THE EFFECT OF TEMPERATURE ON THE CILIATION OF THE EMBRYONIC

AXOLOTL EPIDERMIS.

A Thesis submitted for the Degree of Master of Philosophy of the

University of Southampton.

by

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INDEX.

ABSTRACT

FACULTY OF SCIENCE

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Master of Philosophy

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EPIDERMIS.

by Linda Hansen

The embryos of Amblystoma mexicanum were allowed to develop at temperatures of $7^{\circ}C, 12^{\circ}C, 20^{\circ}C, 27^{\circ}C$ and $32^{\circ}C$. Embryos from just before closure of the neural folds, to about the 18 somite stage were fixed with formaldehyde/glutaradehyde mixture and then prepared either for light microscopy, scanning or transmission electron microscopy.

From the scanning electron microscope observations, it was found that cilia appeared on the epidermal surface after closure of the neural folds, from the 1-3 somite stage, regardless of the temperature of development. This finding is contrary to Twitty's observation of 1928, that embryos developed at low temperatures (i.e. $7^{\circ}C$) show ciliary determination and activity earlier than room temperature ones.

It was found that at $27^{\circ}C$, extra "secondary differentiation" of cilia occured on already differentiated mucus cells. This was in addition to the differentiation of normal ciliated cells. Transmission electron microscope photographs show that this differentiation of patches of cilia on mucus cells appears to be due to the multiplication of the centriolar material which eventually gives rise to basal bodies and cilia.

The sizes of the $27^{\circ}C$ embryonic cells were larger at a given stage than those of other embryos, suggesting they had undergone less mitoses. It is suggested that perhaps D.N.A. synthesis is channelled in some way into the replication of centrioles, and this change is brought about by the high temperature.

INTRODUCTION.

A General Account of Ciliogenesis.

Cilia are widely occuring throughout the animal kingdom, and cilia or flagella are known to be present in all the major animal groups except the Nematoda. However, within all thses extremely diverse groups their mode of formation is very similar in the majority of cases, and they all have a common structural plan. Even the structure of the basal body seems to be consistent. Lengths of cilia, do, of course, vary so much more, especially in cases where many cilia compound into one multi-ciliate structure. Diameters vary less, and usually lie between 0.15-0.3u.

In the process of ciliogenesis, centrioles play a major role. In most somatic cells, however, there are just one or two centrioles, and they are directly involved in mitosis as is widely known. A clear case illustrating the inter-changeability of these mitotically functioning centrioles and centrioles concerned with ciliation, is nicely given by the developing chick neural epithelium. (Sotelo & Trojillo-Cenoz 1958).

Here, the epithelial cells each form one cilium after their formation by the division of an undifferentiated cell. The two centrioles that were associated with cell division, both migrate to the free cell surface, where one makes contact and the other aligns parallel, immediately beneath the first. The contact between the first centriole and the cell surface, initiates the formation of a ciliary bud and a cross membrane then forms between the distal ends of this outer centriole.

Meanwhile, inside the bud formed by cell membrane protrusion, many small vesicles have been formed, and as the bud elongates, the vesicles are carried up and remain in the tip. Following them up into the elongating bud are ciliary fibrils, which are initiated from the ciliary base. The mature cilium then consists of a cytoplasmic shaft with nine peripheral and two central fibrils.

The central fibrils run parallel to each other and appear tubular in T.S., with a total diameter of $150-250°A$. The peripheral fibrils are arranged to form a cylinder of outside diameter of about $1600Å$. Each fibril is double, i.e. being composed of two sub-fibrils, and these too appear to be tubular in T.S., with an elliptical outline of diameter $200-250Å$. Afzelius 1959, 1961; Gibbons & Grimstone 1960, have shown the presence of two longitudinal rows of "arms" on one of the sub-fibrils of each peripheral doublet. They are actually rods about $120-150Å$ long and $50Å$ thick.

The structure of basal bodies as seen in T.S. is different from that of the ciliary shaft. It is a dense cylindrical structure, usually $4-5000Å$ long and $1500-2000Å$ diameter, with a less dense central region and a dense wall containing the continuations of the peripheral fibrils of the shaft. The outer end of the basal body is continous with the ciliary shaft, while the inner end is open to the cell cytoplasm, or runs into opaque material which is usually connected to a root structure. Root structures of cilia do vary, and can be fine or striated etc, - striated in practically all metazoan cilia.

The peripheral fibrils of the basal bodies appear as triplets and not doublets as in the shaft. There is a definite transition zone between the basal body and the main ciliary shaft where there is this changeover and where the central fibrils from the shaft end. In this region, there is usually a transverse membrane or plate, and above it, usually a spherical or discoidal axosome.

This ciliary structure and the account of its formation given above can be applied to most cases of ciliation, with some individual modifications, but basically following this same pattern throughout the animal kingdom. Without much modification, it is definitely the case as it occurs in the amphibian embryo.

The embryonic amphibian epidermis consists essentially of two main cell types, namely mucus-secreting and the somewhat less numerous, ciliated cells. These two distinct cell types provide a simple system in which to study embryonic cell differentiation. Later morphogenesis depends on complex tissue interactions to a greater extent than earlier morphogenesis. The epidermal ciliation occurs early on in development and possibly in evolution, and hence is a good example of cellular differentiation, and also exhibits a possible relationship to D.N.A. synthesis because of the major role played by centrioles.

Ciliation is particularly interesting when the ciliated cells occur within a large, apparently divergent population such as the amphibian epidermis where the major cell component is the mucus cell. How cells become ciliated and which ones from within the epidermal population become ciliated is another intruiging point. The fact that amphibians are easy to keep and breed, and the epidermis is a relatively easy tissue to examine, are still more reasons why the embryonic amphibian epidermis was chosen for the present study: that is, the differentiatio of ciliated cells.

PREVIOUS STUDIES.

Previous studies of <u>Xenopus laevis</u> have investigated the development and the fine structural changes that take place in the mucus-secreting cells during embryonic development, these cells forming the main epidermal type in both the larval and adult animals (Eakin & Lehmann,1957; Chapman & Dawson, 1961; Mercer, 1964; Perry & Waddington 1966; Billett & Gould 1968; Steinman, 1968; Billett, 1970).

A desciption of both the mucus and ciliated cells in <u>X. laevis</u> is given in work on ectodermal explants from the gastrulae (Billett, 1968) and the morphological differences between the fully differentiated mucus and ciliated cells were studied. In this work, it was also noticed that there are approximately 1:10 ciliated:non-ciliated cells. A more detailed study of ciliation in <u>X.laevis</u> epidermis and trachea by the electron microscope is given by Steinman in 1968. Particular attention is paid to the "de novo" formation of centrioles and how the centrioles align themselves in the supranuclear cytoplasm to form the bases from which the ciliary shafts grow out.

Billett & Gould (1970), in a paper on the fine structural changes that take place in the differentiating epidermis of <u>X. laevis</u>, noted that the cells that become ciliated, i.e. ones with many centrioles and associated precentriolar material, did not occupy positions in the outermost layer of cells, but just below these. Thus it seems that these precursor ciliated cells move up into the outer layer during development, and here become ciliated. Scanning electron microscope work on the appearance of cilia on the axolotl <u>Amblystoma mexicanum</u> embryonic epidermis (Billett & Courtenay 1973) seems to confirm this idea, for it was found that cilia first appeared between the 1-3 somite stages in the anterio-dorsal position, and thereafter, the whole

embryonic surface suddenly became spattered with ciliated cells. As development progressed, the ciliated cells became raised up and fairly prominent compared to their non-ciliated neighbours.

Embryo axolotls have essentially the same epidermal cell types as X. laevis, and are therefore directly comparable. However, axolotls have larger cells which are better suited to scanning electron microscopy, and embryos can be developed over a wider temperature range.

One of the earliest works on ciliation of axolotl embryos, was done as long ago as 1928 by Victor Twitty, and it is this work in particular that comes under scrutiny in this thesis. Twitty developed Amblystoma punctatum embryos at room temperature and at $7^{o}C$, and from his observations of their subsequent ciliation, he claimed that the embryos developed at the low temperature obtained ciliary activity and determination earlier than those developed at room temperature. As criteria for determination of ciliary polarity, he rotated rectangular pieces of ectoderm in such a way as to reverse the anterioposterior axis. If the cilia continued to beat in the direction normal for the grafted piece, he regarded ciliary polarity as determined. In the room temperature embryos, ciliary polarity was determined in 12% at stage 18 (neural folds approaching); in the low temperature ones 77% showed ciliary determination. At stage 19 (folds closing), 89% were determined at room temperature, but 100% were determined at the low temperature.

Due to these results, Twitty postulated that low temperature selectively inhibited morphological development, while permitting the determination of ciliary polarity to proceed more rapidly. Not only

determination, but ciliary activity as well it seems. Presuming that cilia become active as soon as they appear on the surface, it must be that the reported earlier ciliary activity in cold embryos was actually due to the earlier appearance of these cilia. It was therefore decided to check the onset of ciliation of embryos reared at low temperatures against that of embryos reared at room temperature.

In this work in fact, embryos of _Amblystoma mexicanum_ were developed at $7^{\circ}C$, and through a range of temperature up to $32^{\circ}C$. This was to test both high and low temperature effects upon ciliation, and to determine the lethal temperature limits of development. This work involved successfully mating axolotls, incubating the eggs at various temperatures, and examining the embryonic ciliation by transmission and scanning electron microscopy and ordinary light microscopy.

It was hoped to find a correlation between the time of appearance of the cilia/stage and temperature of development, and hence postulate how an external, non-genetic factor such as temperature might influence the path of differentiation or sequence of gene activation.

It was found that within the temperature range investigated, the time of appearance of cilia relative to stage did not vary; i.e. was stage dependent. These results appear to be contrary to Twitty's much earlier observations, and low temperatures do not therefore seem to permit earlier differntiation of cilia.

Apart from demonstrating the stage dependence of ciliation, a very interesting effect on differentiation was noted at the higher temperature of development. This seemed to cause secondary differntiation

of cilia in already established mucus cells. An explanation of this
phenomenon is forwarded in terms of re-activated centriole replication,
and the relationship of this phenomenon, if any, to the phases of the
cell cycle, is explored.

MATERIALS AND METHODS.

CARE AND BREEDING OF THE ANIMALS.

Some preliminary work was carried out on <u>X. laevis</u> embryos, for the sake of comparison later with the axolotls. To obtain the necessary embryos, adult Xenopus were induced to produce fertile eggs spontaneous -ly by the following method:- Four pairs were chosen, and isolated in tanks. The females were given a priming injection of 100i.u. of gonadotrophin, the males 50i.u. The following day, the main dose was given, i.e. 350i.u. for the females, and 150i.u. for the males. By the next day, the pairs had coupled up and started laying eggs. The eggs were collected and left at room temperature (18°C) to develop.

This method of breeding, however, was not employed with the axolotls. Axolotls are more sensitive animals and are not suitable for injection. They are not really suitable for artificial fertilisation either, because this involves sacrificing both partners, as the female eggs must not be released into the water before fertilisation. The eggs therefore have to be taken from inside her.

Obtaining embryos was no great problem though, because spontaneous mating occured during the normal breeding season, which is in the winter. Axolotls originate from Mexico, and winter is their normal breeding season there. Under laboratory conditions, however, the breeding season can extend from November/December until June, and they can be encouraged to breed more than once by 'chilling, simulating the onset of winter. Initially, in breeding condition, no preliminary conditioning is usually necessary to insure mating.

Suitable individuals were chosen at the beginning of the breeding season, and paired up in clean tanks of fresh cold water, supplied with plenty of stone and rock. If a change of water and pairing did not induce the animals to mate, slightly more drastic measures were taken. These involved icing down the water to a temperature below that which could be obtained by running tap water. In April, the temperature of the tank water was between $17^{\circ}-18^{\circ}$C and that of the fresh running tap water $11^{\circ}-13^{\circ}$C. If this tap water was iced down, however, the temperatures was taken down to about 8°C. In May, these temperatures were still approximately the same. By June, often the tank water temperature was 19°C and running water 15°C. The fresh tap water was then iced down to about $9^{\circ}-10^{\circ}$C.

An adequate supply of stones in the tanks is fairly essential, because mating involves the initial laying of spermatophores by the males on to a substrata suitable for adhesion. These spermatophores consist of a small white packet of sperm covered and anchored by an inverted U-shaped jelly sheath, so that it is kept in position a few mms. above the substratum level.

Once deposited, the male will lead the female round until she is positioned exactly above the spermatophore, enabling it to be taken up directly into the vagina for internal fertilisation. Spawning may be expected a few hours after mating, i.e. 18-30 hours after they were coupled. Most eggs are usually laid within 24 hours, although this has been known to be lengthened by low temperatures (Humphrey '73).

The colony of axolotls used, consisted of both black and white members, and embryos of both types were obtained and examined. If a pair seemed "incompatible", i.e did not show any signs of mating after a couple of chillings, they were sometimes re-mated with other individuals. All matings were done in the late afternoon/early evening to avoid too much daytime disturbance, as they are quite easily upset. A record was kept of all matings, re-matings and the temperature of the chillings, and the number of eggs laid.

PREPARATION OF THE EMBRYOS.

After the eggs had been laid, (between a few dozen and a couple of hundred) they were removed carefully from the stones with blunt forceps and transferred to small glass or plastic Petri dishes for incubation (about ten per dish). The embryos were then incubated at either $7^{\circ}C, 12^{\circ}C, 20^{\circ}C, 27^{\circ}C$ or $32^{\circ}C$. The temperature was kept constant at $7^{\circ}C$ by putting the eggs in conical flasks and incubating these in an aquarium in the cold room. The aquarium was heated against the background temperature of the cold room, which was $5^{\circ}C$, and kept constant at $7^{\circ}C$. The other constant temperatures were obtained by putting the dishes of embryos into carefully regulated thermostatic incubators. Incubation was attempted at $5^{\circ}C$ in an aquarium in the cold room, but failed because frequent use of the room meant that the temperature fluctuated rather.

All the embryos during incubation were checked and staged at fairly regular intervals, using Laevitts table for <u>Amblystoma punctatum</u> in Experimental Embryology, R. Rugh as a guide. Cleavage is known to begin 6-8 hours after laying, at $20^{\circ}C$. Representative stages from before the closure of the neural folds to about the 18 somite stage were chosen to be fixed and examined.

FIXATION OF THE EMBRYOS.

With both Xenopus and the axolotls, the same method of fixation was used. Initially, the jelly coats were very carefully removed with watchmakers forceps. As the embryos were to be immediately fixed, the decapsulation was done in aged tap water. After jelly coat removal, the freed embryos were picked up in a broad-mouthed pipette, and transferred to cold ($5^{\circ}C$) formaldehyde/glutaraldehyde mixture, buffered at pH 7.0 with 0.1M sodium cacodylate (Karnovsky, 1965).

Double fixation with aldehyde and osmic acid is possible, but permeability of glutaraldehyde is good, whereas that of an osmium tetroxide solution is poor, i.e. 50u/4 hours, hence for large specimens like these embryos, osmium tetroxide treatment would not be of much use. Embryos were fixed in the formaldehyde/glutaraldehyde mixture for at least 24 hours. After fixation, they were either prepared for scanning or transmission electron microscopy.

PREPARATION FOR SCANNING ELECTRON MICROSCOPY.

The embryos were firstly washed with the O.1M cacodylate buffer and then dehydrated by one of two methods. The method most frequently used was acetone dehydration, but freeze-substitution by iso-pentane was also used for comparison.

Acetone dehydration simply involved passing the fixed embryos through a series of acetone/water concentrations:- 25,50,75, & 90% v/v, for two hours each. They then had two treatments in absolute acetone, the first for two hours, the second for 24 hours. The dehydration process leaves the embryos very light and brittle, but very little or no distortion of the embryonic surface occurs.

For the freeze-substitution method, the iso-pentane was carefully decanted into thick glass-stoppered pyrex tubes and cooled in a cannister of liquid nitrogen until it started to become viscous. The boiling point of liquid nitrogen is about $50^{\circ}C$ below the melting point of iso-pentane, which becomes viscous just before it freezes. Liquid air cannot be used as the cooling agent, because it will form an explosive mixture with the volatile iso-pentane (Boyde & Wood 1969).

The washed embryos were pipetted onto small, flattened squares of aluminium foil, the excess buffer drawn off, and the embryos plus foil plunged into the iso-pentane. The pyrex tubes were then stoppered with their ground glass bungs, and put in a deep freezer set at $-40^{\circ}C$, along with a medical flat of absolute alcohol. A few hours later, the tubes were checked to see that the stoppers were not frozen up. After approximately 24 hours, the iso-pentane from the tubes was decanted off and replaced with the already cooled absolute alcohol, and returned to the deep freeze. A week later, the embryos were taken out of the freezer and brought up to room temperature.

This method of freeze-substitution was not often used, because as was subsequently seen, it produced only comparable results with the simpler and quicker acetone dehydration method. Also, the embryonic surfaces were often cracked by expansion of their volume caused by the freezing of moisture in the tissues.

After either dehydration method, the embryos were allowed to dry in air, and then stored in aluminium foil dishes in a vacuum desiccator over phosphorus pentoxide. The next step was to mount the specimens on special stereoscan metal stubs, which fit into a socket in the specimen chamber at the base of the stereoscan column. The mounting material used was fine conductive silver paste (Johnson Matthey Metals London), which was mixed before use, with amyl acetate. The amyl acetate moistened the paste for easier handling. Special perspex stagings were made to hold the stubs steady, and for storage in air-tight glass dishes.

Mounting of the embryos was done under a binocular microscope to enable accurate positioning of the embryos in the paste. The moistened silver paste was spread on the centre of the stub and the embryo carefully positioned on it with the aid of watchmakers forceps. After placing, the specimen was very gently pressed down into the paste, to ensure a secure attachment. The embryos were then left for several hours so that the paste could harden up.

When the paste had hardened, the embryos were then ready for coating. Carbon and/or metal coating is usually applied to stereoscan specimens, to ensure electric conductivity from the specimen surface to the stub holder (Fujita, Tokunaga & Inoue '72). Gold-palladium alloy is highly effective in generating secondary electrons, and since a film can be made on the specimen, it is not easily oxidised (JEOL News May 1973). Gold-palladium was thus chosen to coat all the embryos. Also, as almost all biological specimens are of low conductivity, it is there -fore necessary to coat with vacuum evaporated metal to prevent charge-up and damage by heat.

An Edwards coating unit was used, and two embryos were coated at a time, evaporating three 1cm pieces of gold-palladium wire for each coating. Whilst the wire was being evaporated from the filament, the embryos were rotated around their vertical axis to ensure an even surface coating. The actual thickness of the coating was approximately 30nm. This was all the preparation that was needed for the scanning electron microscope.

PREPARATION FOR TRANSMISSION ELECTRON MICROSCOPY.

Fixed embryos were washed overnight in ice-cold 0.1M sodium cacodylate buffer, and then post-fixed with ice-cold 1% osmic acid buffered with the cacodylate, for two hours. This double stain is very popular, as the glutaraldehyde fixes proteins well, and the osmium fixes membranes and organelles (Mereer & Birbeck 1961). When using this double fixation method, however, a thorough washing with the is essential, because otherwise there is a strong possibility of an interaction between excess aldehyde and osmium, giving a fine, dense precipitate of reduced osmium.

After post-fixation, the embryos were dehydrated through a series of graded alcohols into absolute alcohol, then through propylene oxide, propylene oxide/araldite mixture, and finally into two changes of araldite. The specimens were usually left overnight in 55:45 propylene :araldite mixture, to ensure thorough penetration of the araldite into this rather thick and bulky material. The embryos were embedded into the final araldite on their sides in flat, shallow aluminium dishes. This facilitated easy orientation.

These flat araldite blocks when polymerised, were cut up into smaller pieces containing an embryo, with a small steel hacksaw. The blocks thus obtained were trimmed on a pyramitome and cut on the L.K.B. using glass knives and a flat chuck holder. Silver/ light gold sections were cut and picked up on uncoated grids and stained with uranyl acetate and lead citrate. The grids were examined in the Philips 300 E.M.

LIGHT MICROSCOPY.

1-2 um sections were cut from the araldite blocks using a pyramitome, and they were fixed onto glass slides and stained with a 1% solution of toluidine blue on a hot plate for 5-10 minutes.

These sections were examined to gain a general picture of the differences between the cell types and to do a cell count of them. Using equivalent stages of embryos developed at the different temperatures, a cell count of the ciliated:non-ciliated cells for the different temperatures was done, to see if there was any variation according to temperature.

OBSERVATIONS.

Firstly, an account of the speed of development at the different temperatures is presented, with a series of graphs.

