult, especially where several batches of eggs
from the same fertilized lot are to be compressed at different
stages.   Since it was found that the results obtained by means
of the slide-coverslip method were not due to lack of oxygen or
to hypertonicity, the use of compressorium was soon discontin-
ued.

The amount of pressure applied to the egg was fairly con-
stant enough to increase the diameter of the egg from 96 micra
to about 131 micra.   The diameter of the compressed egg did
not vary noticeably while under compression, but remained about
35% longer than the diameter of the normal egg.   When the eggs
are released from pressure they return to their normal shape.

As has been previously stated, the egg which is to produce
a double embryo can be detected at the first cleavage.   The
first cleavage of this egg gives two equal cells, instead of
a large and small cell (Fig. 2a, b).   The equally cleaving
egg forms no real polar lobe, but a broad flattened bulge,

through which the cleavage plane passes, appears at the anti-pole. At the next cleavage, in most cases, each of the two equal cells cleave slightly unequally, resulting in two pairs of equal sized cells. The equal sized cells may be diagonal to each other or next to one another. Two very small polar lobes are sometimes seen at the second cleavage of these equally cleaved eggs.

The third cleavage is dexiotropic, as in the normal egg, and the fourth leiotropic. The latter two cleavages as well as the succeeding ones appear to be similar to corresponding cleavages of the normal egg. However, some experiments on the egg of the gastropod Ilyanassa seem to indicate that the third cleavage may be quite different from the normal. A small percent of the Ilyanassa eggs may be made to cleave equally, by means of pressure or low temperature. At the 8-cell stage of the normal Ilyanassa egg the four micromeres are considerably smaller than the four macromeres, and practically devoid of yolk; but the 8-cell stage from equally cleaved eggs was found in three cases to consist of practically equal sized micromeres and macromeres, yolk being also present in the micromeres.

In the 8-cell stage of an equally cleaved egg of Chaetopterus the four "micromeres" may thus be of a different nature from the micromere quartet of the normal egg, even though the sizes of the cells do not differ markedly from the normal.

Through cell lineage studies on Annelid eggs it has been

found that one of the cells of the second micromere quartet, the 2d-blastomere, gives rise to the ectoderm and one of the cells of the fourth micromere quartet, the 4d-blastomere, gives rise to the mesoderm. An attempt was made therefore to determine whether two cells of each kind were formed from the equally cleaved eggs. This was done mainly through a study of fixed and stained material. The equally cleaved eggs were picked out in the 2-cell stage, and then preserved at the particular later stage desired. Comparisons of these eggs with normal Chaetopterus eggs of the same stage showed no noticeable differences. Even if there were two 4d-blastomeres in the 64-cell stage of the equally cleaved egg, these would not be readily observed, since in the normal egg the 4d-blastomere is not easily distinguished from the others. This point, however, has already been settled by the observations of Penners ('22, '24) on the egg of the oligochaet, Tubifex rivulorum. Penners reports the occurrence of double embryos in egg capsules which have been brought from a low temperature ($10^{o}C$) where development is entirely normal, to a higher temperature ($15^{o}C$ to $20^{o}C$). Five cases are reported in which the early development has been followed. Two first somatoblasts (2d-blastomeres) and two second somatoblasts (4d-blastomeres) were found to be present.

The nine hours old embryo resulting from the equally cleaved Chaetopterus egg shows a marked difference in shape from the normal. (Fig. 3a, b.) It may be recognized as a double structure at about 20 hours after fertilization (Fig.

4a, b). At this stage the embryo is broader than the normal embryo and its posterior end is bifurcated. One apical flagellum is present, though occasionally embryos with two apical flagella may be found. As development proceeds, the double embryo becomes progressively easier to recognize. The amount of separation of the posterior ends of each embryo increases to approximately half of the length of the body. A mesotrochal band of cilia develops around each partner, at about the region of the bifurcation. As this embryo develops into the later transparent trochophore (Fig. 5b), four eye spots appear anteriorly. Two mouths are usually present which are more difficult to make out than in the normal embryo. The stomach becomes a large clear structure which is sometimes seen to be divided in two by a septum which lies in the plane formed by the anterior tip of the embryo and its two posterior ends. The two trunk ends of the embryo are bent away from each other until they come to lie approximately in a straight line. Each of the mesotrochal bands of cilia is replaced by two lateral flagella, and an intestine develops in each of the two trunks. A second ciliary band is formed around each trunk in the region of the posterior part of the intestine, and a holdfast appears at the posterior tip of each partner. The rate of development of the double is slower than for the normal embryo. Such parts as the eyes and the lateral flagella appear about one to two hours later in the former than in the latter. Many of these double embryos are recognized to be of the Janus type. The composit-

ion and arrangement of parts in a Janus, or "duplicitas cruciata", embryo may, for the purpose of description, be represented diagrammatically as follows: Two normal embryos are slit halfway up their median planes, as represented in Fig. 6a, and the resulting right- and left-halves are bent away from each other at an angle of 180 degrees. The two embryos are then fused along their cut surfaces, the dorsal side of each embryo facing upward (Fig. 6b). The head ends are then bent upward towards each other until their dorsal surfaces unite (Fig. 6c). In the resulting picture the median plane of the fused heads is at right angles to the median plane of the separate trunks. Each trunk is a composite of the right half of one embryo and the left half of the other. Among the Chaetopterus Janus embryos there are sometimes cases in which one or both tail ends show a slight bifurcation, a fact which is probably indicative of their composite origin.

When a batch of Chaetopterus eggs are compressed at the proper stage for the production of double embryos (i.e., the "pear-shaped" stage), the two blastomeres resulting from the first cleavage vary in size from completely normal to exactly equal. A few cases (less than 0.1%) are sometimes obtained in which the two blastomeres are more unequal in size than in the normal 2-cell stage. These eggs do not develop very far, probably because of irregularities in the distribution of the chromosomes. Pressure at the pear-shaped stage results in approximately 15% equal cleavage, 30% normal, and 55% inter-

mediate between equal and normal cleavage. However, this numerical ratio itself cannot be considered as very significant, inasmuch as it is dependent on an arbitrary grouping of a closely graded series of size variations, and on the ability of the observer to exactly determine the group into which a given egg may fall. Nevertheless the development of eggs selected from each of these three groups (Table I), brings out an interesting point. In these experiments pressure was usually applied at the pear-shaped stage and released about five minutes later, just as the first cleavage plane was cutting through most of the eggs.

The results, shown in Table I, may be summed up by the statement that exactly equally cleaved eggs produce perfect double embryos, normally cleaved eggs produce normal embryos, and intermediate types of cleavage result in partially double embryos. The exceptions to this statement occurring in the table may be explained as being due to the inability to judge with perfect accuracy the exact sizes of the two cells through the binocular dissecting microscope under which the eggs are picked out. In the partially double embryo, one partner is usually complete while the other is lacking or imperfect in one or more parts, such as the holdfast, intestine, ciliary band, lateral flagella, eye spots, etc.

The sizes of the blastomeres in the two-cell stage of an egg that had previously been compressed does not apparently depend on the amount of pressure applied. This is shown by the fact that equally cleaved and unequally cleaved eggs may

Table I

	No.of eggs isolated	Normal late trochophores	Partial doubles	Perfect doubles	No. of dead embryos
Equally cleaved	83	0	21	52	10
Intermediates	60	5	45	2	8
Normals	38	34	2	0	2

Note: This represents a summation of
       several experiments.

be seen next to each other on the same slide of a compressed batch of eggs; and also by the fact that even when the pressure is removed just before cleavage, equally cleaved as well as unequally cleaved eggs are obtained. This latter point is brought out very strikingly by some results on the egg of Ilyanassa. In this egg there is formed at the time of the polar body division a large polar lobe (not previously described for this stage), which makes the egg elongate in its primary axis. All the eggs of a given batch may be compressed from the side at this time. When a batch of eggs are compressed at this stage, and the pressure released after the egg rounds up again, a small number of equally cleaved eggs are produced, even though this cleavage comes in about two hours after the pressure is removed.