A large section of cytological observations and quantitative results follow, with lastly a small section dealing with ciliation of Xenopus laevis embryonic epidermis.

OBSERVATIONS.

Comparison of embryonic development at different temperatures.

1 set of readings of the relationship between the length of time of incubation and stage of development reached by the axolotl embryos was chosen as an example for each incubation temperature (7°C, 12°C, 20°C and 27°C), and plotted on separate graphs (1-4). Graphs for 5°C and 32°C are not included, because no consistent readings at 5°C could be obtained due to fluctuation of the cold room temperature, and the embryos incubated at 32°C all died before completing gastrulation.

For ease of comparison, the points plotted on these graphs were all standardised to obtain the stages reached at 20 hour intervals. Originally, the actual recordings were not done every 20 hours, but from graphs plotted from the original readings, the stage reached at each 20 hours was able to be read off, and used as the information for the graphs presented.

One graph of the raw data for a batch of embryos incubated at 12°C is included to show how the original graphs were recorded. The points of the graph correspond to when the embryos were actually checked and staged after laying.

The sixth graph included, is one of graphs of all four temperatures averaged out. Each individual graph on the page was compiled by the records of at least three incubation runs. The limits of the variance of the stage reached at each point is marked on each graph by vertical bars.

From graph 6, it can be clearly seen that the speed of development is directly proportional to increased temperature. One interesting point to note is, however, that there appears to be a break in continuity at the beginning of the 7°C graph at about 80 hours development. The graph then appears to continue linearly, but at a slower rate and there is another smaller break at about 460 hours, and the rate seems to slightly speed up again here. The other three graphs all appear quite smoothly linear in comparison.

From graph 6, generation time can be approximately worked out. For example, at stage 24, generation time is three times longer at 12°C than at 27°C. At 7°C it is 12 times longer :-

From graph 6:

It takes 50 hrs to reach stage 24 at 27°C

" " 100 hrs " " " " at 20°C

" " 160 hrs " " " " at 12°C

" " 600 hrs " " " " at 7°C

Between 27°, 20° and 12°C, there are 7° and 8°C difference respectively, and the difference in the time of reaching stage 24 between these temperatures in the above table, is 50 and 60 hours respectively, which shows a fairly good linear relationship. However, although the difference between 12° and 7°C is only 5°C, the difference from the above table of time of development is 440 hours, and clearly not to the same ratio compared with the other temperatures. Since there is such a great difference at 7°C, this is probably just about the lower limit of development. The upper limit is between 27°-32°C, probably about 28°-29°C.

GRAPH OF EMBRYONIC DEVELOPMENT OF AXOLOTLS AT 7°C.

This graph is of one set of readings obtained from embryos that were incubated at 7°C. Altogether three runs at 7°C were done and their averaged reading appears on graph 6.

Unfortunately, to keep the scale of this graph the same as those at the other incubation temperatures, the graph has had to be cut into three sections 1-3.
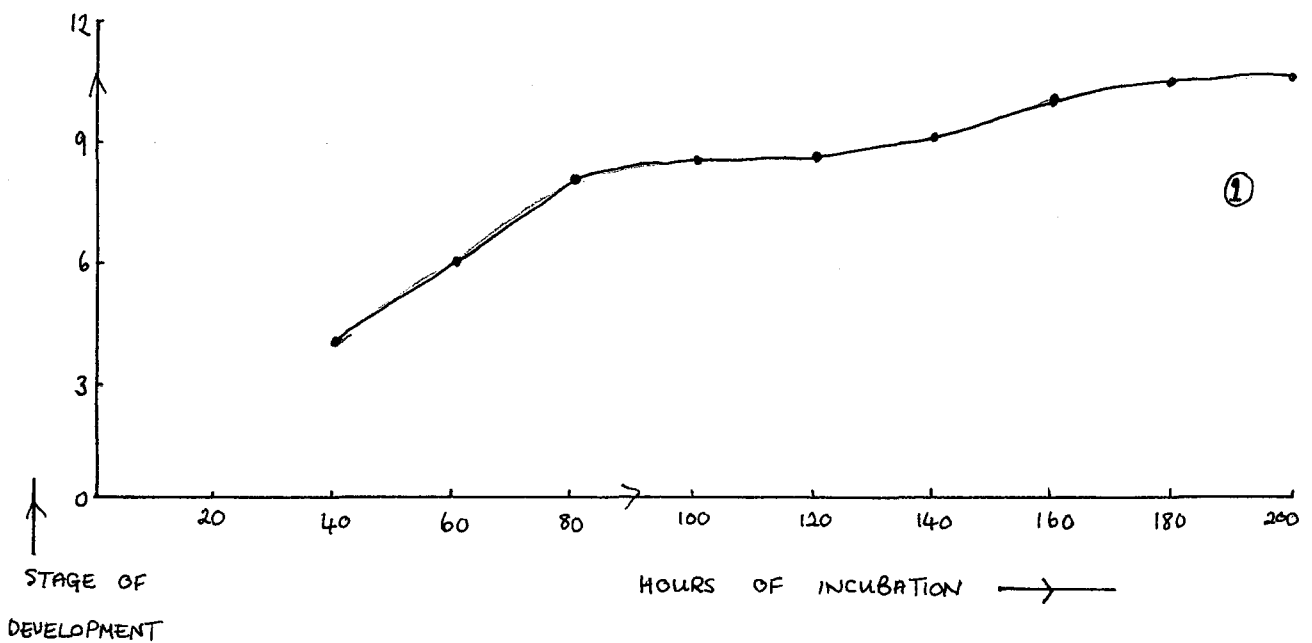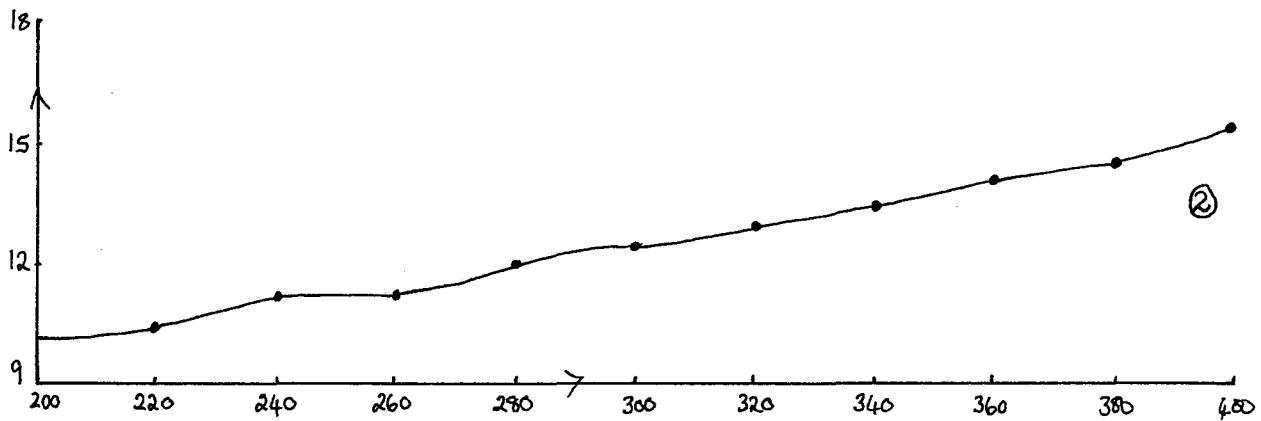
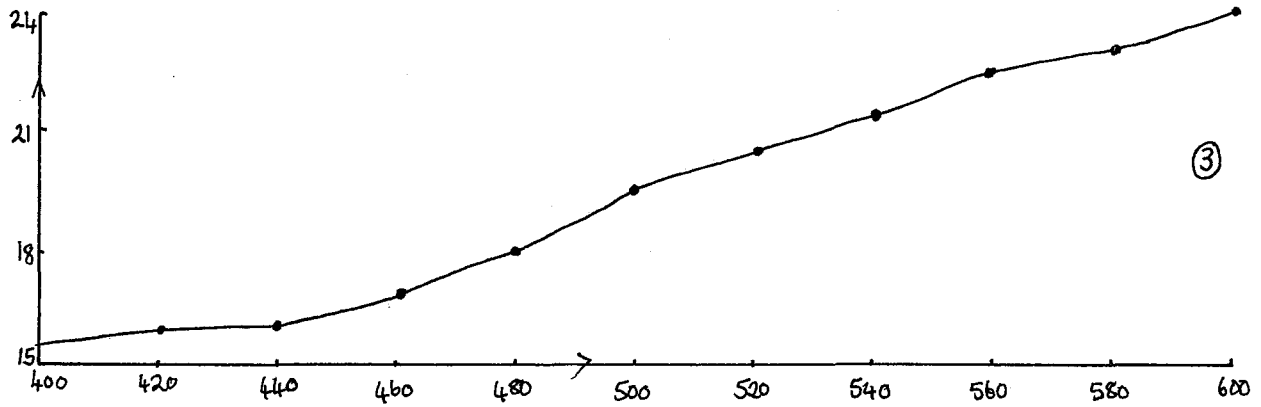   1) is from 0-200 hours of development

        2) " " 2-400 "   "   "

        3) " " 4-600 "   "   "

If put together, the graph would appear thus:-
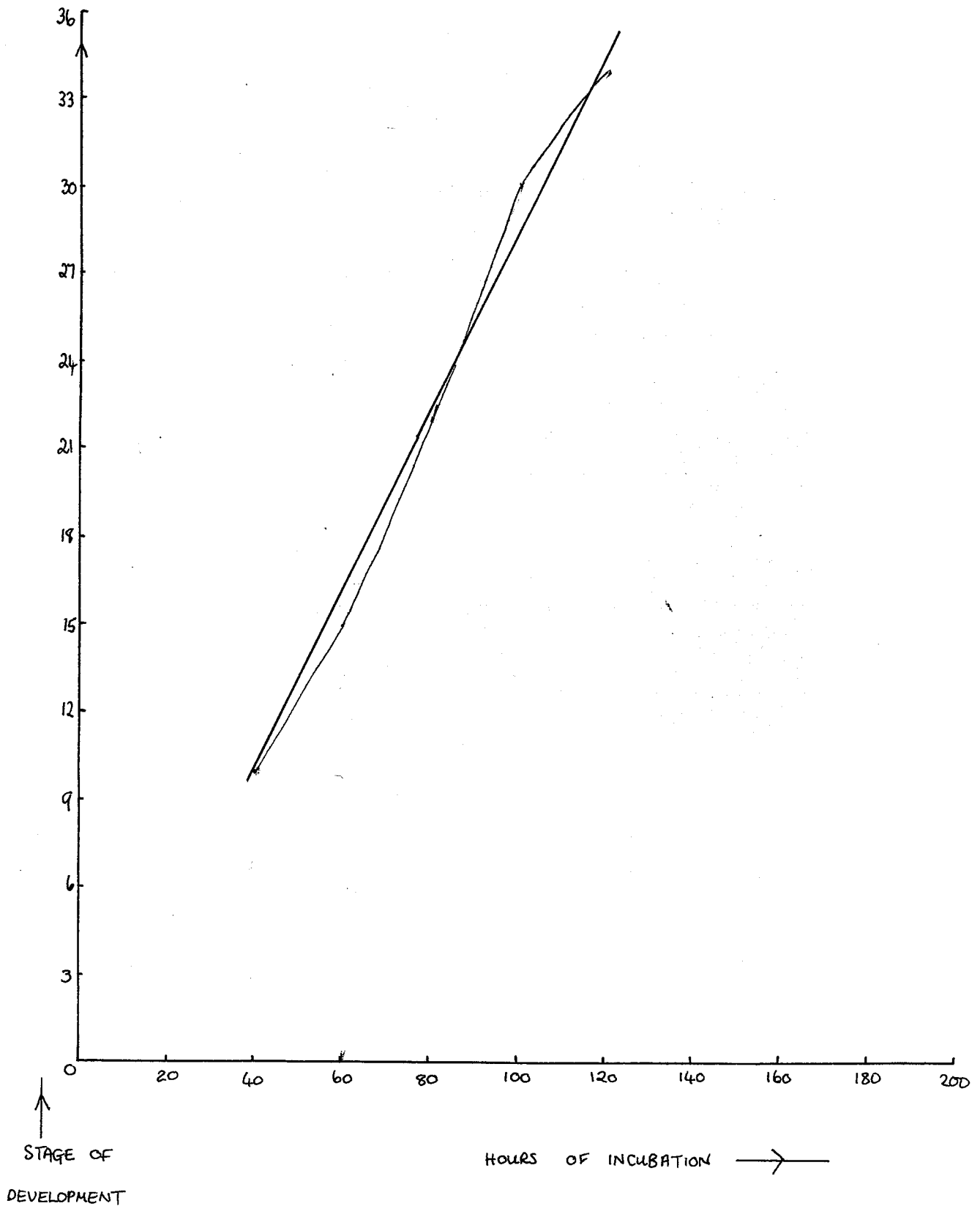
NOT TO SCALE.
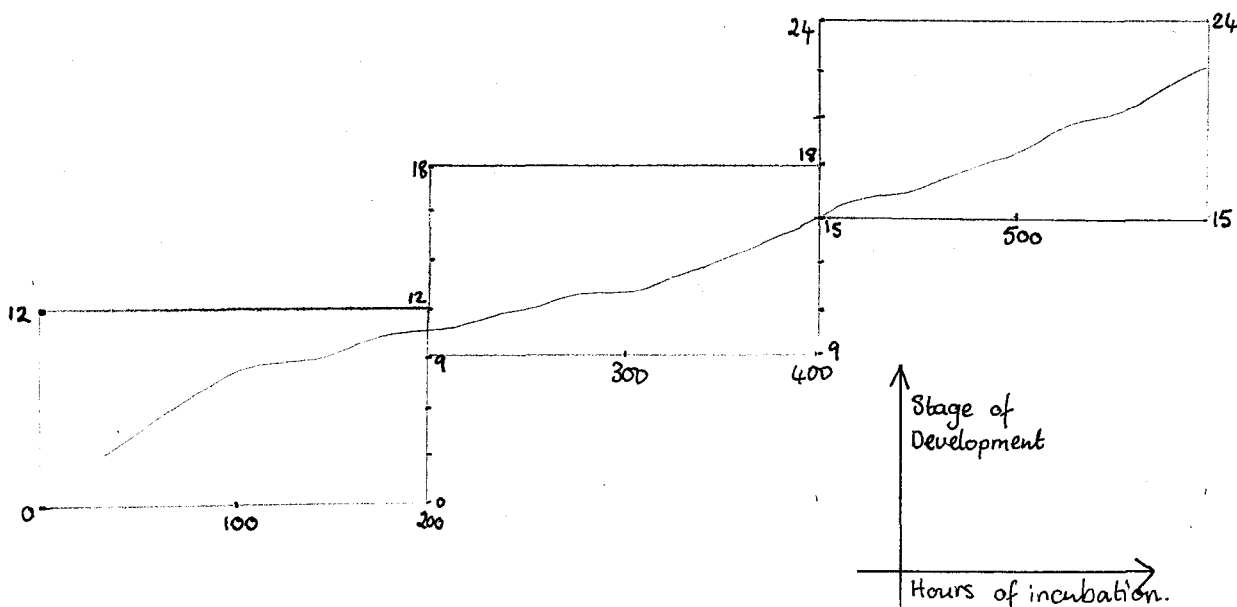
GRAPH OF EMBRYONIC DEVELOPMENT OF AXOLOTLS AT 7°C.

GRAPH OF EMBRYONIC DEVELOPMENT OF AXOLOTLS AT 12°C.

This graph is of a set of readings recorded for one batch of embryos developed at 12°C. As with the 7°C graph, the points plotted on the graph are at 20 hour intervals for standardisation. The points were obtained by reading off the stage of development reached after each 20 hours from the original graphs on which the points were plotted as the embryos were checked, i.e. at irregular intervals.

Four runs were done at this temperature and their average reading is recorded graphically on graph 6.

The faint line indicates the actual line of the graph, and the bold line the nearest straight line through it.

N.B. The calibrations on the vertical axis are not strictly linear in a temporal sense, but represent the conventional stagings as used by other embryologists.

GRAPH OF EMBRYONIC DEVELOPMENT OF AXOLOTLS AT 12°C.
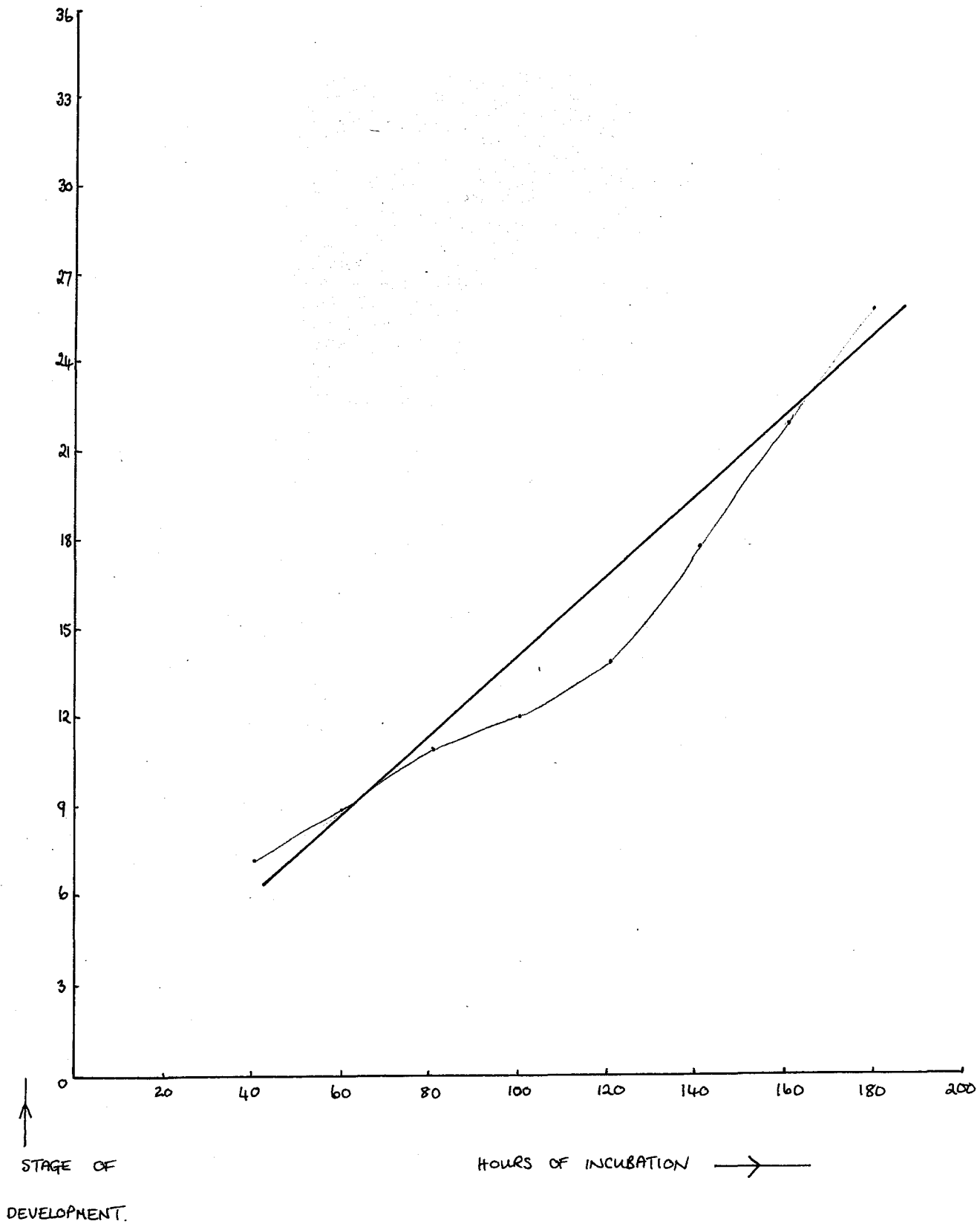
STAGE OF

DEVELOPMENT.

HOURS OF INCUBATION ⟶

GRAPH OF EMBRYONIC DEVELOPMENT OF AXOLOTLS AT 20°G.

This graph is of a set of readings recorded for one batch of embryos developed at 20°C.

Three runs were done at this temperature and their average reading is recorded graphically on graph 6.

The faint line indicates the actual line of the graph, and the bold line the nearest straight line through it.

GRAPH OF EMBRYONIC DEVELOPMENT OF AXOLOTLS AT 20°C.

STAGE OF

DEVELOPMENT

HOURS OF INCUBATION

GRAPH OF EMBRYONIC DEVELOPMENT OF AXOLOTLS AT 27°C.

This graph is of a set of readings recorded for one batch of embryos developed at 27°C.

Three runs were done at this temperature and their average reading is recorded graphically on graph 6.

The faint line indicates the actual line of the graph, and the bold line the nearest straight line through it.

GRAPH OF EMBRYONIC DEVELOPMENT OF AXOLOTLS AT 27°C.

STAGE OF
DEVELOPMENT

HOURS OF INCUBATION ⟶
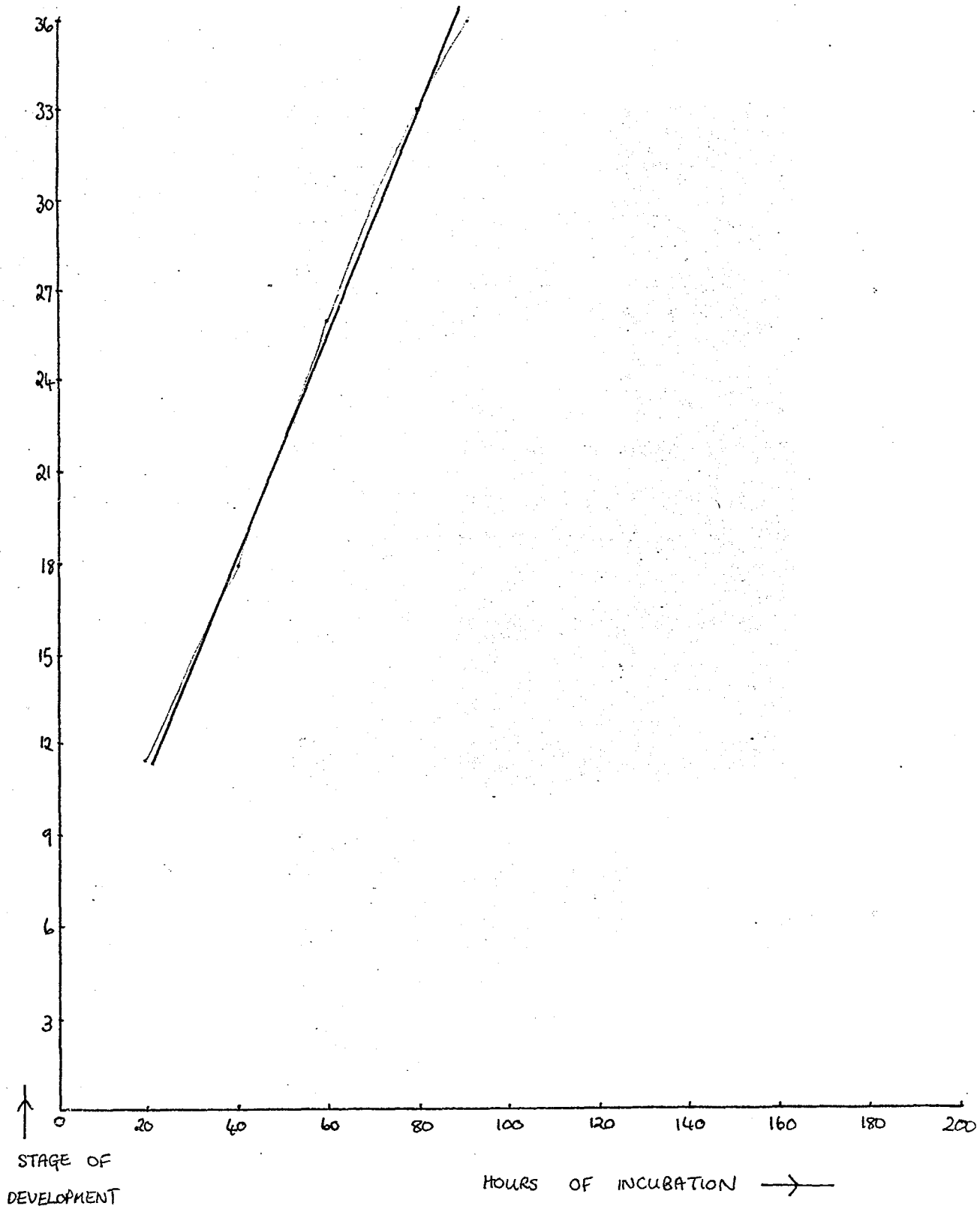
AN ORIGINAL GRAPH OF DEVELOPMENT OF ONE BATCH OF EMBRYOS AT 12°C.

This is an original graph of the record of development. The
points on this graph correspond to when the embryos were actually
checked and staged after laying.

AN ORIGINAL GRAPH OF DEVELOPMENT OF ONE BATCH OF EMBRYOS

AT 12°C.

STAGE OF
DEVELOPMENT

HOURS OF INCUBATION. ⟶
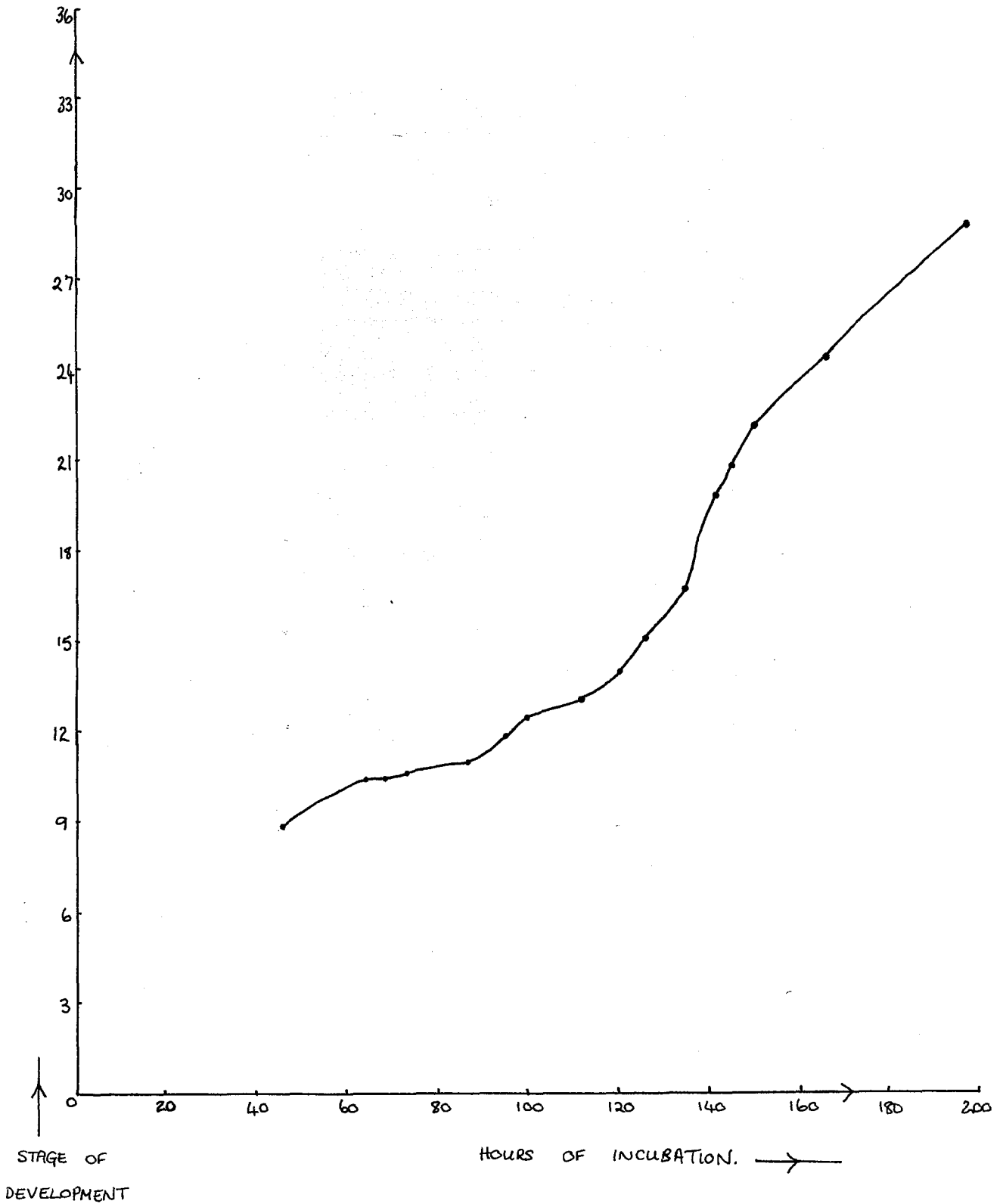
OVERALL GRAPH OF THE EMBRYONIC DEVELOPMENT OF AXOLOTLS AT

DIFFERENT TEMPERATURES.

Here, averaged readings for each temperature are plotted on a
smaller scale to the other graphs, to enable the $7^{o}C$ graph to be
included. The limits of variance of stage reached at each 20 hour
interval is marked on the graphs by vertical bars.

GRAPHS OF THE EMBRYONIC DEVELOPMENT OF AXOLOTLS
AT DIFFERENT TEMPERATURES.

S T A G E   O F   D E V E L O P M E N T

Hours of incubation ⟶

A

B

C

D
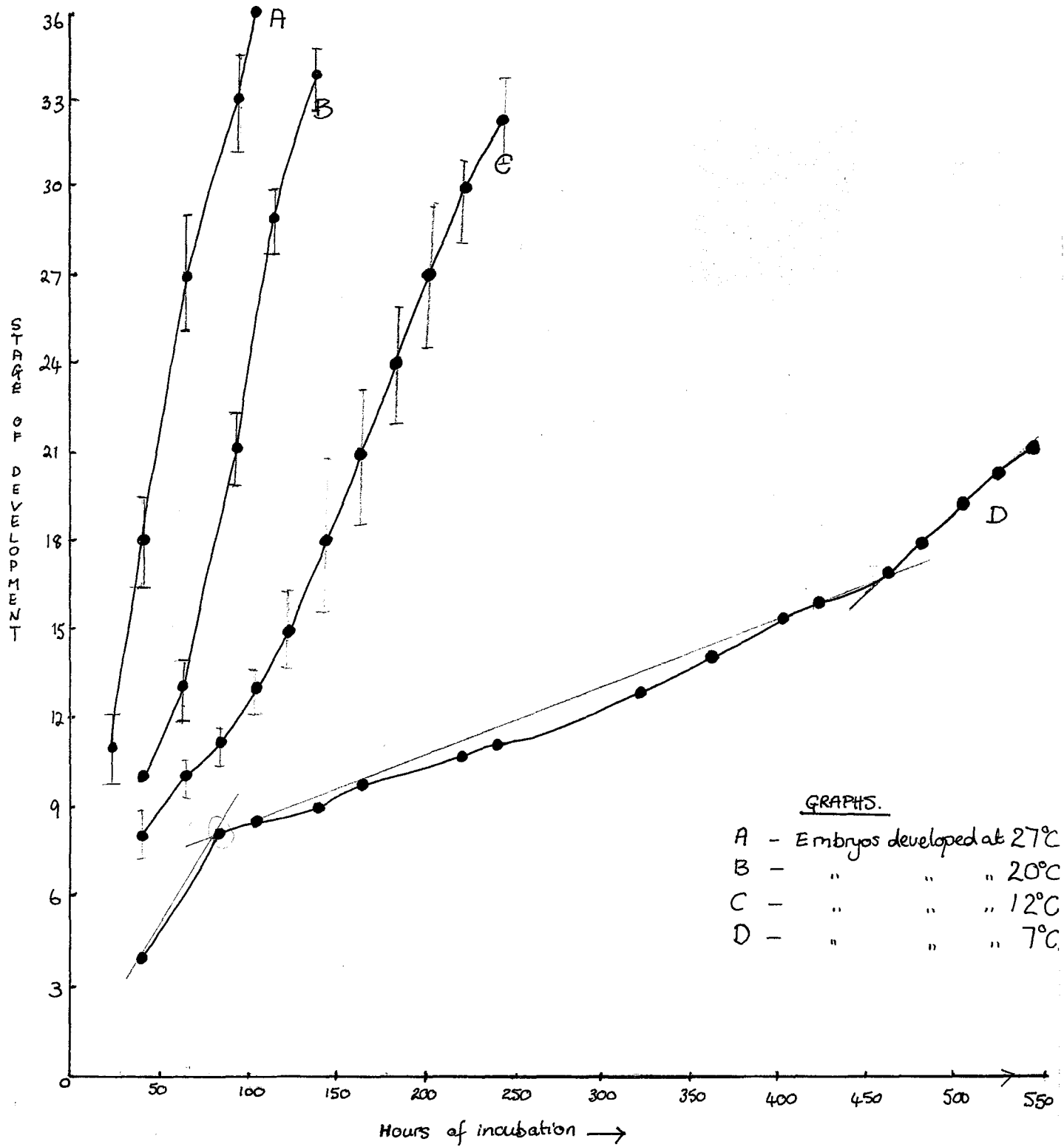
GRAPHS.

A – Embryos developed at 27°C
B – ,,   ,,   ,, 20°C
C – ,,   ,,   ,, 12°C
D – ,,   ,,   ,, 7°C

CYTOLOGICAL OBSERVATIONS.

In the following section, an account is given of the T.E.M. and
S.E.M. work on the embryos developed at $7^{\circ},12^{\circ},20^{\circ}$ and $27^{\circ}C$.

Next are given the morphological observations of the lu araldite
sections of these embryos observed by light microscopy.

Quantitative results follow and the ratios of ciliated:non-ciliated
cells/embryo are given from cell counts made from the lu araldite
sections.

Next are given tables illustrating the difference in cell sizes
between embryos developed at different temperatures, as seen by S.E.M.

Lastly, there is a short account of the ciliation of Xenopus laevis
embryonic epidermis.

SCANNING AND TRANSMISSION ELECTRON MICROSCOPE OBSERVATIONS ON THE

DEVELOPING EPIDERMIS OF AXOLOTLS.

An account is given in the following pages of the observations
firstly of the scanning, and secondly of the transmission electron
microscope work, separately for each incubation temperature. The
order of the accounts is from the $7^{o}C$ incubations to the $27^{o}C$, and
the observations are reported in developmental sequence, i.e. from
when the cilia first appeared, to full ciliation. The table below,
summarises from the observations when the embryos first become
ciliated at each temperature, when they are fully ciliated, and how
many embryos were studied to substantiate these findings.

	Stage 1st cilia	Stage all cilia	No. of observations
$7^{o}C$	1-3 somites	By 8 somites	3
$12^{o}C$	" "	" " "	4
$20^{o}C$	" "	" " "	3
$27^{o}C$	" "	" " "	5

With the scanning electron microscope (S.E.M.) work, every coated
embryo was examined all over, to ensure that no parts of their surface
were non-ciliated, or that no ciliated cells were missed in the early
developmental stages. The whole embryo can be easily viewed at x20
and x50 magnification, and while at this magnification, the specimen
can be tilted or rotated in the column for orientation at practically
every angle. The magnification can then be boosted to x100,x500, and
x1000, by which time the ciliated cells, if present, are usually visible.
Better and closer views of these cells are gained with magnifications
of 2-5000x, x10,000, and x20,000 was also used for finer detail of the
cilia or microvilli.

The embryos were scanned systematically from head to tail at x1,000 to ensure location of the ciliated cells. The actual position of a ciliated cell relative to the rest of the embryo at higher magnifications can easily be found by reducing the magnification right down to x100 or even x50, and the cell in question will be exactly in the centre of the picture.

DEVELOPMENT OF THE EMBRYONIC EPIDERMIS AT 7°C.

Scanning Electron Microscopy.

As mentioned in the table, cilia first appeared when the neural folds had closed, at 1-3 somites, fig. 1. In fig. 2 some cilia can be seen to have emerged already, but not all. Small microvilli are clearly visible on the ciliated cell surface, fig. 3. The neighbouring, smooth surfaced, lighter cells are mucus cells. By the 3-6 somite stage, cilia are longer, figs. 4 and 5, and by the 8 somite stage, the ciliated cells have their full complement of cilia figs. 6,7 and8, and as the embryos grow a little older, the cilia seem to slightly elongate.

The distribution of the ciliated cells appeared to be fairly random, as was expected from previous observations (Billett and Courtenay 1973). They did not appear to occur in groups or clusters but singly and spaced out over the entire surface, figs. 4,9,10 and 11.

The cell boundaries in these specimens can be very well observed figs. 4,9,12 and 13, and there appears to be no distortion of the surface at all. All the cells seem to be fairly rounded up when mature, particularly the mucus cells. The ciliated cells in this material often appear slightly lower than the mucus cells.

ABBREVIATIONS.

ANT   anterior

axo b.   axonemal bodies

b b   basal body

C   centriole

c f   central fibril

c/cilia   cilia

cis   cisternae

c m   cell membrane

D   desmosome

E R   endoplasmic reticulum

G   Golgi apparatus

l b   lipid body

M C   mucus cell

mito   mitochondria

m v   microvilli

M ves   mucus vesicle

N   nucleus

P C   procentriole

p f   peripheral fibril

POST   posterior

R   ribosome

S R   striated rootlet

t f   tonofilaments

t m   transverse membrane

T W   terminal web

ves   vesicle

y p   yolk platelet

Fig. 1.

Lateral view of a 3 somite stage embryo. The embryo is embedded in silver paste and mounted on a stereoscan stub. x 150.

Fig. 2.

A cell from one of the early ciliated embryos. The cilia have not emerged completely, but the cell surface is already covered in microvilli. x 6000.

1

POST

ANT

2

cb
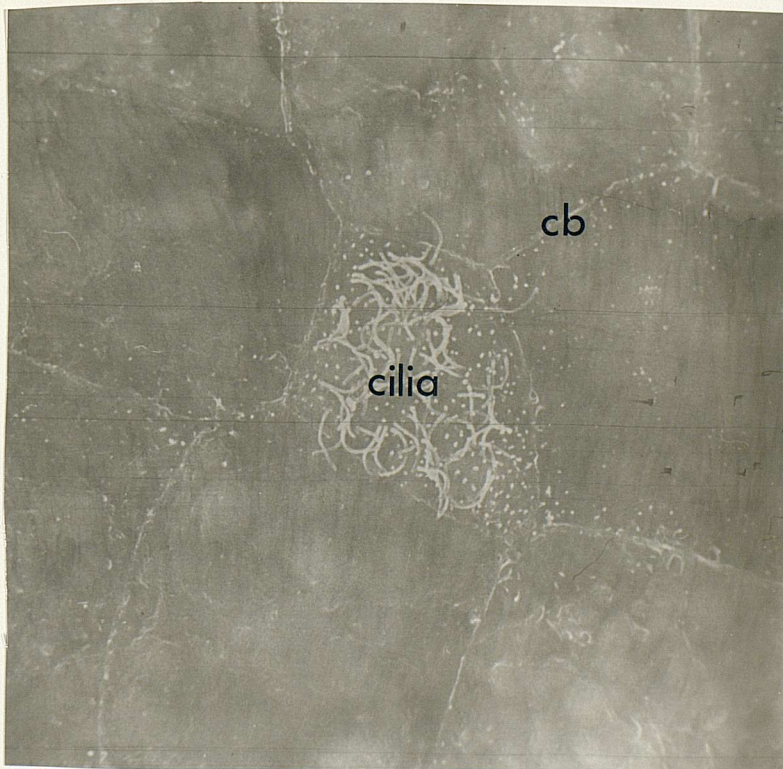
cilia

7°C EMBRYO.

Fig. 3.

A clear picture of the microvilli on an early ciliated cell.

x 15000

Fig. 4.

3-6 somite embryo where the cilia are longer, and the mucus
cells more rounded up.

x 6000.

3

mv

4

MC
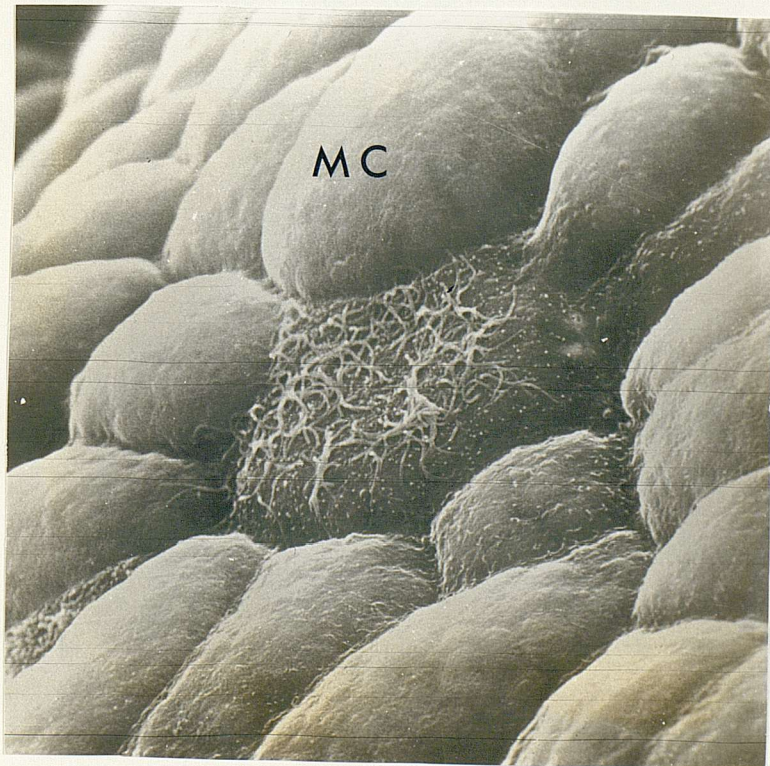
7°C EMBRYO.