The fact that only a certain percent of a batch of compressed eggs cleave equally is obviously not due to differences in age among the eggs at the time of compression; for the variation in rate of development of eggs from the same fertilized lot is far smaller than the range of stages over which pressure may effect equal cleavage.

Effect of Pressure at Different Stages

In order to learn more about the factors which cause equal cleavage, and the production of double embryos, detailed series

of pressure experiments were made on eggs in varied stages of development, and for various lengths of time. These experiments were also instructive in other respects. The terms used to describe the various stages refer to the changes in shape of the normal egg up to the first cleavage (Fig. 1). Their sequence is in the order given in the tables.

Table II represents one such series of experiments. Here each batch of eggs is compressed at that time when the preceding batch is released from pressure (or within one minute of that time). One hundred eggs from each of the first four compressed batches, and two hundred eggs from each of the last four batches were counted out into separate dishes irrespective of how they cleaved. The number of doubles were counted in the four-day trochophores, partially double embryos being counted in with the doubles.

In Table III several similar series are summarized. The results show that the optimum time for production of double embryos is about the pear-shaped stage. If, however, pressure is applied at a preceding stage and maintained through the pear-shaped stage up until cleavage, the number of doubles obtained from the preceding stages is increased, as shown in Table IV.

In order to obtain more accurate information as to the stage at which pressure produces double embryos most frequently, three series of experiments were run at a slightly lower temperature than usual. This increased the time between the formation of the second polar body and the first

Table II

Stage at compression	Time after fertiliza-tion minutes	Length of time com-pressed minutes	No. of eggs isolated	No. of double embryos
Before first polar body	2	5	100	0
Before first polar body	7	5	100	0
First polar body	13	4	100	0
First polar body (round)	17	5	100	0
Second polar body (flattened)	22	5	200	1
Second polar body (round)	26	5	200	3
Pear-shaped stage	30	5	200	80
Polar lobe stage	34	5	200	0

Table III

Stage at compression	Approx. duration of pressure	No. of eggs isolated	No. of double embryos
First polar body	6 min.	325	0
Second polar body	6 "	425	3
Pear-shaped stage	5 "	450	143
Polar lobe stage	4 "	425	7

Table IV

Stage at compression	Time of compression	No.of eggs isolated	No. of double embryos
First polar body	30 min.	250	23
Second polar body	25 "	348	56
Flattened	22 "	250	38
Pear	17 "	250	69

cleavage by about fifty percent.   Table V shows the result of one such series.

The time interval between each of eight stages was approximately three minutes.   The results place the optimum time for production of doubles by pressure at the middle pear-shaped stage, which occurs 57 minutes after fertilization at 19° C.

The amount of mortality in the experiments tabulated was extremely low.   There occur, however, in some experiments - mainly in the later stages - equally cleaved eggs in which the first cleavage plane disappears.   Some of these eggs undergo subsequent irregular cleavages and produce abnormal embryos, not recognizable as doubles.   The others show no subsequent cleavages, although nuclear divisions may occur, but undergo a process which has been called differentiation without cleavage.   Both of these types of embryos die within two days.   Another type of abnormal embryo is sometimes produced by the fusion of two young trochophores.   Eleven such combinations were isolated.   None of these lived beyond two days.   They gave no indication of being related to the type of a double embryo described above.

Pressure experiments were also performed on eggs prior to fertilization, and at two- and the four-cell stage.   When a batch of eggs is inseminated while under pressure or compressed just at the time of insemination, some of the eggs often remain unfertilized or may be polyspermic.   In one case a batch of eggs compressed at the time of insemination for about ten minutes gave mainly normal cleavage, but a

Table V

Stage at compression	Time of compression	No. of eggs isolated	No. of double embryos
Second polar body	5 min.	250	0
Flattened	$3\frac{1}{2}$ "	500	1
Later	$3\frac{1}{2}$ "	500	4
Round	5 "	500	5
Begin. pear	3 "	500	10
Pear	4 "	500	184
Late pear	$4\frac{1}{2}$ "	200	31
Polar lobe	4 "	200	0

great many of the resulting late trochophores were bent backwards. One double was isolated from this batch, but its presence may have been due to contamination from a pipette used for some previous transfer.

When pressure is applied from the two- to the four-cell stage a small percentage of the eggs cleave normally while the rest show wide departures from the normal type. A few normal embryos are produced from these eggs, and many abnormal ones, most of which die within two or three days. Some of these abnormal forms arise from eggs in which the cleavage plane between the C- and D-blastomeres disappears. Other forms arise from eggs in which the second cleavage plane in one or both of the first two blastomeres comes in parallel to the first. In some of these cases the CD-blastomere may be divided equally, and in others unequally. None of the eggs compressed between the two- and four-cell stage gave rise to the perfect type of double trochophore produced by the equally cleaved 2-cell stage. There were some that looked like double structures, but none of these developed far enough to be identified with certainty.

When pressure is applied at the 4-cell stage, the eggs all orient with their poles against the compressing plates. The cleavage plane of a compressed egg is always at right angles to the compressing plates. Consequently the third cleavage in this case is at right angles to the normal third cleavage. This results in a flat plate of eight cells being formed. The D-blastomere as well as each of the other three

are seen to cleave equally, but the cleavage plane disappears frequently in the D-blastomere.   No normal embryos develop from these eggs, and they all die within two to three days. Some of these embryos look double, although here again they do not differentiate far enough to be accurately judged.

It is obvious from the above experiments that the alterations in the nature of the first cleavage, and the production of double embryos are due to the effect of pressure on the internal changes going on in the egg, between the time of the pear-shaped stage and the first cleavage.

Cytological Details

In order to learn something about the nature of these changes, normal Chaetopterus eggs were fixed at various stages from prior to fertilization up to cleavage: equally cleaving eggs and later cleavage stages were also fixed.   Sections and total mounts of these eggs were made, stained with Delafield's or Heidenhain's haemotoxylin, erythrosin being used in most cases as a counter-stain.

The internal structure of the Chaetopterus egg at various stages has been described in some detail by Lillie ('06). His description has been checked, and only one additional feature has been made out: that is, the presence within the spherular endoplasm of three or four groups of spherules,

which stain intensely with erythrosin.   However, since the
history of these groups of spherules has not been completely
traced, they will be omitted from the present report.

At the time of the formation of the first polar body, the
sperm pronucleus has advanced into the non-spherular endoplasm
(Fig. 7a).   It remains there until the second polar body is
given off (Fig. 7b, c) and then proceeds to fuse with the egg
pronucleus at about the time of the "round stage" (Fig. 7d).
The sperm asters are not very distinct until this time, but
at the beginning of the pear-shaped stage they show up clear-
ly and enlarge during this time (Fig. 7e).   At the polar lobe
stage (Fig. 8a) the first cleavage spindle is fully formed and
the egg has elongated in the direction of the spindle.   The
cleavage amphiaster is markedly heteropolar, the aster and
centrosome on the side that is to form the CD-blastomere being
larger than on the other.   The spherular endoplasm has been
noted by Lillie to be broader on that same side (Fig. 8c).   In
the equally cleaving eggs (Fig. 8b, d) it is seen that the two
poles of the spindle are of the same size, and that the spheru-
lar endoplasm is of the same thickness on both sides.   The
main points shown by a study of these slides are: first, that
at the pear-shaped stage (which is the optimum time for pro-
duction of doubles) the cleavage amphiaster is just forming in
the egg;   secondly, that in equally cleaved eggs the spheru-
lar endoplasm is of the same thickness on both sides and the
spindle is equipolar.   (This seems to indicate that the spin-
dle has been made to elongate at right angles to its normal

position.) Thirdly, that at the polar lobe stage the spindle and also the egg have already begun to elongate preparatory to division. Thus in this latter case the egg will generally take such a position between the compressing plates that the first cleavage plane will appear in its normal position, and the resulting normal development should naturally be expected. It may be noted that the egg in the pear-shaped stage almost invariably lies on its side. Thus the egg at this stage cannot be compressed from the pole, but can be compressed in any one of an infinite number of planes passing through its primary axis. It is possible at other stages, however, to compress the egg from the pole. In those cases where the egg is about to give off a polar body, when placed under pressure, the polar body appears in the same position that it would normally occupy.