Fig. 5.
_____

Another ciliated cell from a 3-6 somite embryo, where the cilia

are not quite full length.

x 6ooo

Fig. 6.
_____

The cilia from this 8 somite embryo have attained their full

length practically.

x 15000.

5

6
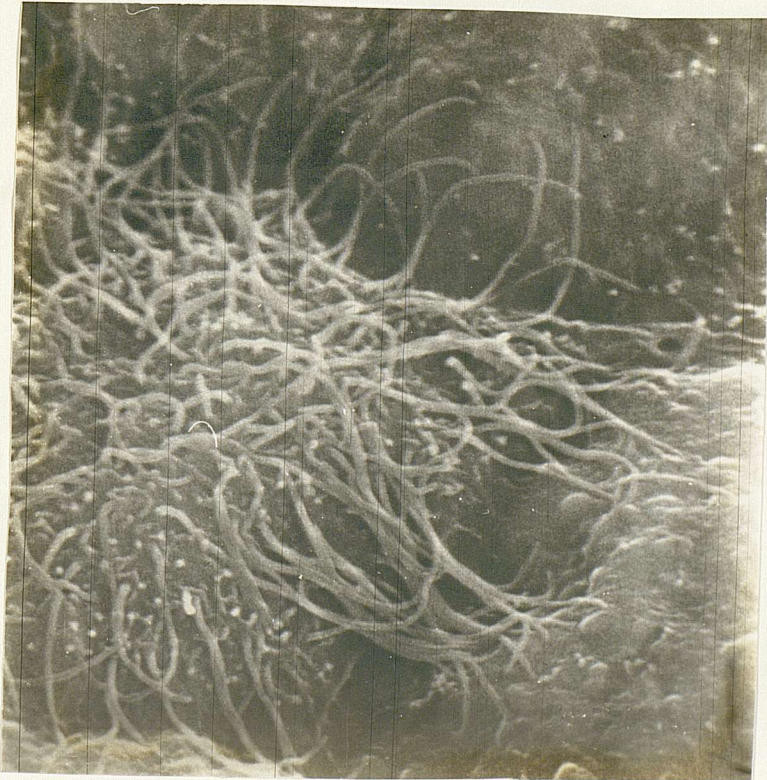
7°C EMBRYO.

Figs. 7 and 8.

Fully ciliated cells

x 15000 and x 6000 (respectively).

7

8
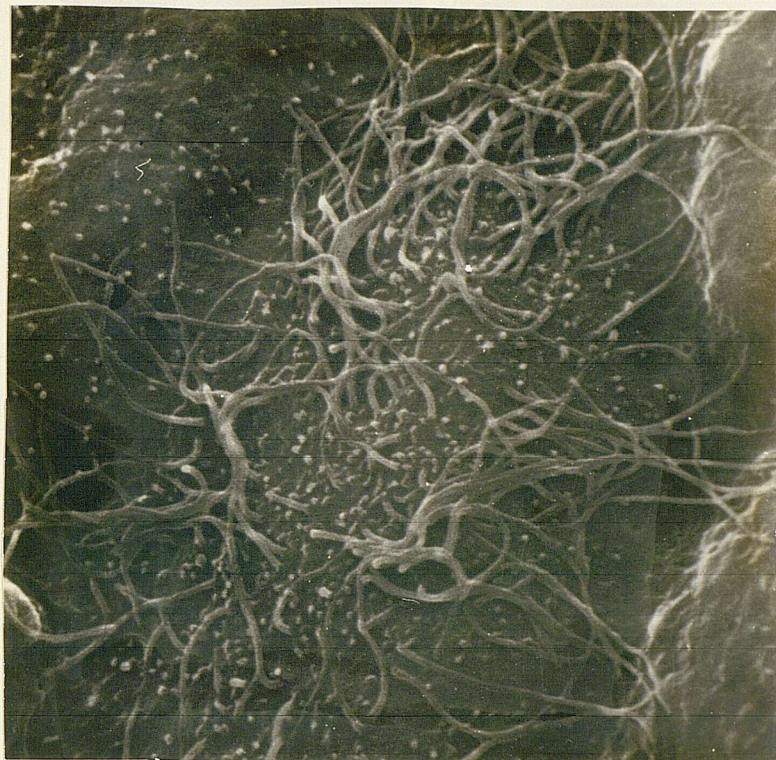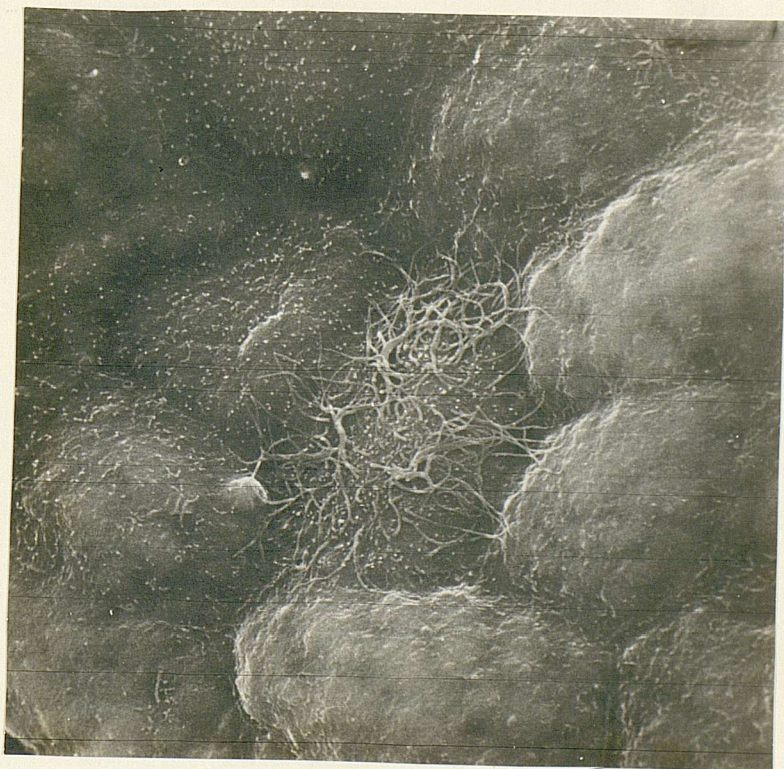
7°C EMBRYO.

FIGS. 9 and 10.

These photographs show the distribution of ciliated cells over
the embryonic epidermis.

x 3000 and x 1500 (respectively).

9

10

7°C EMBRYO.

Figs. 11 and 12.

Both these photographs illustrate the ratio of ciliated:non-ciliated cells , and how clearly the cell borders are defined.
x 3000 and 3000.

11
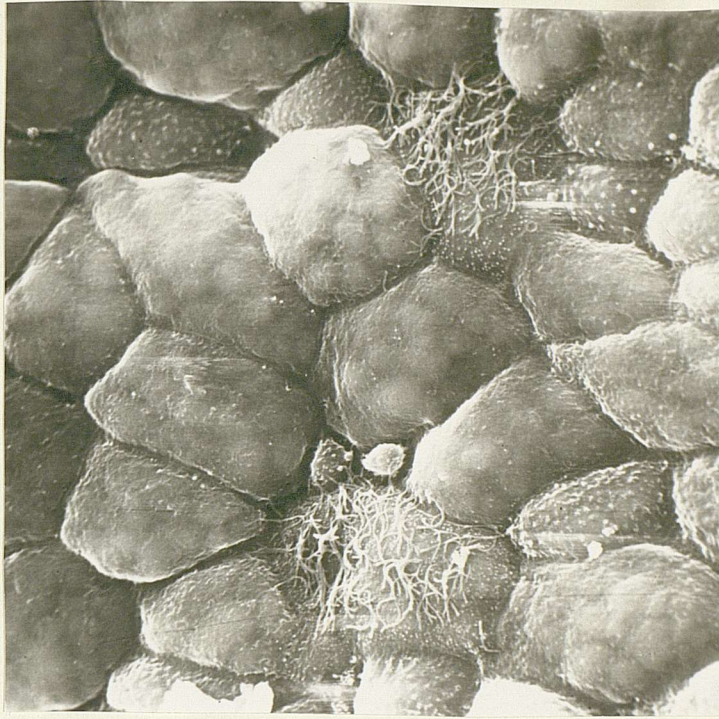
12

<u>Transmission Electron Microscopy.</u>

The cilia observed in the 7°C specimens, were the same as those developed at other temperatures, figs 1 & 2 . The basal body runs up into the main ciliary shaft via the transverse membrane, and from this membrane upwards into the shaft, the two central fibrils appear. The peripheral fibrils run from the ciliary shaft down through the transition zone, into the basal body and down to where the ciliary roots are, fig 1 . In figs. 3 & 4 can be seen what Steinman (1968) refers to as axonemal bodies. He describes how, in cilia formation, centrioles from the supra-nuclear cytoplasm align perpendicularly to the cell surface, a ciliary shaft then grows out from this now basal body, and simultaneously, small dense bodies termed "axonemal precursor bodies" appear. Their function is probably to provide raw material for the new ciliary shaft.

The photographs shown are of embryos just at the beginning of ciliation ; some cells have a few cilia, as in fig 2 but others are still forming. Figs. 5,6 & 7 are other pre-ciliated cells, i.e. these cells have raeched the surface and are about to become ciliated but unfortunately, no centrioles or basal bodies have been cut through. These cells will definitely become ciliated however, because of the particular zoned distribution of the cell organelles, and the cut through the microvilli. Differentiated ciliated cells typically have the larger yolk platelets and lipid bodies zoned in the lower region of the cells, whilst the apical, supra-nuclear region has a distinct layer of mitochondria and small vesicles, plus of course, all the basal bodies of the cilia. (Steinman claims that these small vesicles are secretory vacuoles, but less dense than the secretory droplets in mucus cells). This zoning is particularly seen in 1u sections of the material, stained with toluidine blue.

Mucus cells on the other hand, have an almost complete layer of mucus vesicles immediately under the free cell surface. According to Billett and Gould (1971) on work with <u>Xenopus laevis</u>, mucus vesicles seem to be generated in the supra-nuclear region, where most of the Golgi apparatus and rough endoplasmic reticulum of the cell is concentrated. Characteristic of all the epidermal cells seen in T.E.M., however, are the large numbers of desmosomes between the cells. Fibrils can very often be seen running through these desmosomes from one cell to the next, providing some sort of communication.

Fig. 1
_____

This is a section through a cilium showing the component fibrils

running down the cilia via the transverse membrane, and terminating

at the base where the ciliary rootlets pass down into the cytoplasm.

x 50,000.

7°C EMBRYO

Fig. 2.

Another section through a cilium showing central and peripheral

fibrils quite clearly.

x 80,000.

7°C EMBRYO

Fig. 3.

A basal body surrounded by axonemal bodies, just below the cell
surface.

x 120,000.

bb

axo
b

R
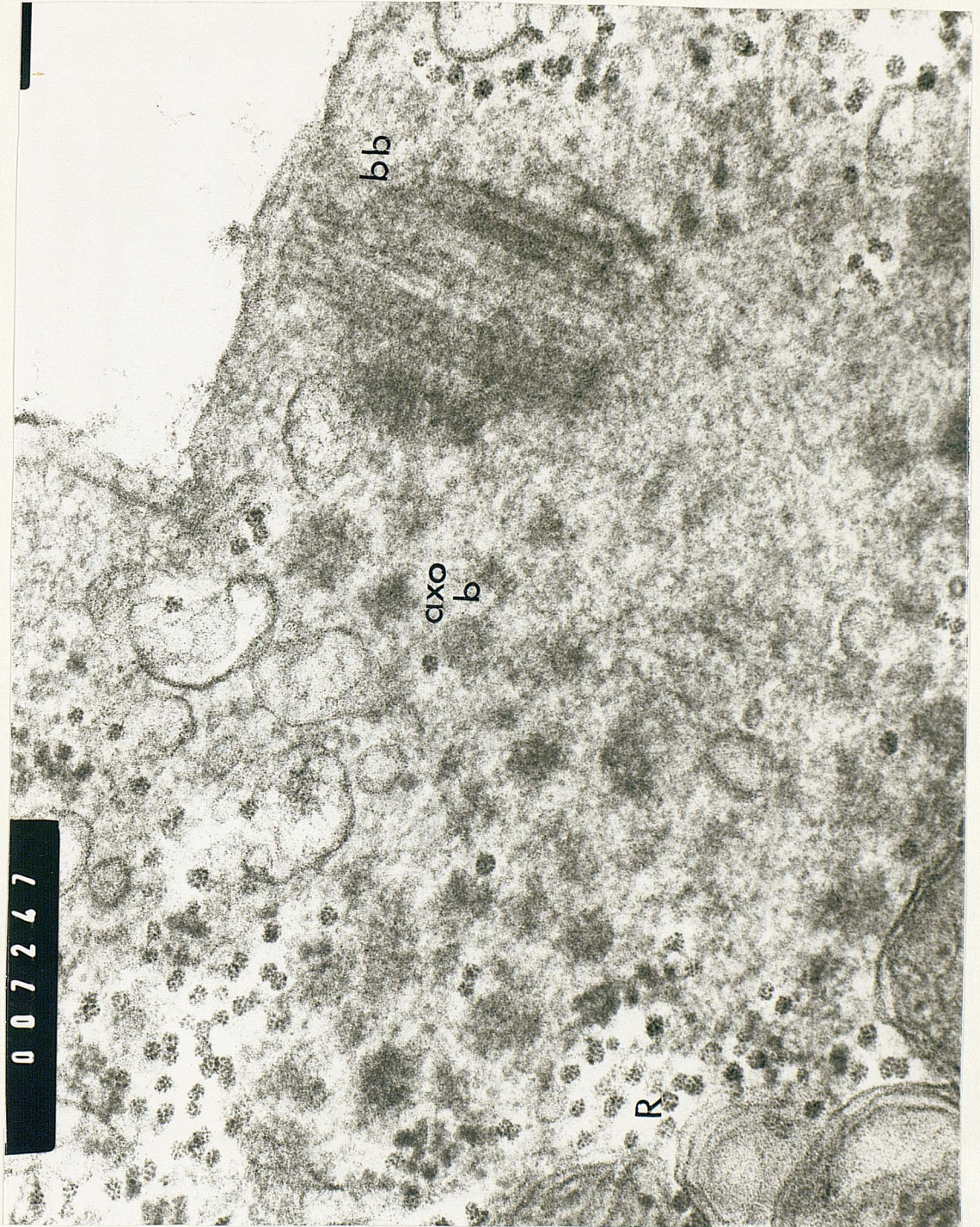
7°C EMBRYOS.

Fig. 4.
_____

This one is fig. 3. at x 50,000.

axo
b

Fig. 5,6, & 7.

The following figures are all of suspected pre-ciliated cells. They all show cut through microvilli and many mitochondria zoned just beneath the surface layer. Also, quite a lot of active Golgi apparata are present, as would be expected in pre-ciliated cells.

Fig.5 = x32,000.

Fig.6 = x25,000.

Fig.7 = x32,000.
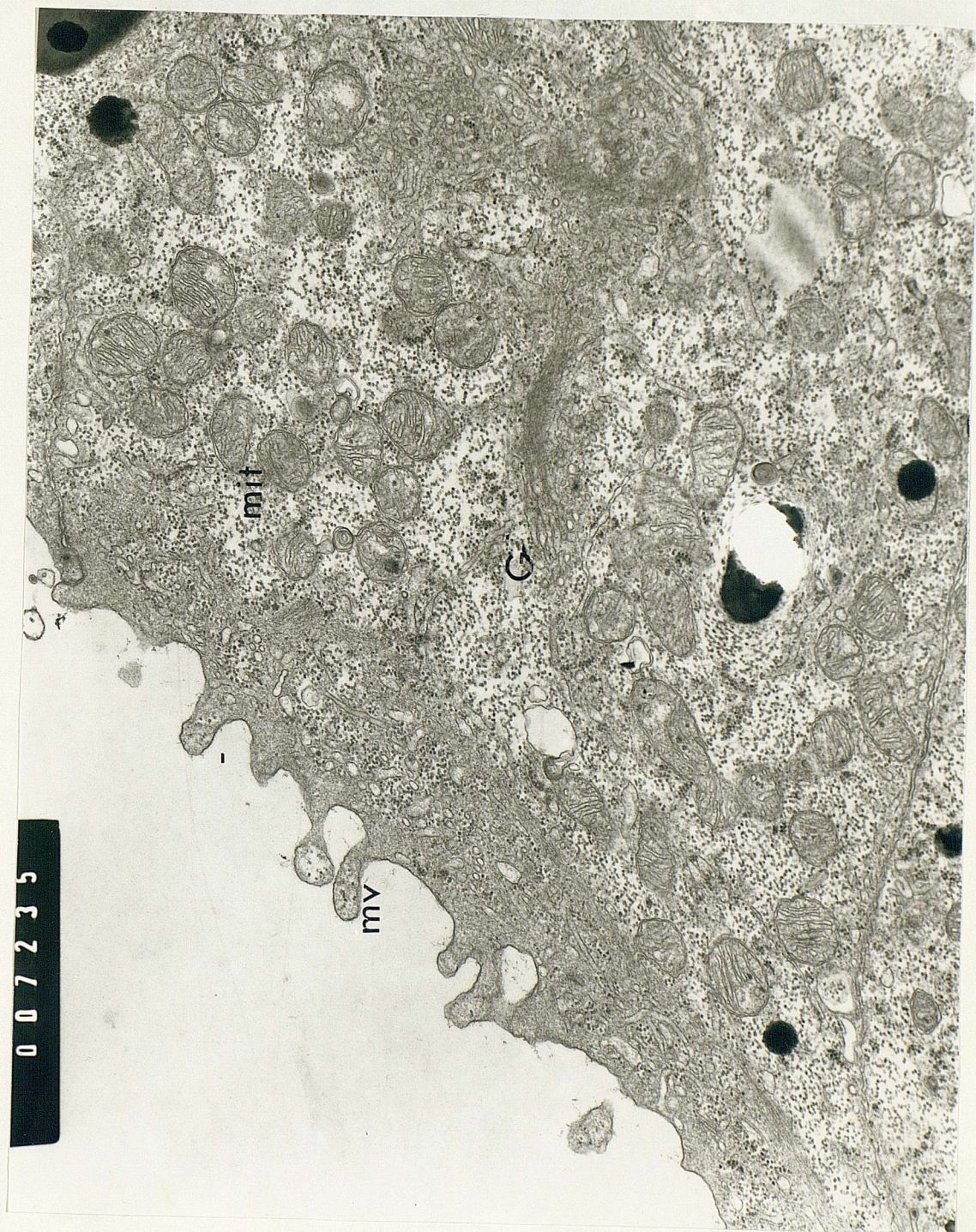
DEVELOPMENT OF THE EMBRYONIC EPIDERMIS AT 12°C.

Scanning Electron Microscopy.

Cilia appeared at the same time in these embryos, as with the 7°C ones, i.e. between 1-3 somites. The fully differentiated ciliated cells are formed by about 8 somites, and clearly show the associated microvilli, fig. 1. These photographs also demonstrate how these cells appear slightly raised up, figs. 2,3,4 and 5. The distribution of these cells over the surface is also well demonstrated by some of these photographs, figs. 5 and 6. Fig. 7 shows how, if not careful, the surface of the material may become contaminated, and contaminating particles be deposited, especially on the cilia.

Twice, and twice only, were the olfactory type of cilia noticed on the embryos. Once was on an embryo developed at 12°C, figs. 8 and 9, and the other time, on an embryo developed at 20°C (see 20°C figs 9 and 10). A similar type of cilia can be seen on the goldfish olfactory epithelium (Barber & Boyde 1967). There the cilia, like those seen here, intertwine and hence it is difficult to determine their length. Those olfactory cilia of goldfish are bordered by cells which possess free motile cilia, to provide the necessary olfactory current. In the two cases where these olfactory type of cilia were observed on the axolotl embryos, they were actually positioned about mid-flank region, and their function remains obscure. They may just be mutations of the normal ciliary type found else-where on the embryos.