The evidence for the alteration of the position of the first cleavage spindle, cited above, can hardly be taken by itself to be sufficient inasmuch as cytoplasmic rearrangements may have occurred as a result of pressure which would give the observed picture.

There are two so called laws which attempt to explain the effect of pressure on the position taken by the spindle. The one of Pfluger ('84) states that the spindle develops in the direction of least resistance; and that of Hertwig, that the spindle develops in the direction of greatest protoplasmic mass. However there are several cases which seem to contradict these statements, such as the fact that the

polar spindle even under pressure always takes its position
at the pole, although that is apparently not the direction of
least resistance or of greatest protoplasmic mass, and also
the case of the fourth cleavage of the sea-urchin egg which
obeys the law in the upper hemisphere and contradicts it in
the lower.

Compression of an egg of Chaetopterus at the 4-cell stage
gives a flat plate of eight cells at the next division. This
indicates that the spindle may be made to assume a position
at right angles to its normal position, and thus cause the re-
sulting cleavage plane to come in at right angles to its nor-
mal direction. An attempt was made, therefore, to further
test the hypothesis that the first cleavage plane can also be
made to come in at right angles to its normal position, and
that equally cleaving eggs result from such an alteration in
the direction of the cleavage.

Other Evidence for
Right Angle Shifting of the Spindle

In the egg of the ascidian, Styela partita, the first
cleavage invariably passes through the middle of a colored
region called the yellow crescent. When these eggs are com-
pressed before the first cleavage some unequal first cleav-
ages, along with the normal equal cleavage, are obtained. The

yellow crescent in some of these cases was found to lie en-
tirely in one of the first two blastomeres (usually the smal-
ler one) showing that the first cleavage plane had been turn-
ed at right angles to its normal position.

The first cleavage of the egg of Nereis limbata is une-
qual. The first plane has been shown by Just ('12) to pass
through the entrance point of the sperm. This point can be
easily found by means of the funnel left in the jelly layer
that pours out after fertilization. The funnel shows up
clearly when the eggs are fertilized in sea-water to which
Chinese india ink has been added. Using this method of
marking the entrance point, Nereis eggs were compressed at
various stages before the first cleavage. The pressure
must be applied slowly and the eggs watched closely under
the microscope since shifting of the funnel very frequently
results. In most of the cases observed the cleavage plane
passed right through the entrance point, and was unequal. In
two cases the cleavage plane was observed to come in almost
at right angles to the entrance point, dividing the egg into
two practically equal-sized blastomeres. The fact that the
Nereis egg so orients itself that in general its primary axis
is at right angles to the compressing plates probably accounts
for the small percent of equal cleavage obtained in this egg.

In the egg of Chaetopterus some unpublished data of Whit-
aker and Morgan has shown that the first cleavage plane usual-
ly coincides with the entrance point of the sperm.

In order to determine the relation between the entrance

point and the cleavage plane in compressed eggs, eggs in which
the sperm was seen to enter at the side were compressed at a
later stage.   The presence of other sperm around the periphery
of the egg served as markers.   In two cases in which the po-
sition of the compressed egg was such that the entrance point
was towards the side, the eggs were observed to cleave equally.
Uncompressed eggs in such a position would have been expected
to cleave unequally and in a horizontal instead of a vertical
plane.

Taken as a whole the evidence shows that the first cleav-
age of an equally cleaved egg of Chaetopterus is at right angles
to its position in the normally cleaved egg.

Compression of Other Eggs

The effect of pressure on the development of other types
of eggs has also been studied.   When the eggs of the small
pelecypod, Cumingia tellinoides are subjected to pressure from
the second polar body stage to the first cleavage a large pro-
portion of equally cleaved eggs are obtained.   The trocho-
phores obtained from these eggs were abnormal and those that
did go as far as the veliger stage were not recognizable as
doubles.   Miss Browne ('10) has described the early cleav-
ages of compressed Cumingia eggs, and since no very signifi-
cant differences were observed from her description, the de-

tails of these experiments may be omitted.

Compression experiments on the egg of the gastropod Ilyanassa obscura, and the ascidian, Styela partita, have been discussed above in other connections.

In the normal cleavage of Ilyanassa a large polar lobe is formed, which goes to one of the blastomeres of the 2-cell stage. It has been possible by means of pressure to divide the polar lobe between both blastomeres, although in many cases the cleavage plane fades before it cuts all the way through the lobe. The resulting eggs do not go much beyond the early cleavage stages. The development of equally cleaved Ilyanassa eggs should prove interesting inasmuch as it has been definitely shown (Crampton '96) that the material of the polar lobe is necessary for the development of the mesoderm.

In the pressure experiments on Styela, one tadpole was obtained that had a short extra piece of tail. It resulted from an unequally cleaved egg. (The normal first cleavage of this egg is equal.)

Pressure experiments on the egg of Nereis limbata were performed by Wilson ('96) and by Morgan ('10). They obtained in their experiments abnormal embryos, some of which showed duplication of parts. An attempt was therefore made by the author to determine whether equally cleaved Nereis eggs would produce double embryos. In several series of experiments eggs were compressed at various stages from the first polar body formation to cleavage. Nine embryos were obtained from equally cleaved eggs, which both Wilson and Morgan identified

as perfect doubles (Fig. 9).   Partial doubles were also ob-
tained.   As has been mentioned before, the difficulty in
producing double Nereis embryos is probably due to the fact
that the egg orients with its pole against the compressing
plate.

Isolated Blastomeres

Isolated blastomeres of the equally cleaved and of the
normal Chaetopterus egg were also studied.   The blastomeres
of this egg are difficult to separate since the egg is enclos-
ed in a fairly close fertilization membrane.   If this mem-
brane is not cut completely through the blastomeres tend to
fuse together again.   In effecting a complete separation,
therefore, one or both blastomeres may be seriously injured.
This would account for the fact that isolated blastomeres
rarely go beyond a very early trochophore stage.   In only
a few instances did both blastomeres from the same egg de-
velop.   In forty-three cases examined the AB-cell produced
a swimming mass of mainly ectodermal cells.   Of the embryos
obtained from fifty-one isolated CD-cells, some outwardly re-
sembled the normal early trochophore.   Two cases (one of
which may have been due to contamination) were obtained in
which a normal late trochophore was obtained from the CD-
blastomere.   The isolated blastomeres of eggs made to cleave

equally by means of pressure were followed in forty-six cases. The first cleavage (second of whole egg) of such blastomeres is usually slightly unequal, and the second cleavage comes in dexiotropically as in the normal third cleavage. Most of the young trochophores obtained appeared to be whole embryos. In one case a normal late trochophore was obtained. The evidence indicates that isolated blastomeres from the two-cell stage of equally cleaved eggs are capable of producing whole embryos.

Low Temperature

Cases have been reported in which double embryos have been produced by exposing eggs to low temperature. These cases were obtained mainly from fish eggs (Stockard '21). The eggs which are to produce doubles cannot be detected until a late stage, so that the initial effect of low temperature has not been made out.

The eggs of Chaetopterus can be made to produce double embryos by exposure to low temperature before the first cleavage. The double embryos arise from equally cleaving eggs, as in the pressure experiments. One series of low temperature experiments is shown in Table VI. A fertilized lot of eggs was distributed approximately equally among nine similar sized dishes containing the same amount of sea-water. Eight of these dishes were placed directly on a cake of ice at the

Table VI

Stage	Duration of exposure	Normal embryos	Abnormal embryos	Double embryos	Dead embryos
First polar body	$4\frac{1}{2}$ min.	300	0	0	0
Second polar body	$4\frac{1}{2}$ "	85	90	0	125
Late flattened	$4\frac{1}{2}$ "	246	30	4	20
Round	$4\frac{1}{2}$ "	99	45	6	150
Early pear	$4\frac{1}{2}$ "	140	35	15	110
Pear	$4\frac{1}{2}$ "	0	0	0	300
Polar lobe	$4\frac{1}{2}$ "	0	0	0	300
Just before first cleavage	$4\frac{1}{2}$ "	0	0	0	300

stages noted in the table. They were returned to room tem-
perature after four and a half minutes. Three hundred eggs
were later counted out of each dish, and examined for double
embryos at the late trochophore stage. As shown in the ta-
ble low temperature applied at the early pear-shaped stage
produces the largest number of double embryos. This was
true in general in other series of low temperature experi-
ments. Table VII represents data taken from experiments
performed at temperatures between 0° and 6° C., and for time
intervals of five to ten minutes. The stage noted there as
second polar body includes the early pear-shaped stage. One
case is recorded in which a double embryo was obtained from
a batch of eggs fertilized at low temperature. Most of the
eggs inseminated at low temperature are unfertilized, and
where fertilization does occur it is often polyspermic, giv-
ing rise to abnormal larvae.

Eggs that have been cooled in the late pear-shaped stage,
in the polar lobe stage, or just before cleavage, show a high
percent of mortality (100% in Table VI). In these eggs the
first cleavage plane usually disappears or may not even be
formed, while the nucleus divides. The next cleavage comes
in at right angles to the faded first cleavage, forming two
equal sized cells. In some of these eggs this cleavage and
the subsequent ones may also disappear giving rise to a so
called unicellular trochophore that has "differentiated with-
out cleavage". In other eggs cleavages may occur, abnormal
embryos usually resulting. In some cases the egg may break

Table VII

Stage	Approx. No. of embryos	Double embryos
Fertilization	1000	1
First polar body	1000	0
Second polar body	3800	32
Pear	2200	15
Polar lobe	2000	0

up into four or more cells after a faded first cleavage. All of these types of behavior result in abnormal embryos which die early.

The position of the faded first cleavage plane may easily be made out. The fact that when the next cleavage plane comes in at right angles to the faded one two equal-sized cells result is a most convincing argument in favor of the hypothesis that equal first cleavage occurs when the spindle has been made to elongate at right angles to its normal position.

In one series of experiments the eggs were placed in dishes of sea-water that had been previously cooled. The amount of mortality was very high. Many abnormal but no double embryos were seen.

Other Agents

Two other methods of causing equal cleavage in Chaetopterus have also been found. Thus equally cleaved eggs result from batches of eggs exposed for fifteen seconds to the action of ultraviolet light, at about the pear-shaped stage. Parthenogenetic activation also results in some equal cleavages. The activation was induced by exposing the unfertilized eggs to a high temperature ($33^{\circ}C$) for about forty-five minutes according to the method of Allyn ('12). Most of the embryos obtained in these experiments were abnormal and

died early.   Since double embryos have been obtained in Fun-
dulus by exposure to ultraviolet (Hinrichs '25) it seems pos-
sible that they may also be produced in Chaetopterus by means
of that agent.

Discussion

The fact that eggs compressed at the pear-shaped stage
give both equal and unequal cleavage may be explained as fol-
lows:

That side of the uncleaved Chaetopterus egg which is to
go into CD-blastomere has been found to be distinctly differ-
ent from the side that is to go into the AB-blastomere.   The
larger centrosome and aster of the heteropolar spindle occurs
on the CD-side.   A cross-section (at right angles to the spin-
dle) of the egg before cleavage would show the same picture on
both sides.   If, then, the spindle is made to elongate at right
angles to its normal position, its two centrosomes and asters
will lie in regions that are apparently alike.

This similarity in the regions at the two poles of the am-
phiaster may account for an equipolar spindle and the resulting
equal cleavage.   The polar lobe material, which normally goes
to the CD side of the egg would be expected to be split between
both cells of the equally cleaved egg, since the CD side has been
divided between both blastomeres.   Whether an egg will cleave

equally or not would therefore depend on the position of the CD side of the egg in relation to the compressing plates. The Chaetopterus egg lies on its side (i.e., its polar axis horizontal) at the pear-shaped stage. We should therefore expect pressure at that time to result in more equal cleavages than at other stages, since according to the above hypothesis pressure from the side may cause a shift in the direction in which the spindle elongates.

In the low temperature experiments the pear-shaped stage (allowing for the time of cooling) is again found to be the optimum stage for producing equal cleavage and double embryos. This indicates that a similar mechanism is involved. When a dish of eggs is placed directly on a cake of ice, it is probable that one side of the egg is cooled more than the other. Since the viscosity of protoplasm increases rapidly at low temperature (Heilbrunn '25) the progressive gelation on cooling the egg might effect an orientation of the spindle. Thus in some eggs the spindle may be made to elongate at right angles to its normal position. The fact that only a small percent of a given batch of eggs can be made to produce double embryos by means of low temperature seems to be better explained by a theory based on the alteration of the cleavage plane than one based on developmental retardation (Newman '23, Stockard '21). In the latter it must be assumed that there is great variation in the stage of development of different eggs in the same lot, or that different eggs vary in their susceptibility to the effect of cold. In regard to the first assumption one

may observe that, in a lot of eggs properly inseminated and in good condition the variations from perfectly synchronous cleavage are very slight. As for the second assumption, it does not seem probable that the female, whose eggs are being treated, should be genetically heterozygous for factors determining susceptibility to cold.

Ultraviolet light may also affect the orientation of the first cleavage spindle. It is known that ultraviolet coagulates proteins, and it is not improbable that the side of the egg facing the source would be first affected.

That parthenogenetic activation should give abnormal cleavages might be due to the absence of some factor, such as the entrance point of the sperm, which determines the meridian which the first cleavage plane will take. The effect of pressure and low temperature on the entrance point of the sperm may account for the few cases (beside the polyspermic ones) of abnormal development obtained from eggs treated at this stage.

The main interest in the production of double embryos probably lies in the bearing of the results on the problem of localization. The Chaetopterus double embryo arises from an egg in which the polar lobe material has been cut in two. The question might therefore be raised as to whether this material represents a "formative stuff". Such an interpretation has been given to the pole-plasma of the Tubifex egg by Penners and by von Parseval, who regard these substances as "organ-forming materials". Penners assumes that these sub-

stances give rise to the germ bands, so that when each pole plasm is divided between two blastomeres (in the two- or four-cell stage) a double set of germ bands develops. However, the evidence for the pole-plasms forming the germ bands does not yet seem conclusive, and there may very well be other factors involved in the formation of a double set of germ bands. In the equally cleaved Chaetopterus egg it appears that the cleavage plane has been made to come at right angles to its normal position, dividing the entire egg as well as the polar lobe into equal parts. Thus the production of a double embryo cannot be said to be due to the behavior of the polar lobe alone. In fact, without appealing to definite substances within the egg, it may be possible to work out an explanation of double-embryo production in terms of "axial relations" or "nature of the division". But such abstract explanations do not serve very well as working hypotheses. It seems simpler, and more in line with certain experiments on amphibian eggs, to interpret the type of development exhibited by equally cleaved eggs in terms of an abnormal distribution of certain specific materials (either in the polar lobe or in the CD-side of the egg).

The fact that certain eggs (even of the so called mosaic type) can be made to produce double embryos indicates that the fate of different regions of the uncleaved egg has not been irreversibly (if at all) determined. In this abnormal type of development different parts of the egg must have certainly gone into other structures than those of which

they would normally have formed a part. Therefore if there were any predelineation of embryonic structures it would have to be quite amenable to alteration. It seems more reasonable to suppose that the egg processes something similar to the material of the dorsal lip of the amphibian blastopore ("organizer" of Spemann, Mangold, etc.), in the form of the 2d- and 4d-blastomeres, which initiates the development of the germ bands. The behavior of the dorsal lip of the blastopore furnishes a clear explanation of the origin of amphibian double embryos (Spemann, Ney, Wessel, Schleip and Penners). An explanation along similar lines would fit in very well with the results obtained on Tubifex, Chaetopterus and Nereis.

S u m m a r y

1. When pressure is applied to uncleaved eggs of Chaetopterus, Nereis, Cumingia, and Ilyanassa, and released at the time when the first division is about half completed, some of the eggs divide into equal parts; others into nearly equal or unequal (normal) parts.