12°C EMBRYO.

Fig. 1.
——————

This photograph clearly shows the microvilli associated with the cilia on the cell surface.
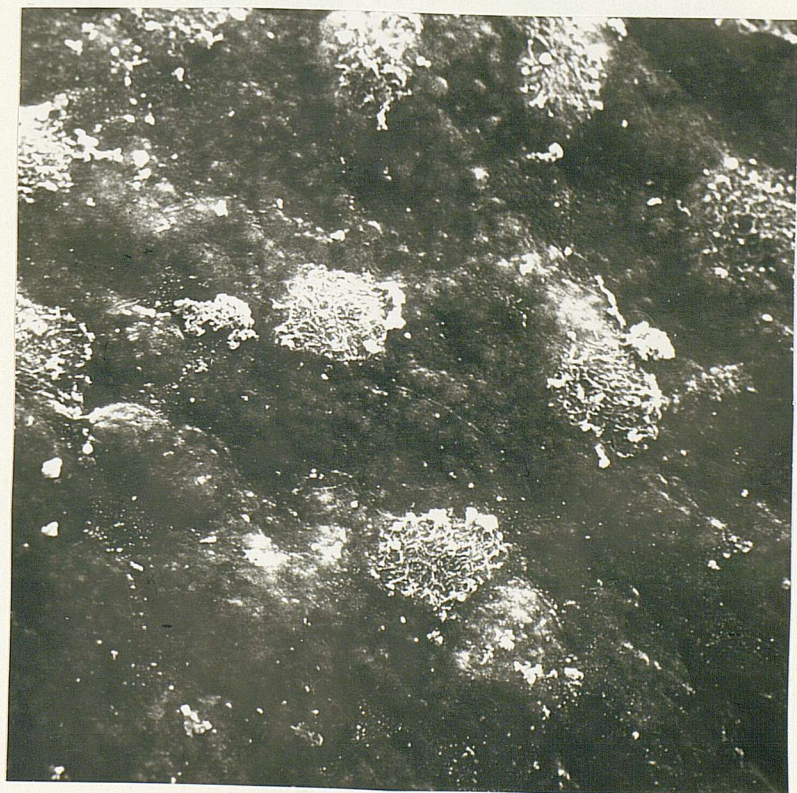
x 6000

Fig. 2.
——————

The prominence of the ciliated cells is clearly demonstrated against the background of mucus cells.
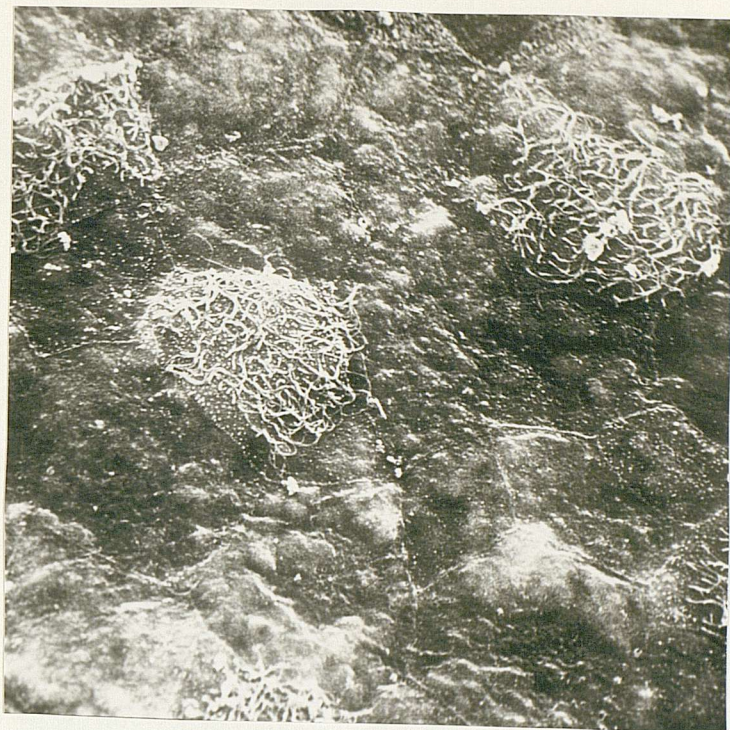
x 1500.

1
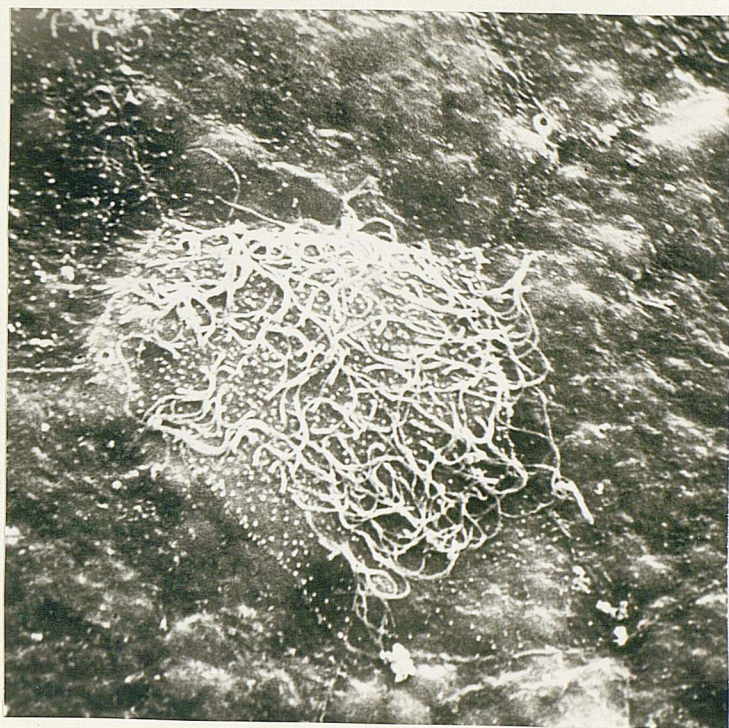
2

12°C EMBRYO.

Figs. 3 and 4.

These photographs again show how the ciliated cells are raised up against the surface, and fig. 4 shows the distribution of these cells.
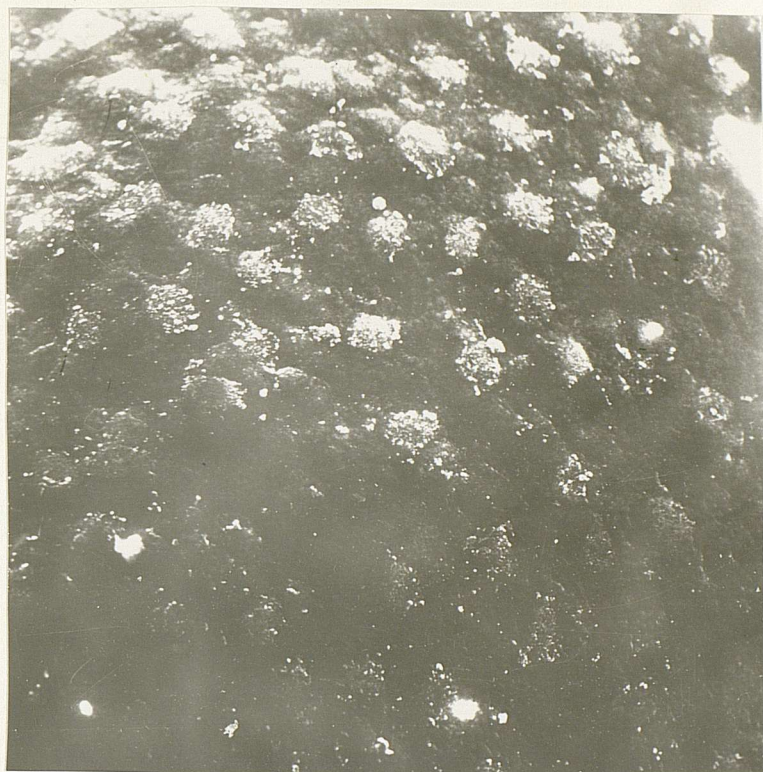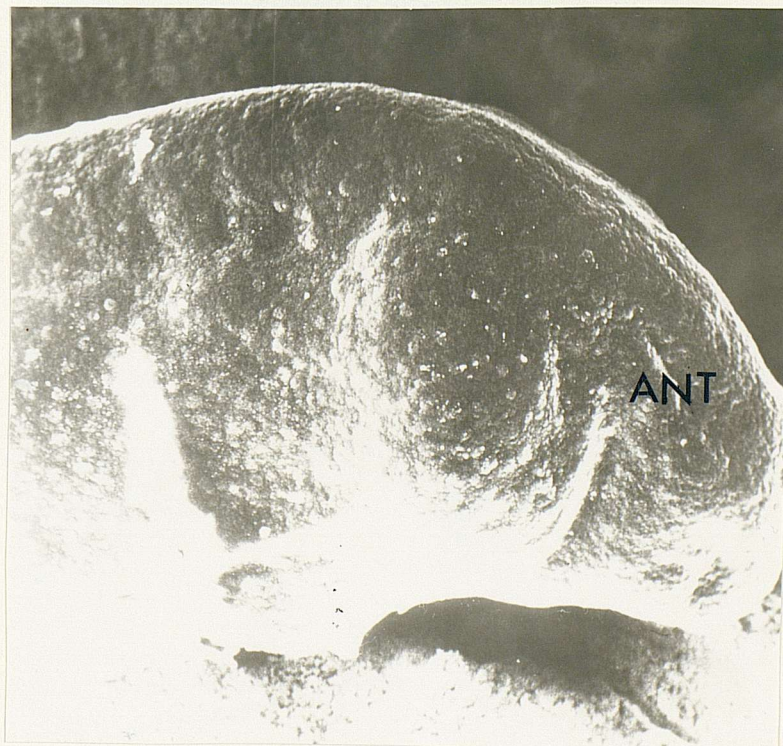
x 3ooo and x 15oo (respectively).

3

4

12°C EMBRYO.

Figs. 5 and 6.

More illustration of the distribution of ciliated cells over

the embryonic surface.

X 600 and x 150 (respectively).

5

6

ANT

12$^{\text{o}}$C EMBRYO.

Fig. 7.
———————

   The cilia in this photograph are fused together at the end and
contaminating dirt particles have adhered to them.
x 6000.

dirt
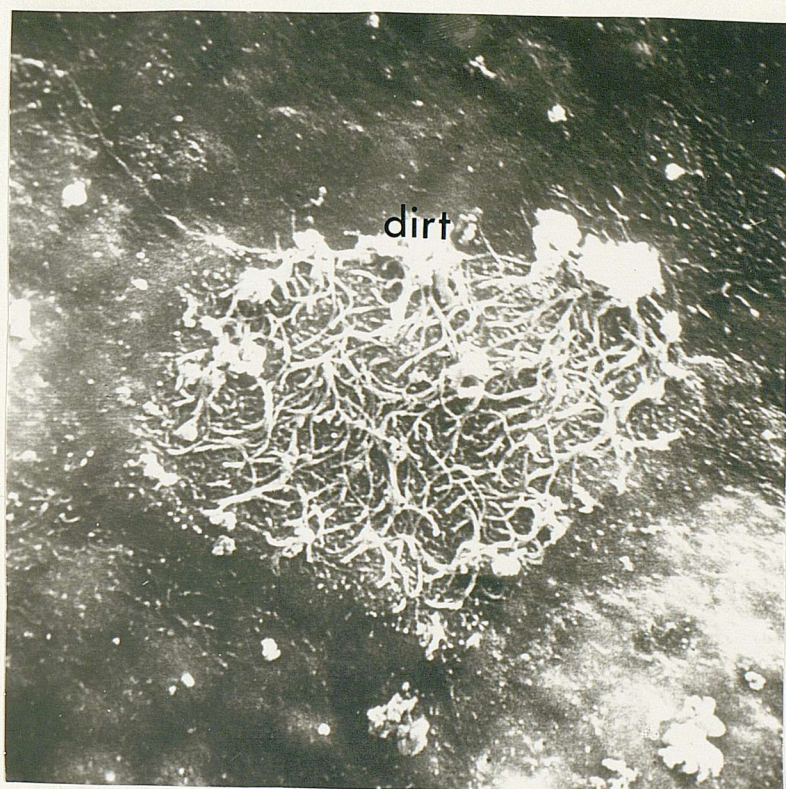
12°C EMBRYO.

Figs. 8 and 9.

The OLFACTORY type of cilia found on the mid-flank region of
an embryo developed at 12°C. This ciliated cell is clearly in a
depression.

x 6000 and 15000 (respectively).

Transmission Electron Microscopy.

8
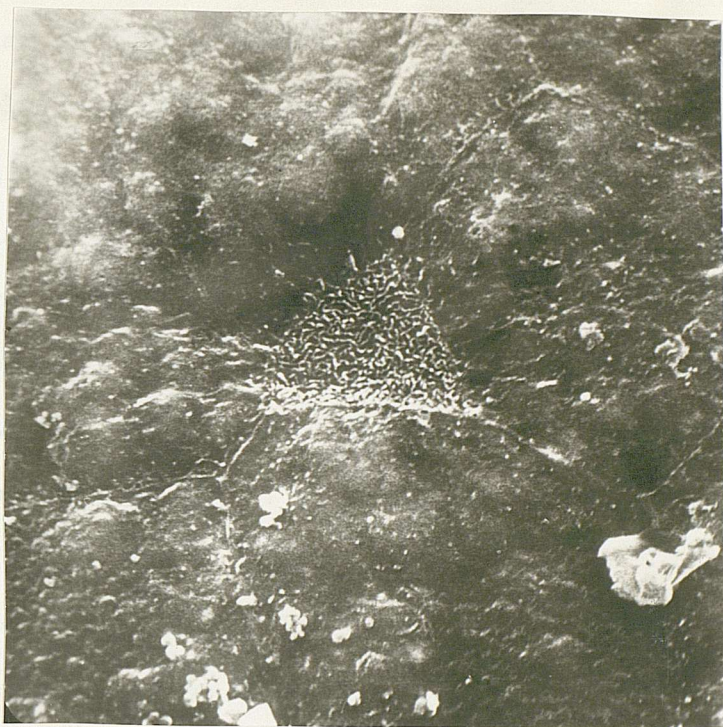
9
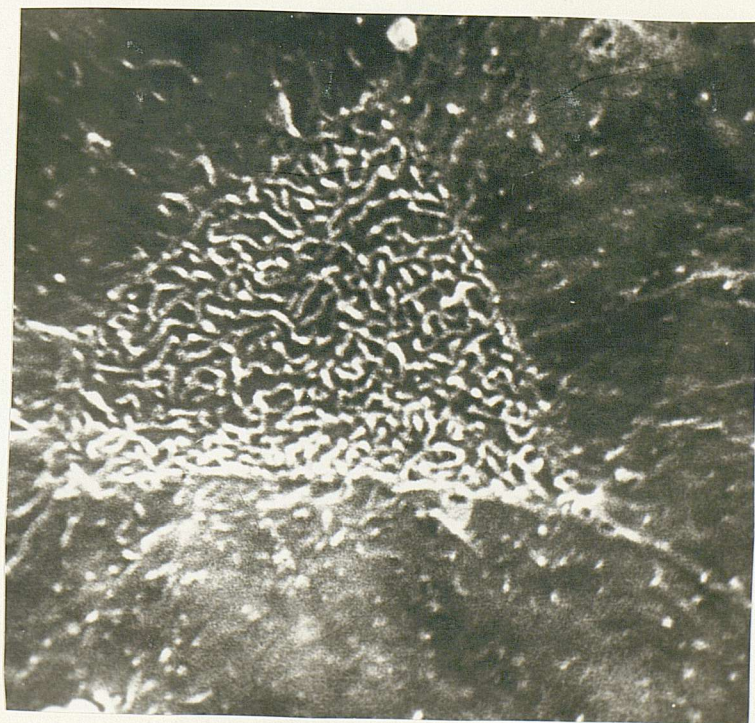
Transmission Electron Microscopy.

Transmission electron microscopy (T.E.M.) revealed the same pattern as for embryos developed at $7^{o}C$. From fig. 8, it is possible to visualise the internal structure of the cilia well, plus the axonemal bodies and two cut through centrioles. Fig. 9 shows nicely the striated rootlets coming off from the basal body, and the quite well developed Golgi apparatus. Golgi apparatus was often found in fairly close association with centrioles and basal bodies. This correlates well with Steinmans idea that Golgi apparatus may bud off vesicles which contribute to the ciliary membrane around the out-growing shaft. Figs. 10 and 11 also emphasise the cellular zoning just beneath the free cell border, to provide energy for the beating of the cilia.

12°C EMBRYO.

Fig. 8.

Section of a cilium showing clearly the internal structure, and

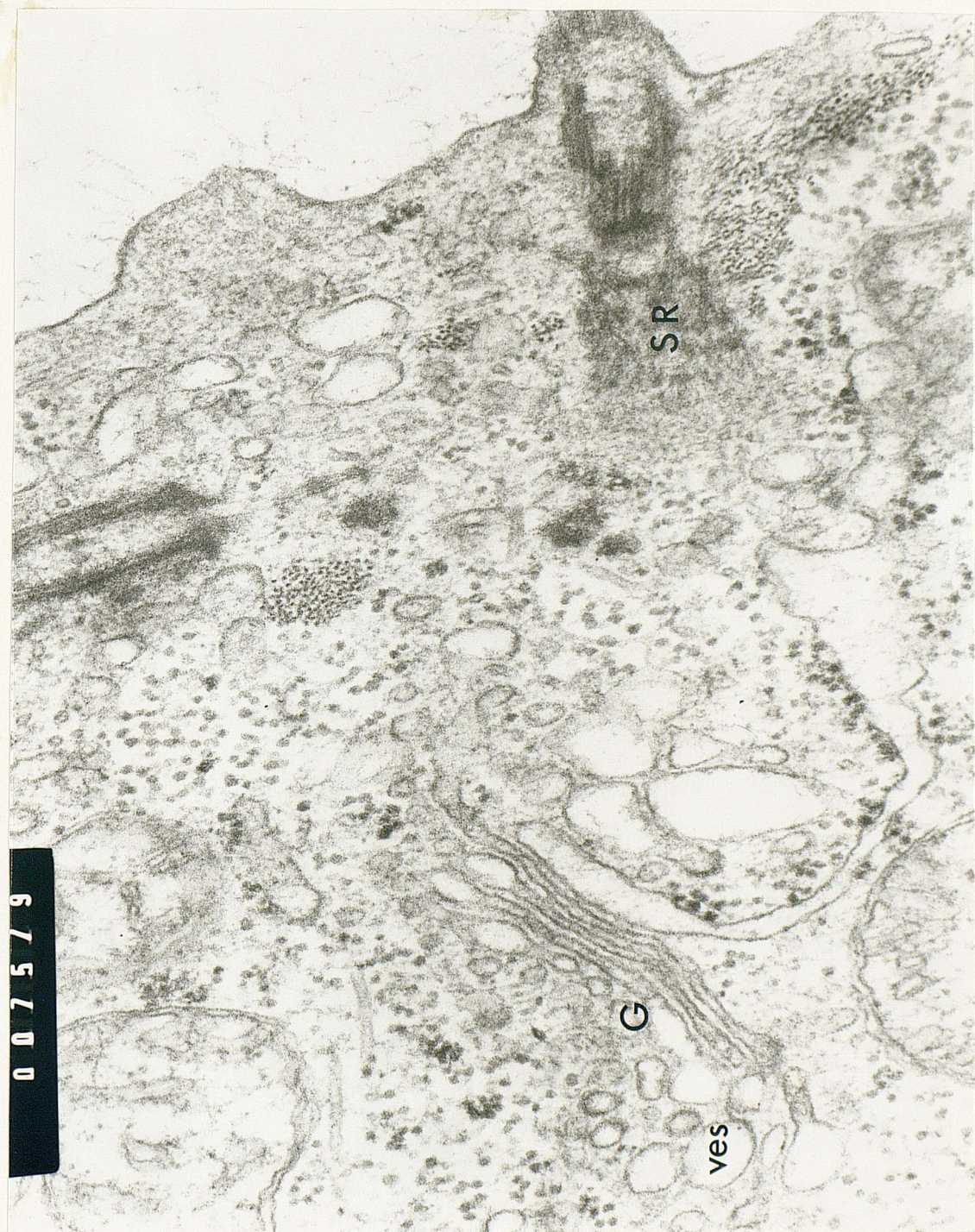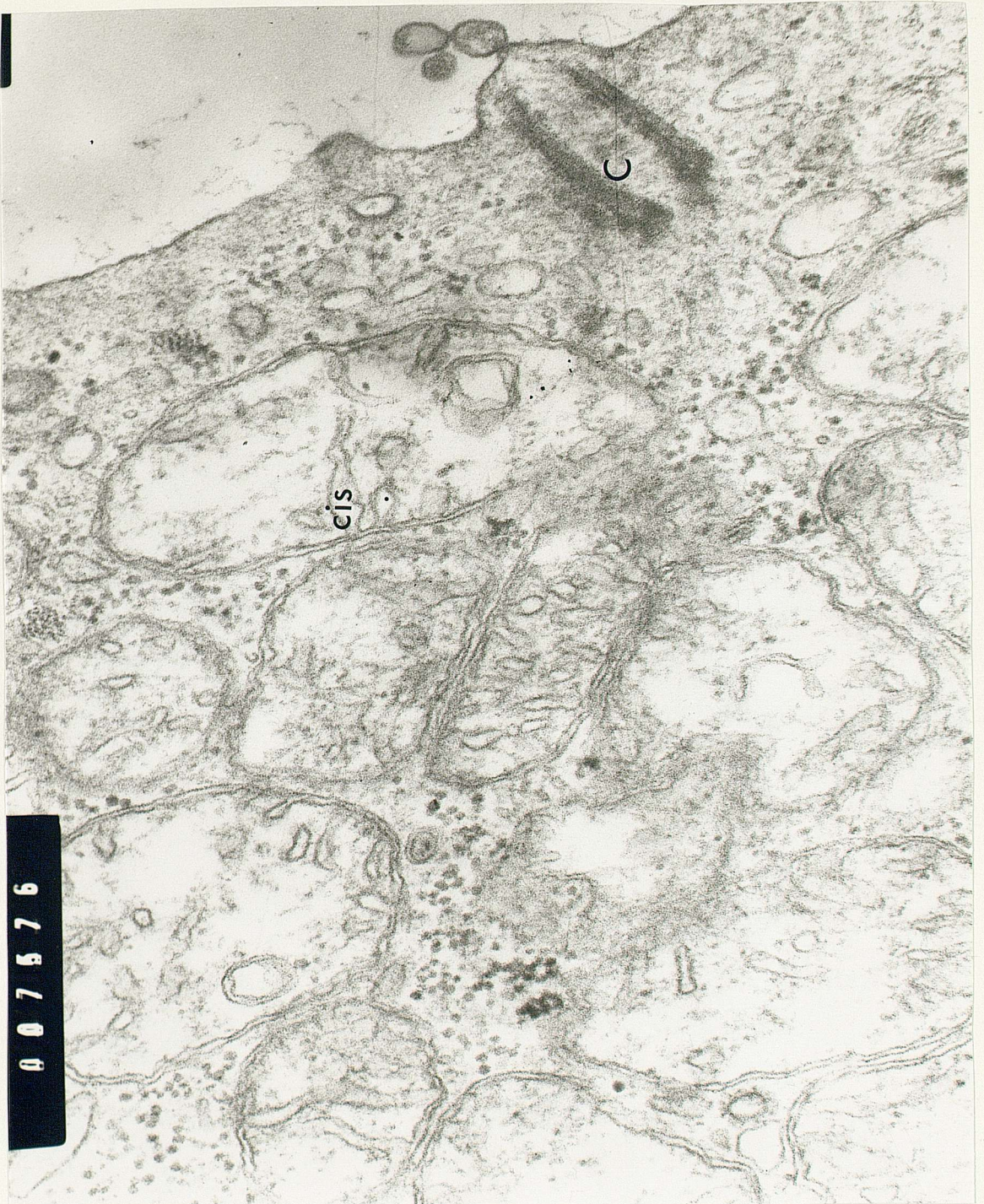also a cut through centriole and axonemal bodies.