2. Those eggs of Chaetopterus and of Nereis that have cleaved equally produce double embryos, many of which are of the duplicitas cruciata (Janus) type. The normally cleaved eggs from the same compressed batch form normal embryos, and the intermediate (or nearly equal) types result in partially double embryos.

3. The largest number of double embryos is obtained from Chaetopterus eggs that are compressed from the pear-shaped stage until the time of the first cleavage. At this stage the cleavage amphiaster is enlarging in the egg.

4. The amount of pressure applied does not determine the type of first cleavage obtained, since in a uniformly compressed batch of eggs some may cleave equally and others unequally.

5. Sections of eggs that have cleaved equally compared with sections of normal eggs show that the first cleavage amphiaster has elongated at right angles to its normal position. That shifting of the spindle may occur under pressure is shown by compression of eggs at the four-cell stage. Flat plates of eight cells are thus produced. That a right angle shifting of the first cleavage plane can be effected by means of pressure is shown by experiments on eggs of Styela, where the first cleavage plane can be located by the yellow crescent.

6. Pressure at the two- to four-cell stage results in a large percentage of abnormal embryos in Chaetopterus and pressure at the four- to eight-cell stage produces only abnormal embryos. No recognizable doubles are obtained from these embryos.

7. The eggs of Chaetopterus subjected to low temperature, ultraviolet radiation or parthenogenetic activation may also divide into equal parts at the first division. The equally cleaved Chaetopterus eggs obtained from low temperature experiments give rise to double (Janus) embryos.

8. The optimum stage for production of the double by means of cold is again found to be the pear-shaped stage.

9. Chaetopterus eggs in which the first cleavage spindle disappears as a result of pressure or low temperature give rise to abnormal embryos.

Bibliography

Allyn, H. M. 1912. The initiation of development in Chaetopterus. Biol. Bull. XXIV.

Browne, E. N. 1910. Effects of pressure on Cumingia eggs. Erch. Entw. Mech. XXIX.

Crampton, H. E. 1896. Experimental studies on gasteropod development. Arch. Entw. Mech. III.

Heilbrunn, L. V. 1924. The colloid chemistry of protoplasm. The viscosity of protoplasm at different temperatures. Am. Jour. Physiol. LXVIII.

Hey, A. 1911. Ueber künstlich erzeugte Janusbildungen von Triton taeniatus. Arch. Entw. Mech. XXXIII.

Hinrichs, M. A. 1925. Modification of development on the basis of differential susceptibility to radiation. Jour. Morph. and Physiol. XLI.

Just, E. E. 1912. The relation of the first cleavage plane to the entrance point of the sperm. Biol. Bull. XXII.

Lillie, F. R. 1906. Observations and experiments concerning the elementary phenomena of embryonic development in Chaetopterus. Jour. Exp. Zoöl. III.

Mead, A. D. 1895. Some observations on the maturation and fecundation in Chaetopterus pergamentaceus Cuvier. Jour. Morph. X.

----- 1897. The early development of marine annelids. Jour. Morph. XIII.

----- 1898. The origin and behavior of the centrosomes in the Annelid egg. Jour. Morph. XIV.

Mangold, O., and H. Spemann. 1927. Ueber Induktion von Medullarplatte durch Medullarplatte im jungeren Keim, ein Beispiel homogenetischer oder assimilatorischer Induktion. Arch. Entw. Mech.CXI.

Morgan, T. H. 1910. The effects of altering the position of the cleavage planes in eggs with precocious specification. Arch. Entw. Mech. XXIX.

Newman, H. H. 1921. The experimental production of twins and double monsters in the larvae of the starfish, Patiria miniata, together with discussion of the causes of twinning in general. Jour. Exp. Zoöl. XXXIII.

Bibliography (cont.)

Parseval, M. 1922. Die Entwicklung zentrifugierter Eier von Tubifex rivulorum Lam. Arch. Entw. Mech. L.

Penners, A. 1922. Die Furchung von Tubifex rivulorum Lam. Zool. Jahrb. Ab. Anat. u. Ontog. XLV.

------ 1922. Ueber Doppelbildung bei Tubifex rivulorum Lam. Verh. Dtsch. Zool. Ges. XXVII.

------ 1923. Die Entwicklung des Keimstreifs und die Organbildungen bei Tubifex rivulorum. Zool. Jahrb. Ab. Anat. u. Ontog. XLV.

------ 1923. Experimentelle Erzeugung der Duplicitas cruciata am Keim von Tubifex als Mittel zur Bestimmung organbildender Substanzen. Verh. Dtsch. Zool. Ges. XXVIII.

------ 1924. Doppelbildungen bei Tubifex rivulorum. Zool. Jahrb. Zool. XLI.

------ 1926. Experimentelle Untersuchungen zum Determinations problem am Keim von Tubifex rivulorum Lam. II. Die Entwicklung teilweise abgetöteter Keime. Zeit. Wiss. Zool. CXXVII.

Penners, A., and W. Schleip. 1928. Die Entwicklung der Schultzeschen Doppelbildungen aus dem Ei von Rana fusca. Zeit. Wiss. Zool. CXXX, CXXXI.

Spemann, H. 1903. Entwicklungsphysiologische Studien am Triton Ei. III. Arch. Entw. Mech. XVI.

------ 1924. Ueber Organisatoren in der tierischen Entwicklung. Naturwissenschaften.

Stockard, C. R. 1921. Developmental rate and structural expression. An experimental study of twin, double monsters and single deformities, and the interaction among embryonic organs during their origin and development. Am. Jour. Anat. XXVIII.

Wessel, E. 1926. Experimentelle erzeugte Duplicitas cruciata bei Triton. Arch. Entw. Mech. CVII.

Wilson, E. B. 1882. Observations on the early developmental stages of some polychaetous annelids. Stud. Biol. Lab. Johns Hopkins Univ. II.

------ 1896. On cleavage and mosaic work. Arch. Entw. Mech. III.
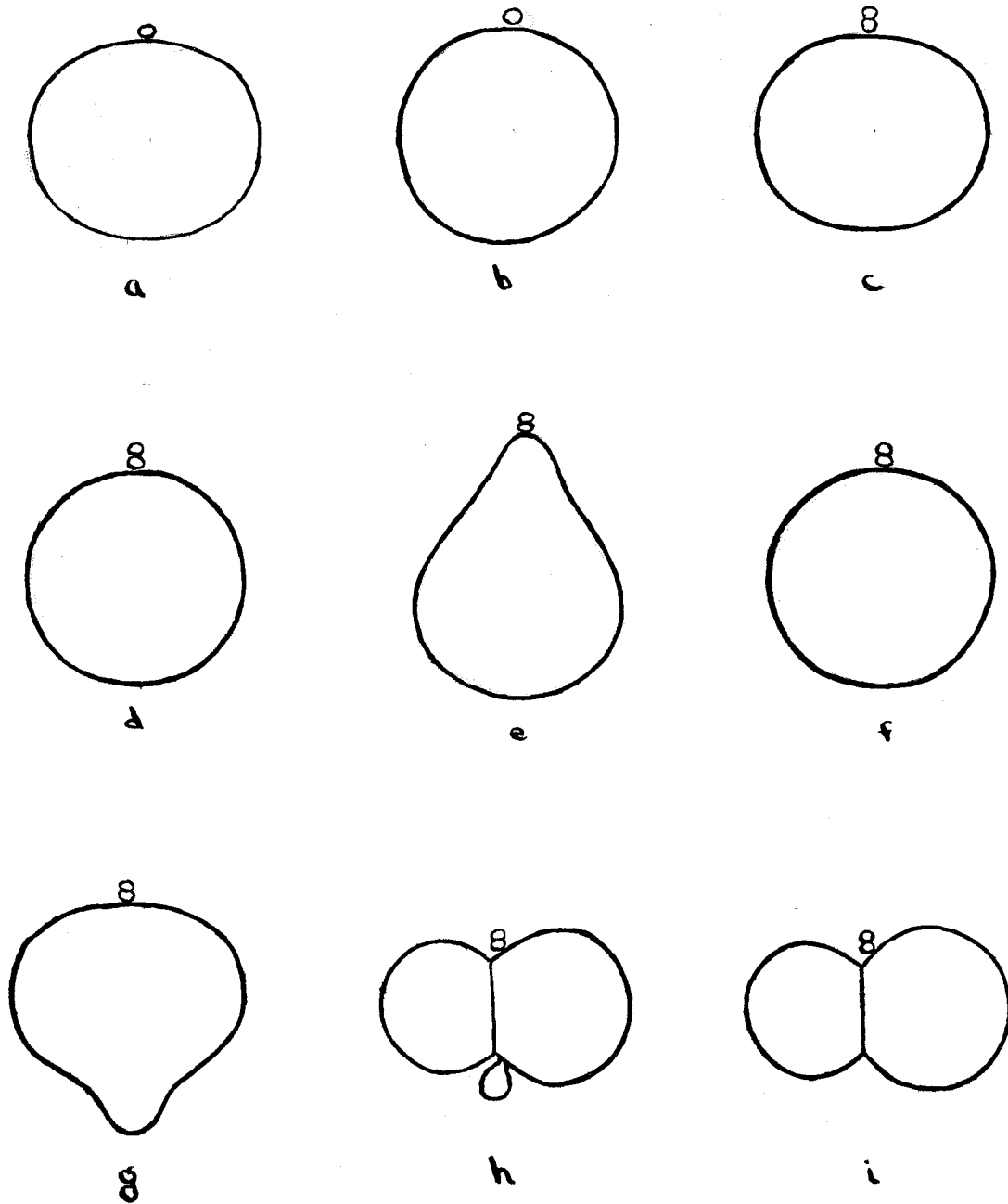
Fig. 1. Normal changes in shape of the egg of Chaetopterus
between fertilization and the first cleavage.
a. First polar body (flattened)
b. First polar body (round)
c. Second polar body (flattened)
d. Second polar body (round)
e. Pear-shaped stage
f. Round   g. Polar lobe-stage
h. First cleavage   i. First cleavage completed

a single apical flagellum.   Two mouths lead into a single large stomach which connects with the intestine of each embryo.   Each embryo has two lateral flagella, a second ciliary band about each intestine, and a "holdfast."   The head-ends are fused back to back and the trunks are separate, the median plane of the fused heads being at right angles to the median plane of the two separate trunks, giving the Janus type.   In most cases one embryo was usually complete, while the other ranged from completeness to various stages of imperfect development of the holdfast, intestine, ciliary band, two flagella, eye spots, etc.

Eggs, compressed for different lengths of time and at other stages up to the first cleavage occasionally give double embryos but in much fewer numbers.   When pressure is applied from the two- to the four-cell stage the *CD* cell frequently divides equally, but the embryos usually do not

a   *b*
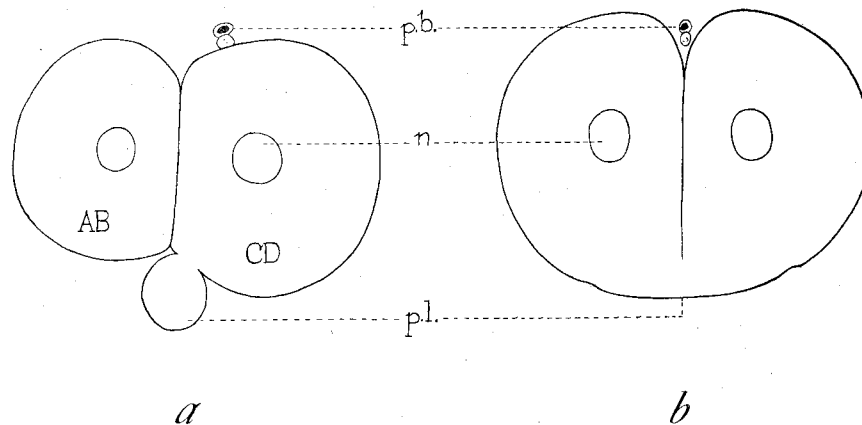
FIGURE 1

Fig. 1(*a*) Normal two cell stage; (*b*) equally cleaving egg; n, nucleus, p.b., polar bodies, p.l., polar lobe.

develop beyond the early trochophore stage, at which time double embryos are very difficult to distinguish.   When pressure is applied at the four until the eight-cell stage at right angles to the polar axis, the *D* cell, as well as each of the others, divides equally, and though some of the larvae resemble the double forms they do not develop far enough to be identified with certainty.   In all cases the cleavage planes were at right angles to the compressing coverslip.

The formation of the double embryos appears here to be correlated with the equal distribution of the substance of the "polar lobe" to the two blastomeres in the first cleavage.   Penners ('24) interpreted his double embryos in Tubifex as due to the distribution of the "pole plasms" to each of the first two blastomeres.   But for the present, the more probable interpretation is that perfect Janus embryos develop only in those cases
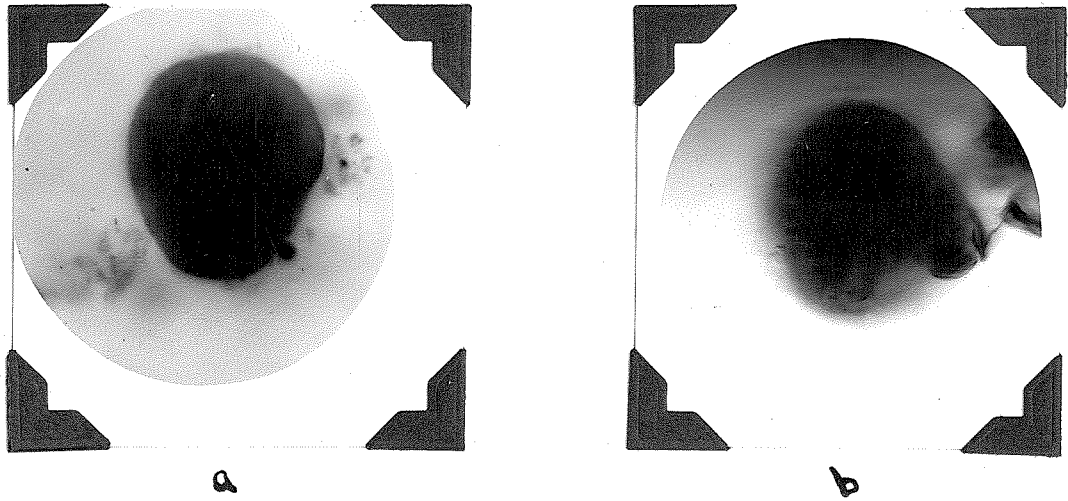
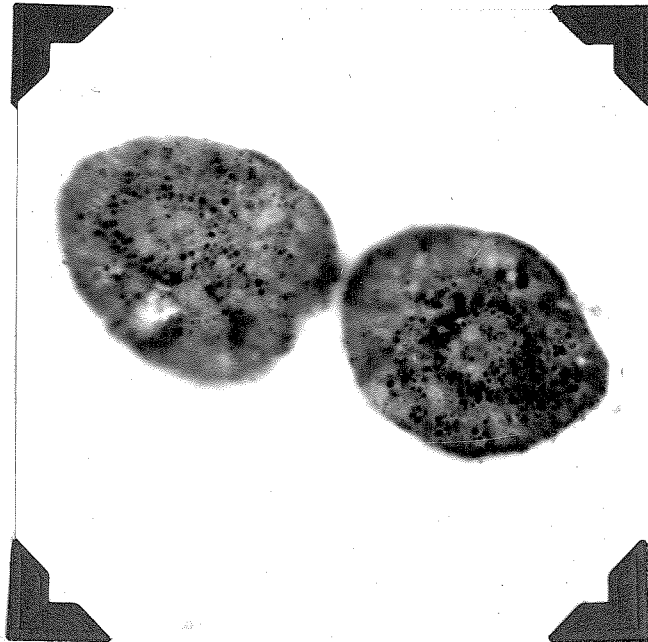Fig.3. Nine-hours old Chaetopterus embryos.
a. Normal embryo
b. Double embryo