x 41,000.
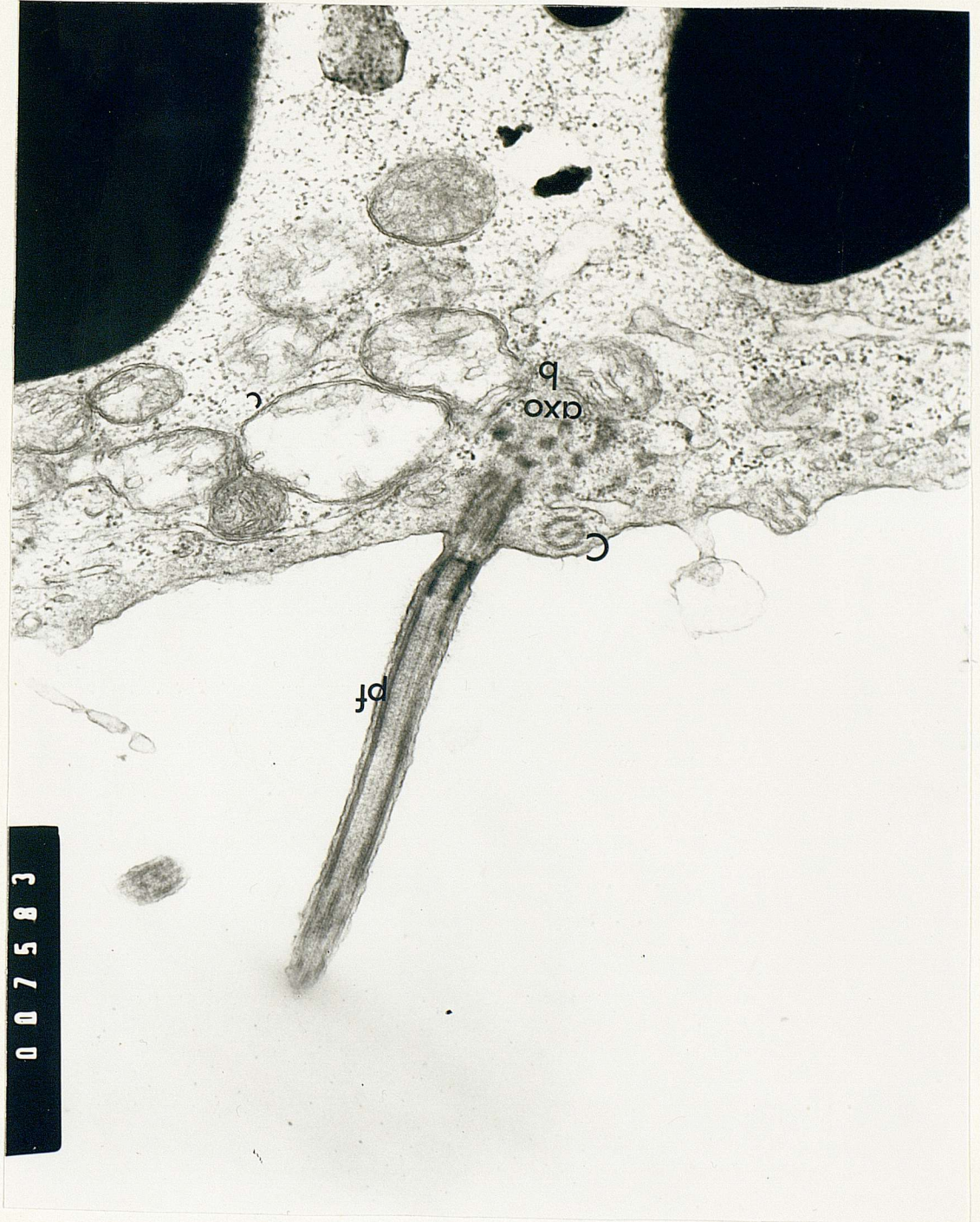
12°C EMBRYO.

Fig. 9.

This photograph shows striated rootlets coming off from a ciliary base and quite a large vesicular system originating at the Golgi apparatus.

x 80,000

SR

G

ves

12°C EMBRYO.

Fig. 10.
_____

   This photograph emphasises the concentration of mitochondria

beneath the ciliated cell surface, to provide the necessary energy

for the beating of cilia.

x 80,000.

12°C EMBRYO.

Fig. 11.

This is a lower magnification of fig. 10, and also shows a cut
through cilia.

x 41,000.

007580

mito

cilia

11

Scanning Electron Microscopy.

Cilia again appeared between the 1-3 somite stage, and were fully formed at the 8 somite stage. Figs. 1 and 2 show early ciliation from the embryo in fig. 3. These early ciliated cells usually look flatter than their mucus neighbours, giving the impression that they might have come up between the mucus cells, from just beneath the surface, fig. 1. The fully ciliated cells are more rounded up. Unfortunately, contaminating particles have been deposited on some of the cilia, fig. 4. The intermediate, elongation stage of the cilia can be seen in figs. 5 and 6, and by the stage of embryo reached in fig. 7, the cilia have reached their full length. Figs. 8 and 2 show how a) the cilia can fuse together, and b) a bad area of contamination, respectively.

Transmission Electron Microscopy.

This work was done on fully ciliated cells, i.e. the 10 somite stage, and older embryos, stage 36-37, where it appeared that the cilia were just beginning to fall off. Figs. 12 and 13 show low power plans of the ciliated cells, with numerous cut through cilia, mitochondrial banding, and lipid and yolk bodies in the lower portions of the cells. Fig. 12 also shows the presence of extensive Golgi apparatus and some of the small, empty-looking vesicles just below the free cell border. Figs. 14 and 15 show a long striated rootlet and basal bodies.

20°C EMBRYO.

Figs. 1 and 2.

An early ciliated cell around the head region of a 20°C embryo.
The cilia are quite short as they have only just erupted.
x 15000 and 30000 (respectively).

1
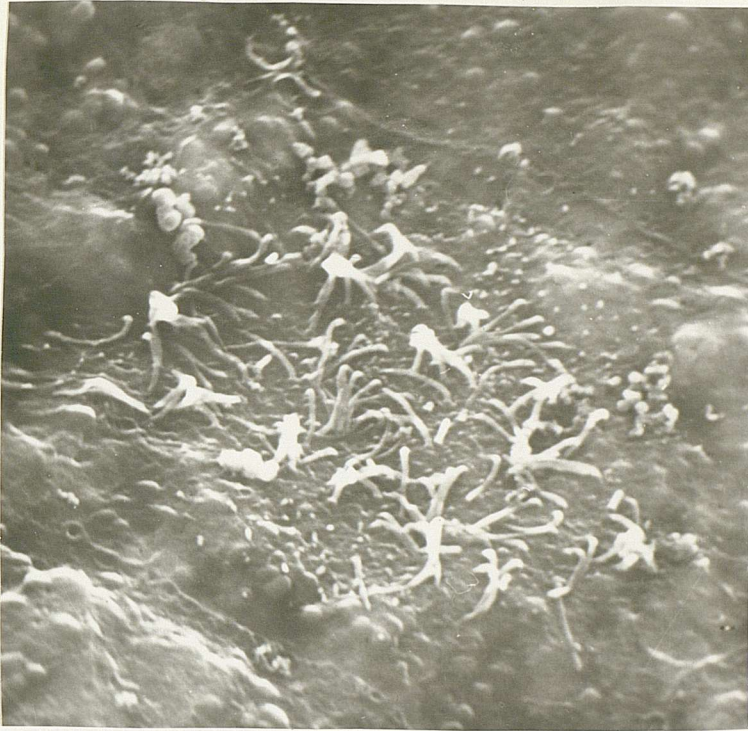
2

20°C EMBRYO.

Fig. 3.

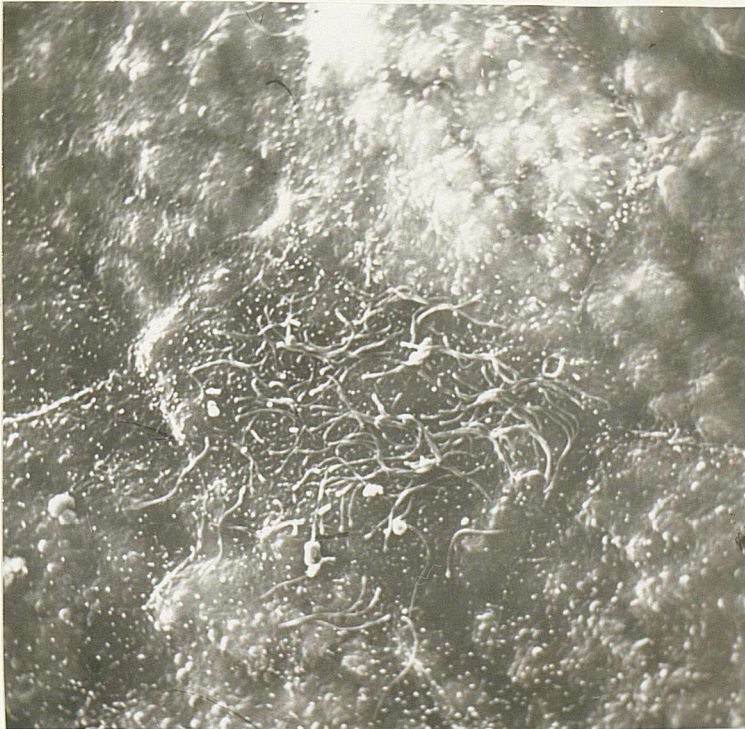Dorsal view of an embryo of the six somite stage.

x 150.

Fig. 4.

An early ciliated cell with contaminating particles attached
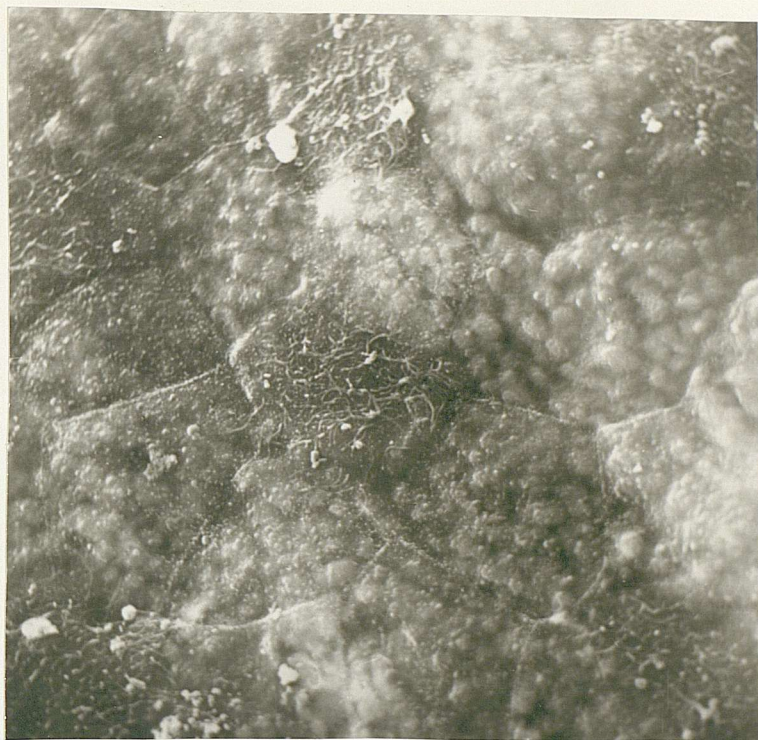
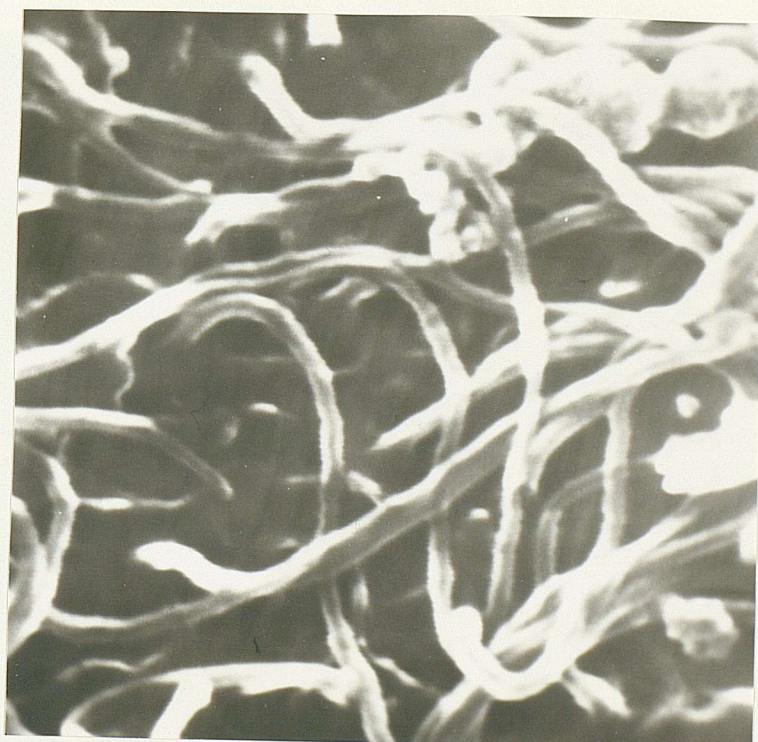to the cilia.

x 6000.

3

ANT

4

20°C EMBRYO.

Figs. 5 and 6.
_____

Cilia of the intermediate elongation stage.

x 3000 and x 60000

5

6

$20^{\circ}C$ EMBRYO.

Fig. 7.
_____

Embryo (12 somite stage) embedded in silver paste on a stereoscan stub.
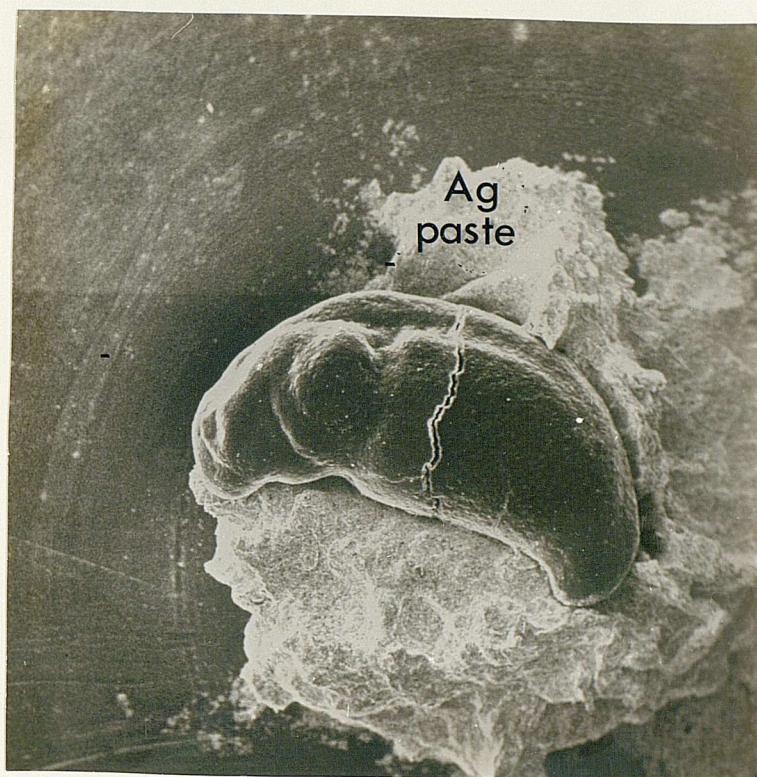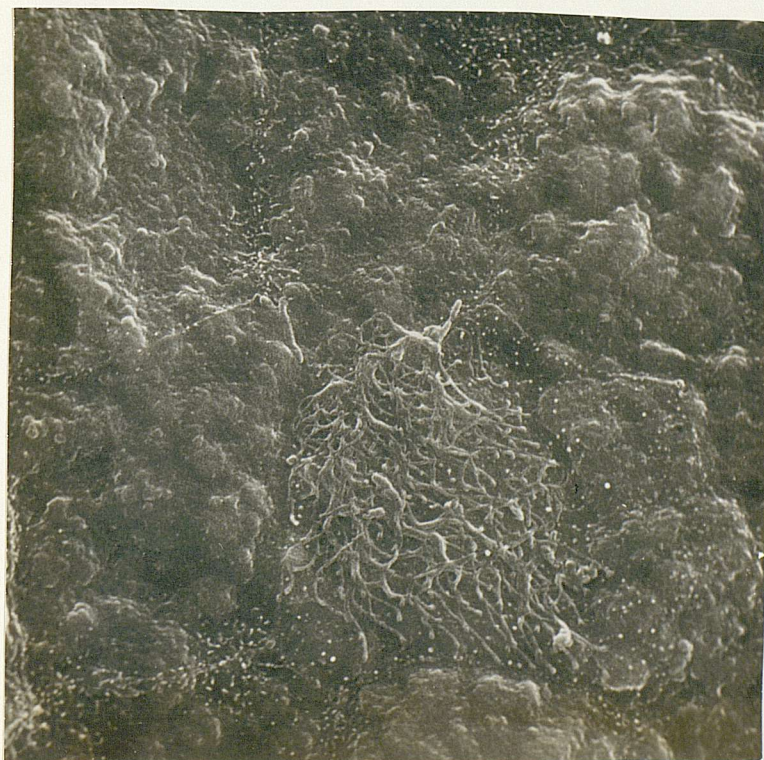
x 60.

Fig. 8.
_____

A bad example of cilia fusion.

x 6000.

Ag
paste

20°C EMBRYO.
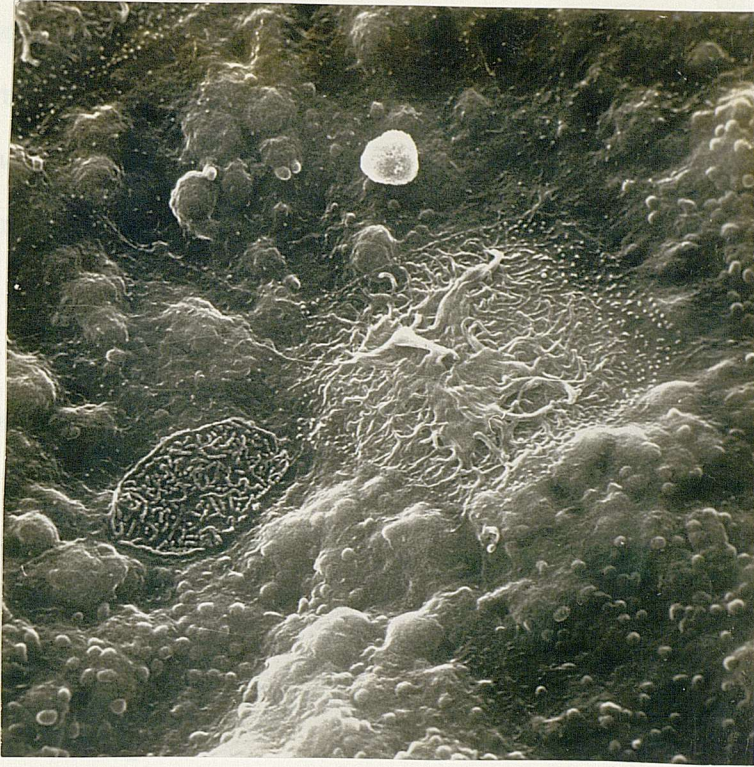
Figs. 9 and 10.

Another example of an olfactory type of ciliated cell.
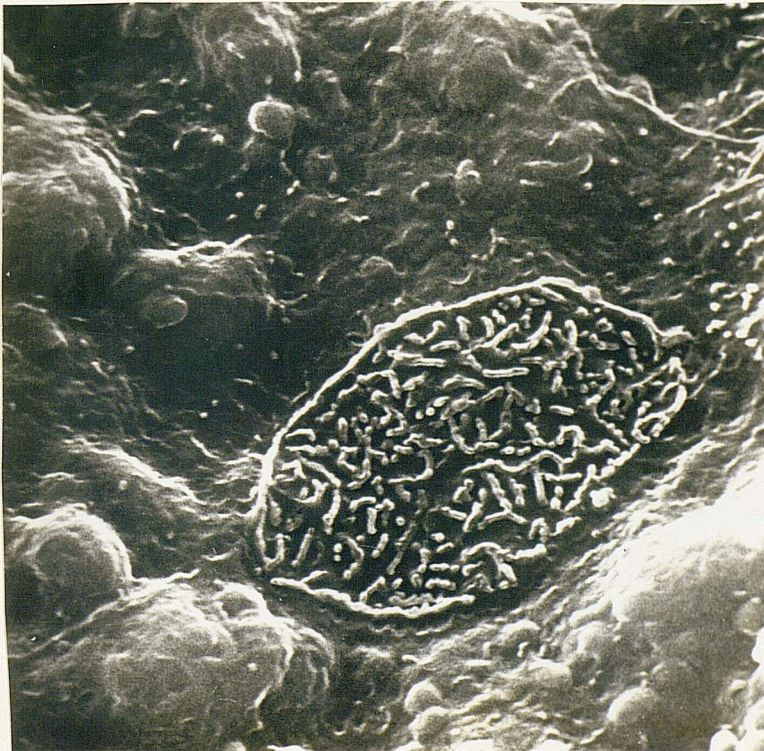
x 6000 and x 15000 (respectively).

Fig 16 is a photograph of the surface of a typical mucus cell from the same embryo. Mucus vesicles with quite densely staining mucus packets (shrunk away from their membrane during fixation probably), plus well developed rough endoplasmic reticulum are two of the typical features illustrated here. A few tonofilaments are also present.

A greatly increased tonofilament content in mucus cells is noticed in the later embryo, producing a fairly extensive terminal web, figs 17-18a There is also an increased terminal web in the ciliated cells. These ciliated cells, however, are just coming to an end as the cilia fall off. Figs 18 & 19 show these cells with just a few cilia left.

A striking feature of these cells here is the absence of mito-chondria, which have presumably disappeared because they are no longer required. The area where they were, is mostly terminal web by this time. Also, a few small mucus type vesicles have appeared under the free cell surface. These vesicles are considerably smaller than those of the normal mucus cells (see fig 20). Consequently these cells may continue to a lesser extent as mucus cells after all the cilia have fallen off.

$20^{O}G$ EMBRYO.

Fig. 12.

Low magnification photograph of a mature ciliated cell showing many cilia, mitochondria and Golgi apparata.

x 11,200.
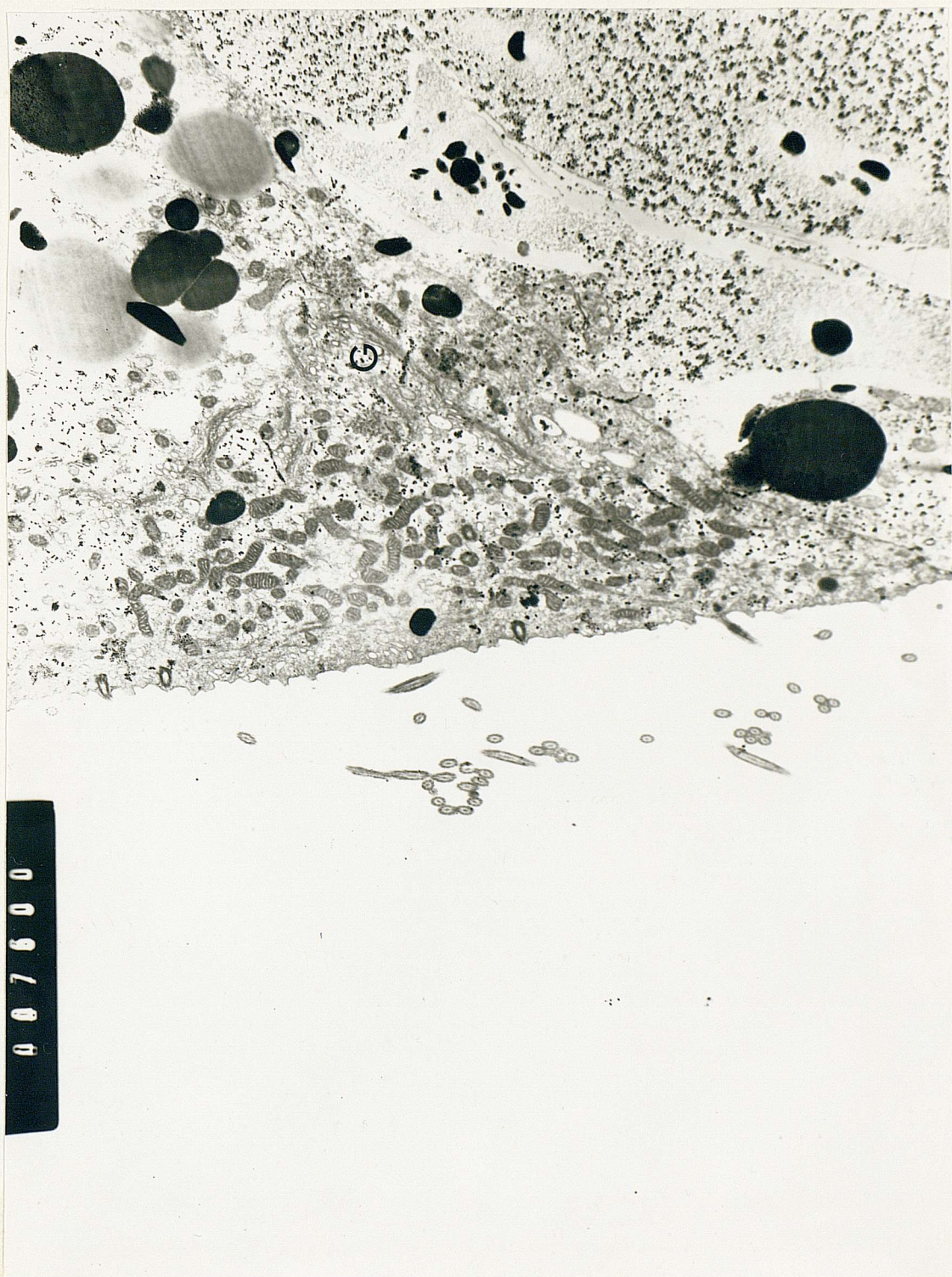
20°C EMBRYO.

Fig. 13.
_____

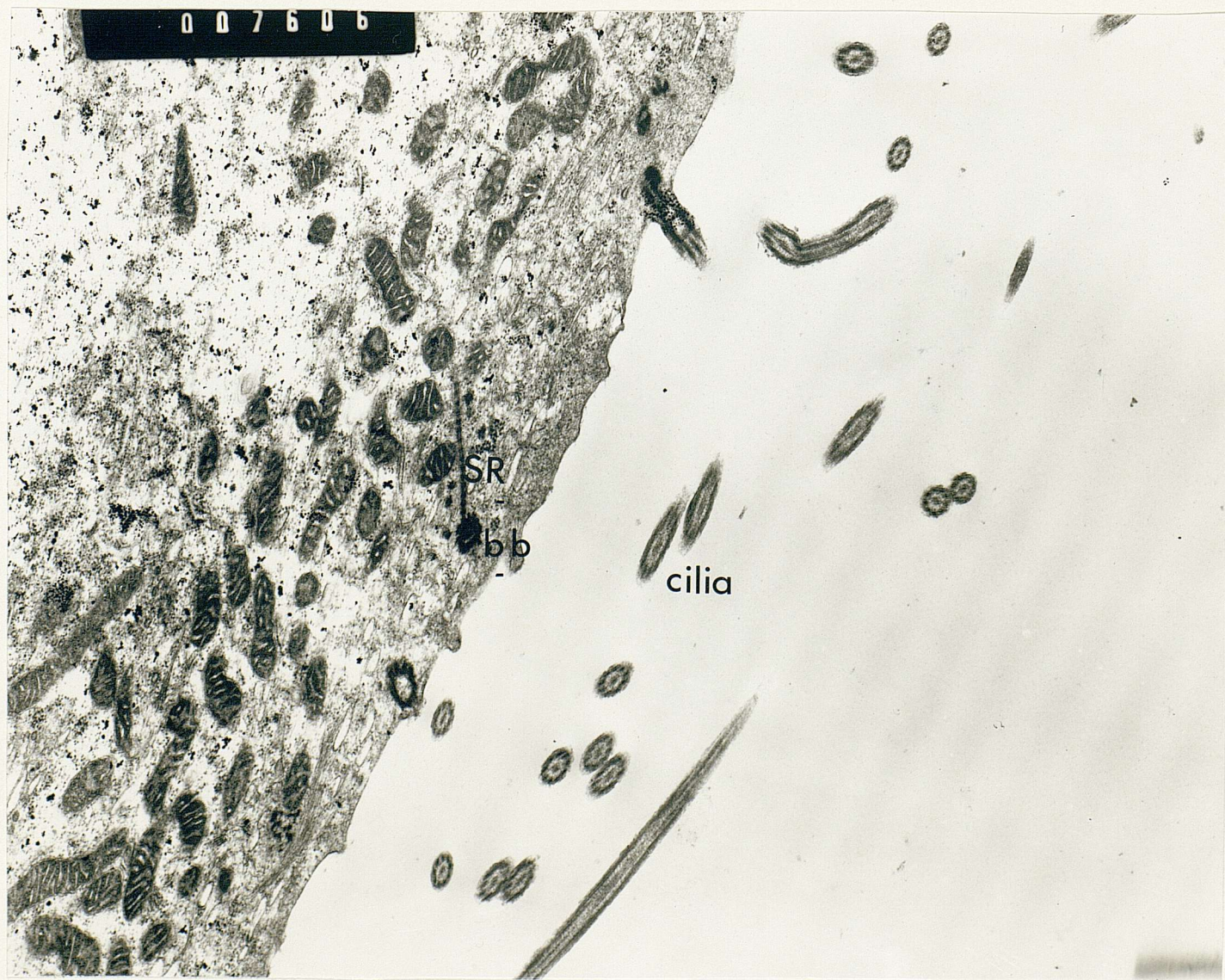Another low magnification photograph of a mature ciliated cell.

x 6,400.

20°C EMBRYO.

Fig. 14.

Illustrartion of the length and form of the striated rootlets
that anchor the ciliary basal bodies.
x 25,000.

SR

bb

cilia

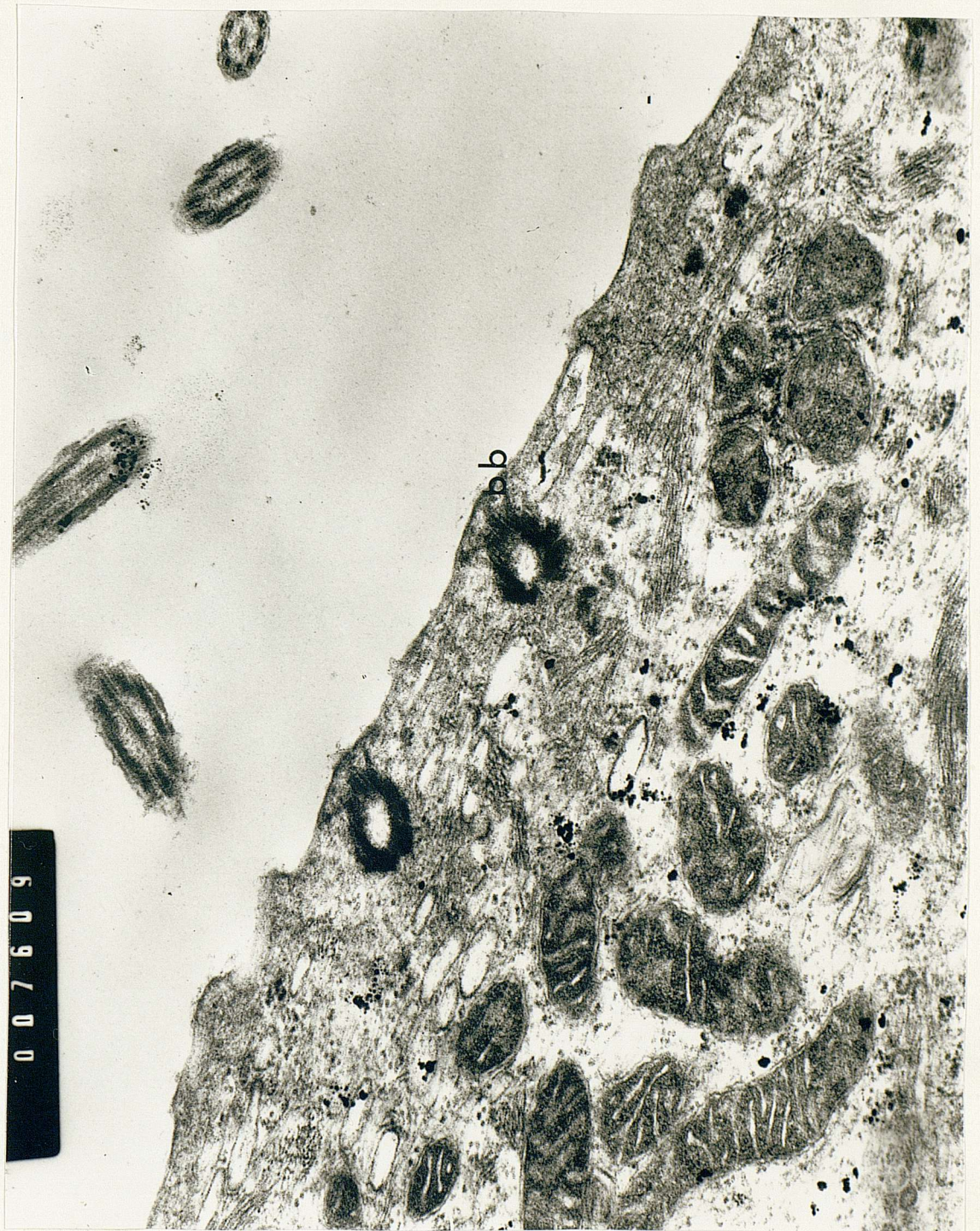20°C EMBRYO.

Fig. 15.

Cut through basal bodies and cilia.
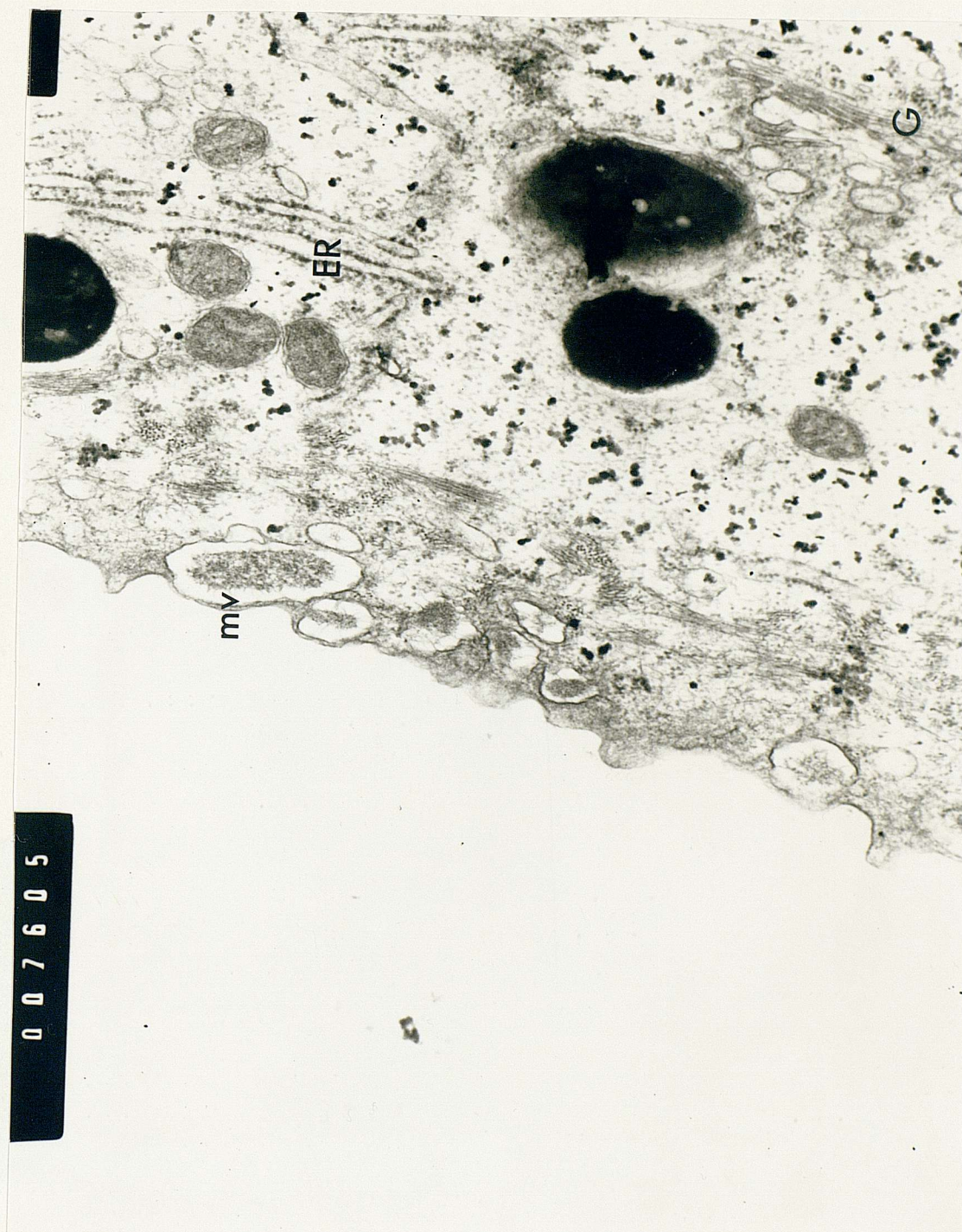
X 50,000.