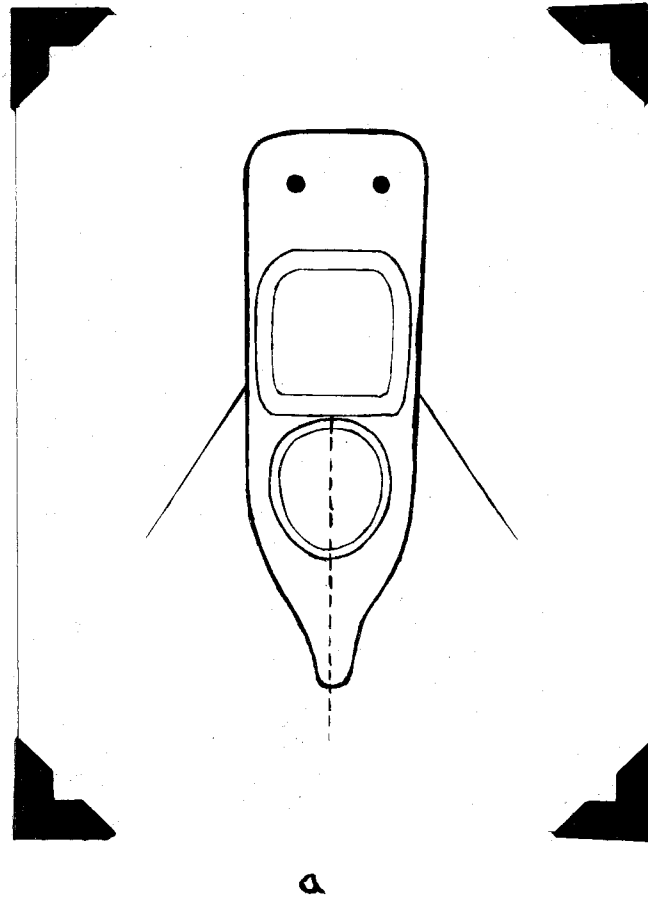Fig.4. Normal 20-hour old embryo, and double embryo
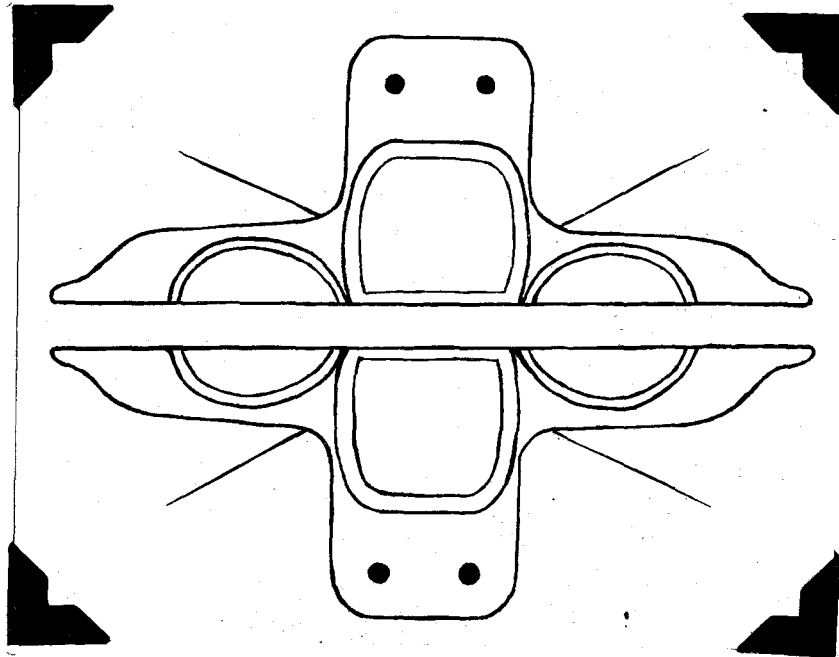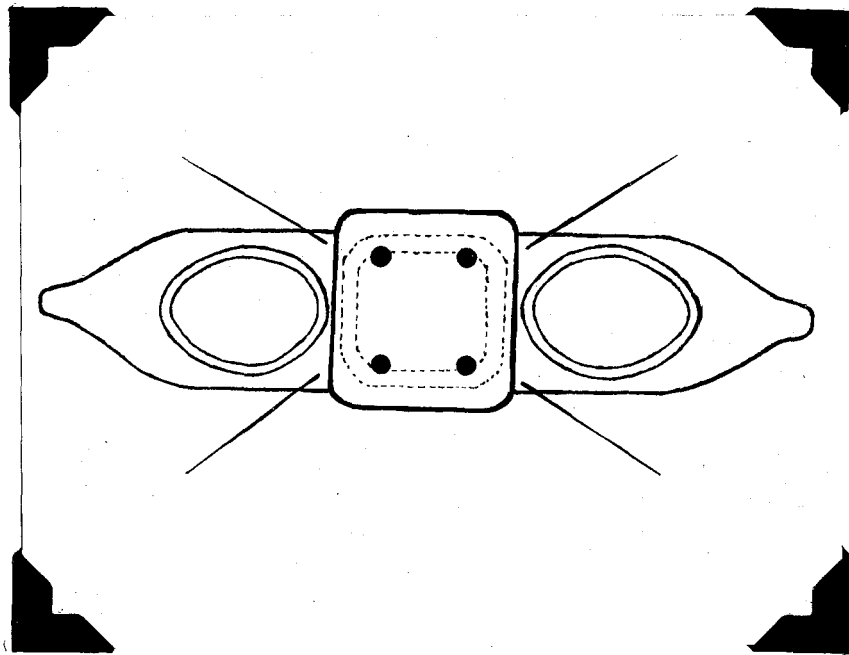of same age.

Fig. 6.   Diagrammatic representation of the composition
          of a Janus-embryo.
          a. Normal embryo cut as indicated by dotted line.
          b. Right and left halves separated and two such
             embryos placed opposite each other, dorsal
             side up.
          c. The two embryos fused along the cut surfaces
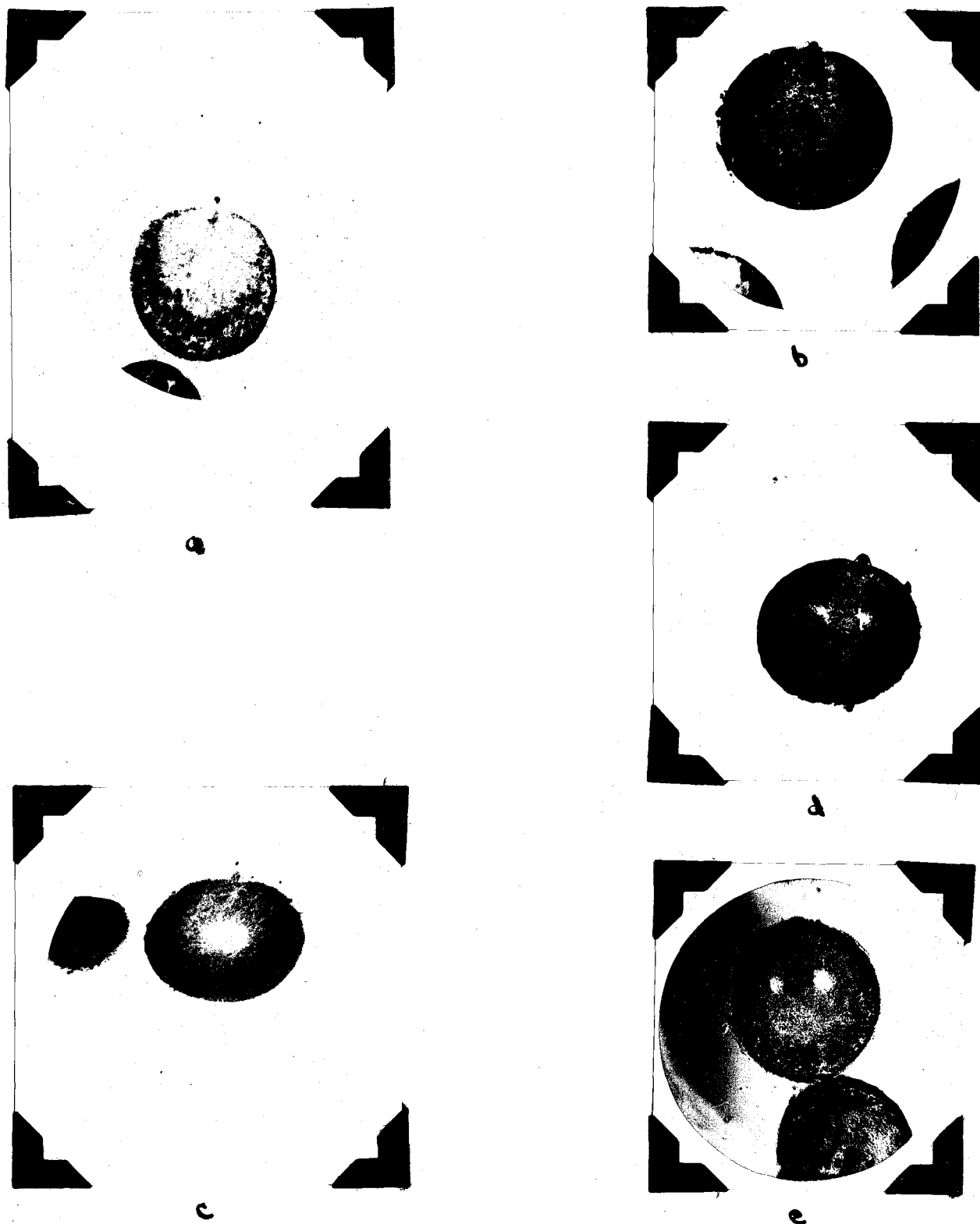   and the head ends fused back to back.

6 b

6 c

Fig. 7a. First polat division.
  b. Anaphase of 2d polar division
  c. Completed 2d polar division
  d. Fusion of the pronuclei, just before
    pear-shaped stage
  e. Pear-shaped stage

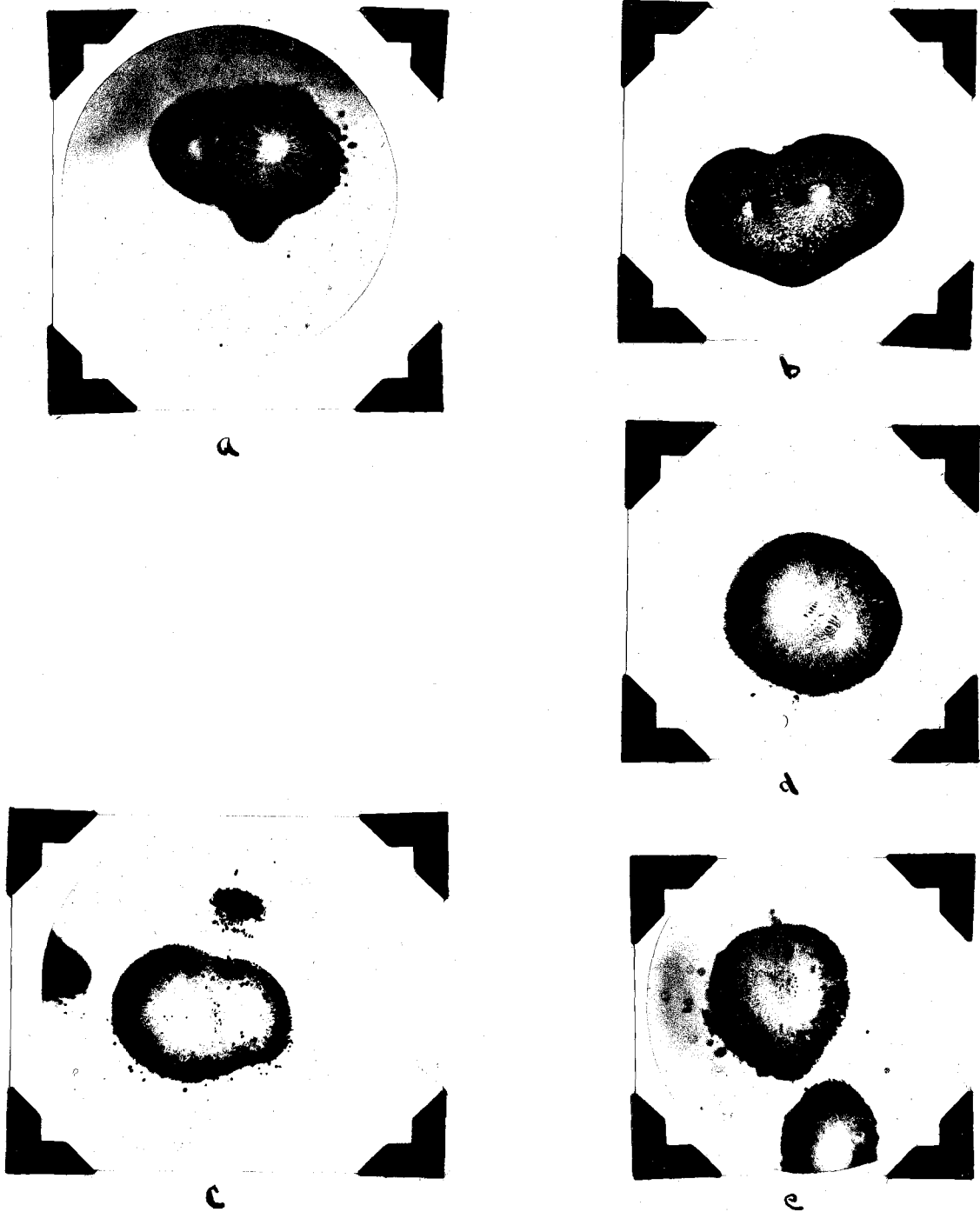Fig.8. a. Sagittal section of normal egg at time of 1st
             division
        b. Sagittal section of equally cleaving egg
        c. Horizontal section of normal egg
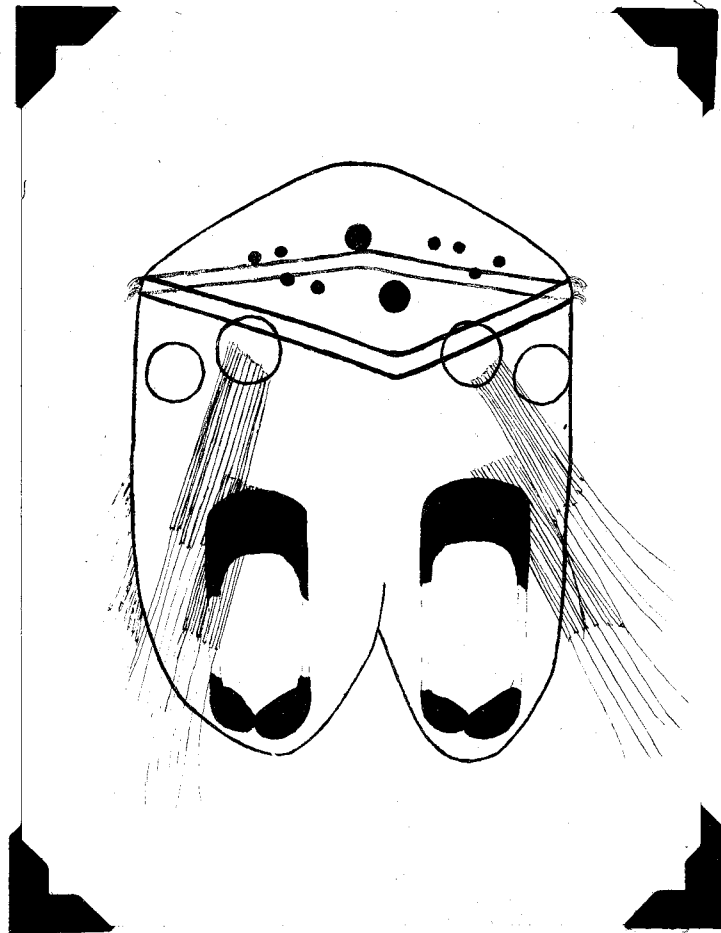   d. Horizontal section of equally cleaving egg
        e. Cross-section of normal egg

Fig. 9.   Nereis double-embryo.