20°C EMBRYO.

Fig. 16.
_____

This is a typical mucus cell, with mucus vesicles just below the surface and hardly any mitochondria in the vicinity.

x 41,000.

20°C EMBRYO.

Fig. 17.

This photograph shows the terminal web beneath the surface of a
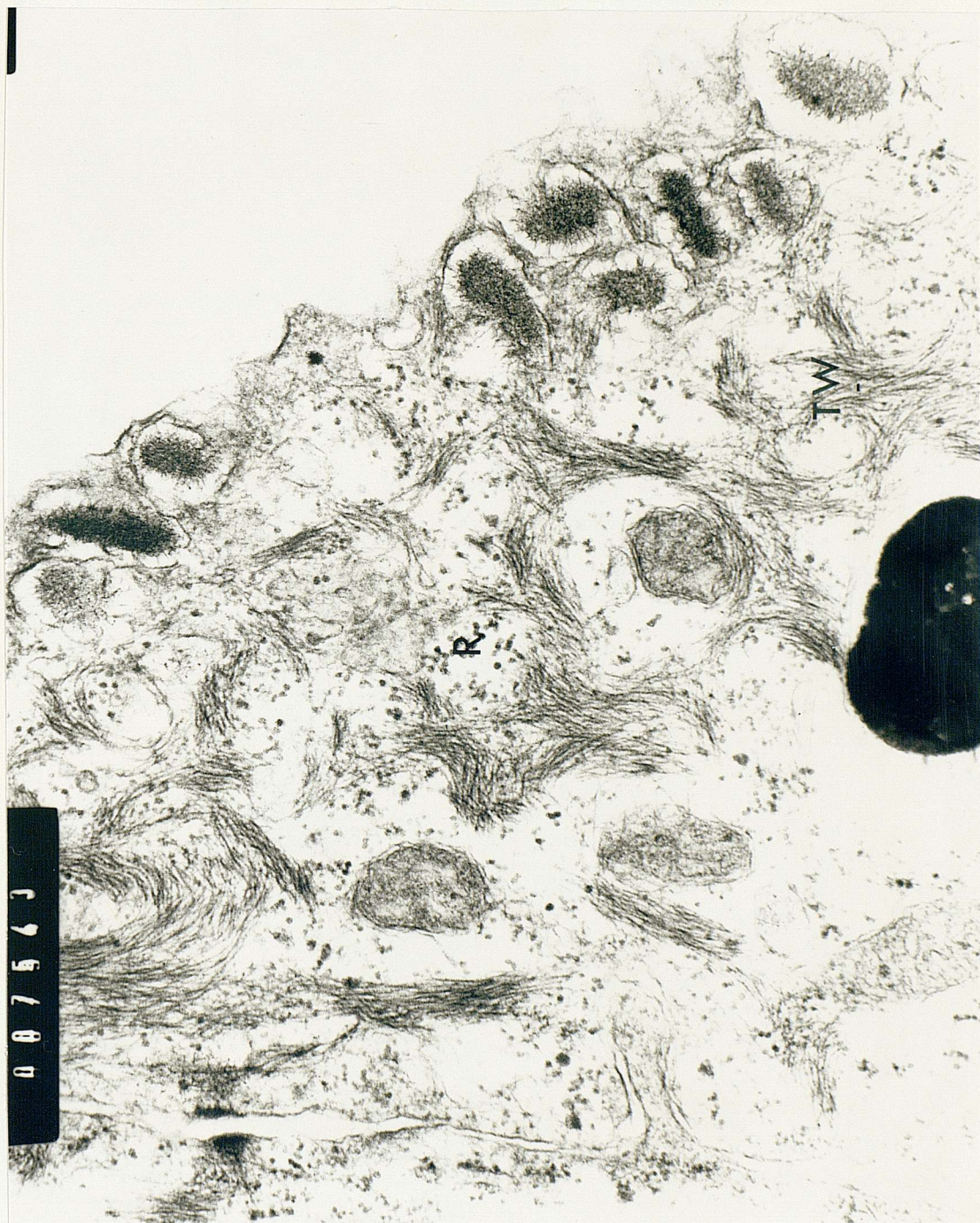mucus cell.

x 32,000.

$20^{\circ}C$ EMBRYO.

Fig. 18.

Another photograph of terminal web in a mucus cell.

x 50,000.

$20^{\circ}C$ EMBRYO.

Fig. 18a.

More terminal web in mucus cells, and this time showing three
very distinct desmosomes between the two cells.
x 41,000.

18a
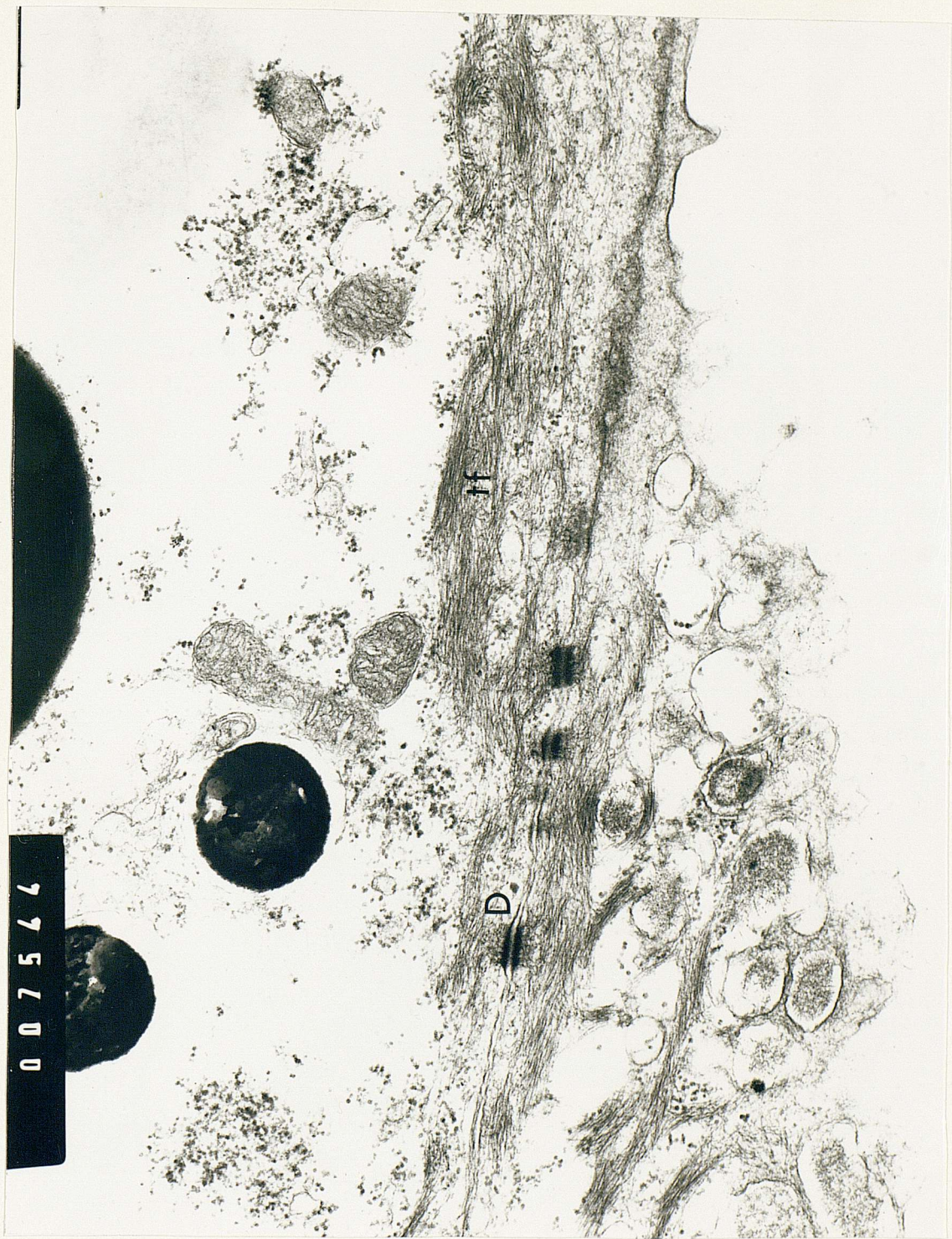
20°C EMBRYO.

Fig. 19.

Two ciliary bases of the few remaining cilia left on these older 20°C embryos.

x 80,000.

Fig. 20.

Cell (1) is a normal mucus cell, with fairly large mucus packets just beneath the cell surface. Cell (2) was originally a ciliated cell, but the cilia have fallen off and small mucus vesicles are forming in their place. The cell function will be mucus secreting from now on.

x 50,000.

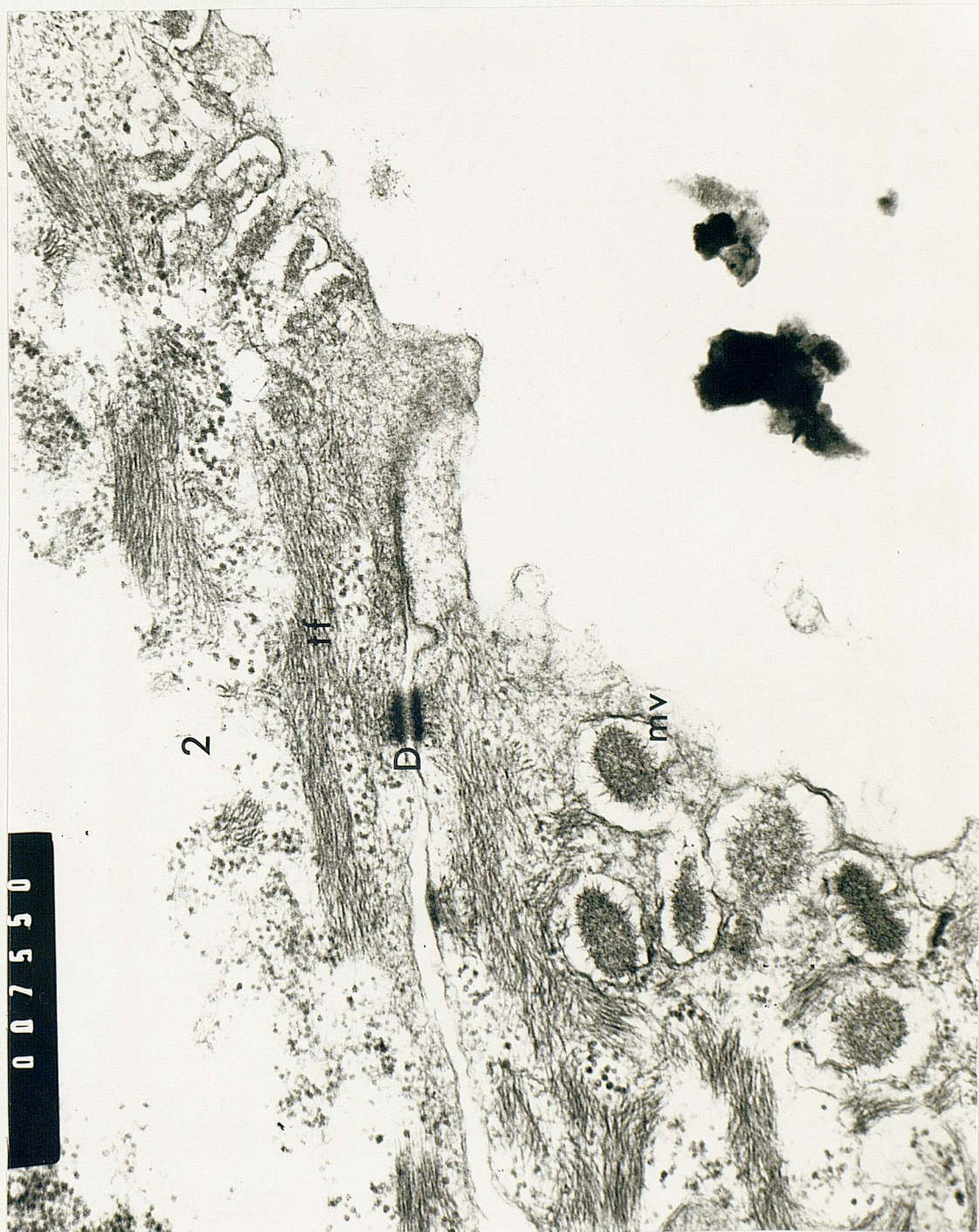# DEVELOPMENT OF EMBRYONIC EPIDERMIS AT 27°C.

Scanning Electron Microscopy.

As with the other embryos, these developed at 27°C first appeared ciliated between 1-3 somites, and were fully ciliated by 8 somites,- but there was one very important difference. This was the patchy appearance of the cilia on what otherwise seemed to be mucus cells. There were a few cells which seemed fully ciliated, but most of the photographs were taken of the unusual "patchy" cells. The surface of these cells seemed quite smooth, and not peppered with micro-villi as ciliated cells usually are. The surface in fact, was identical to the neighbouring mucus cells, figs. 1,2 and 3.

The patches occured in the middle of the cells, or to one side or even occasionally round the edge, as in figs. 2,3,4,5 and 6. Sometimes cells were seen, as in fig.7, to have darker patches in them, suggesting that these cells might have been about to form cilia there. Fig. 8 could presumably be a photograph of an inter-mediate stage, where cilia have indeed formed in patches, but the patches have not yet raised to the level of the rest of the cell. Even in the cells with obviously mature cilia, however, sometimes the cilia appear as if they are in a slight depression. See figs. 9, 10, and 11.

27°C EMBRYO.

Figs. 1 and 2.

These show how the surface of these "ciliated" cells is identical

to the neighbouring mucus cells', and also the patchy distribution

of the cilia.

x 6000

1

2
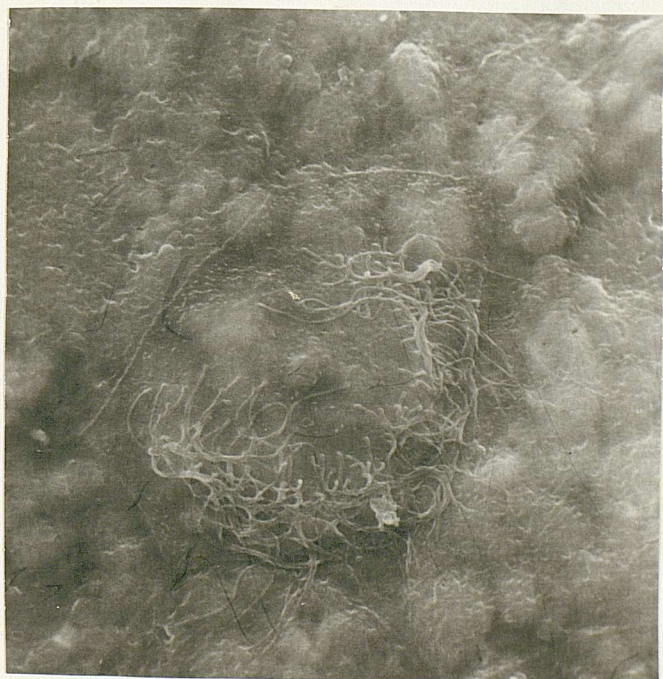
27°C EMBRYO.

Fig. 3.
_____

x 15000 magnification of the surface of a patchy ciliated cell.

The surface is identical to the neighbouring mucus cell.

Fig. 4.
_____

A patchy ciliated cell, just one half of the cell being ciliated

x 6000.

3
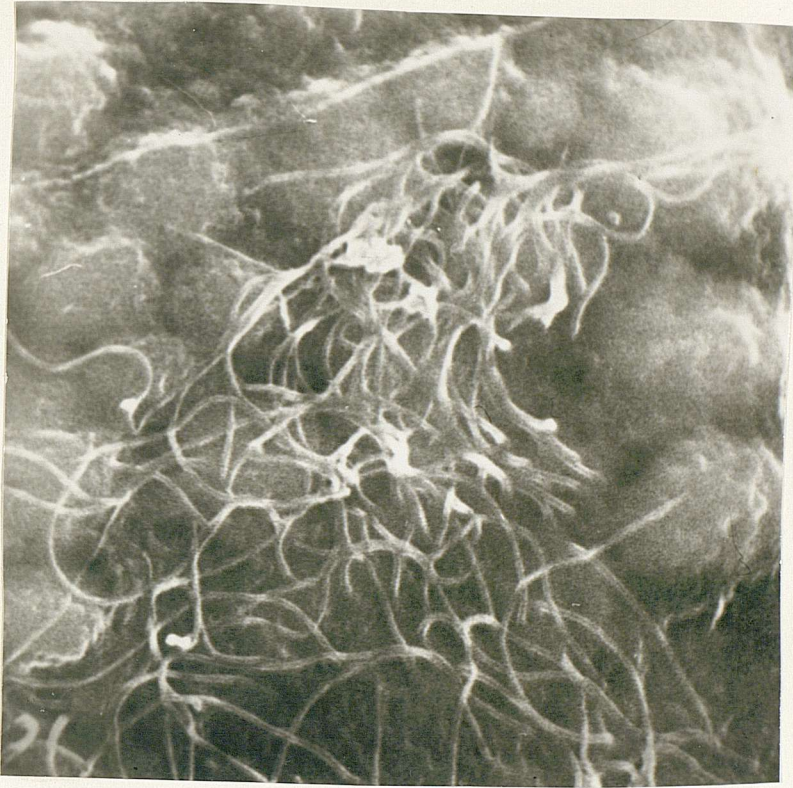
4
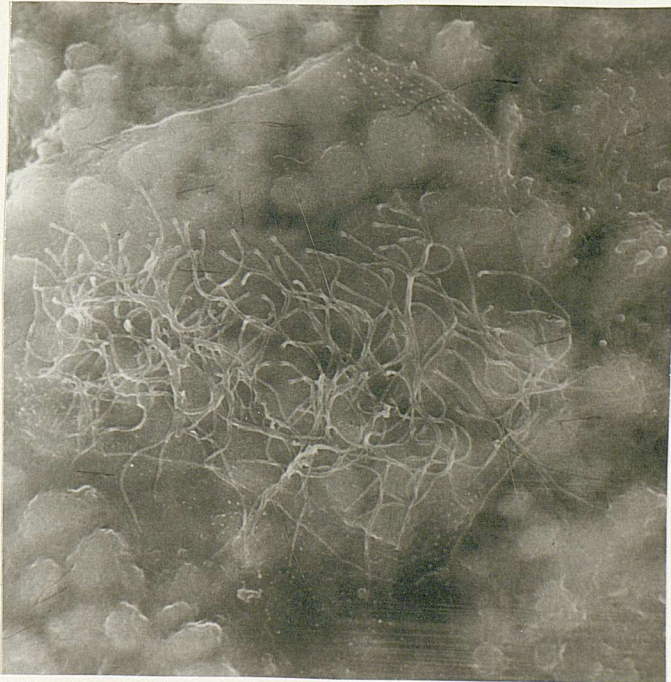
27°C EMBRYO.

Figs. 5 and 6.

Further illustrations of patchy ciliated cells.

x 6000 and x 15000 (respectively).

5
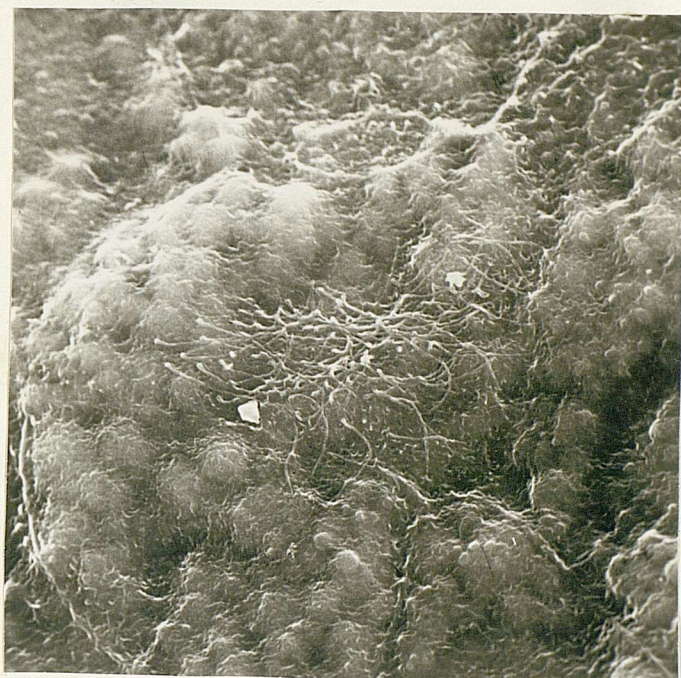
6
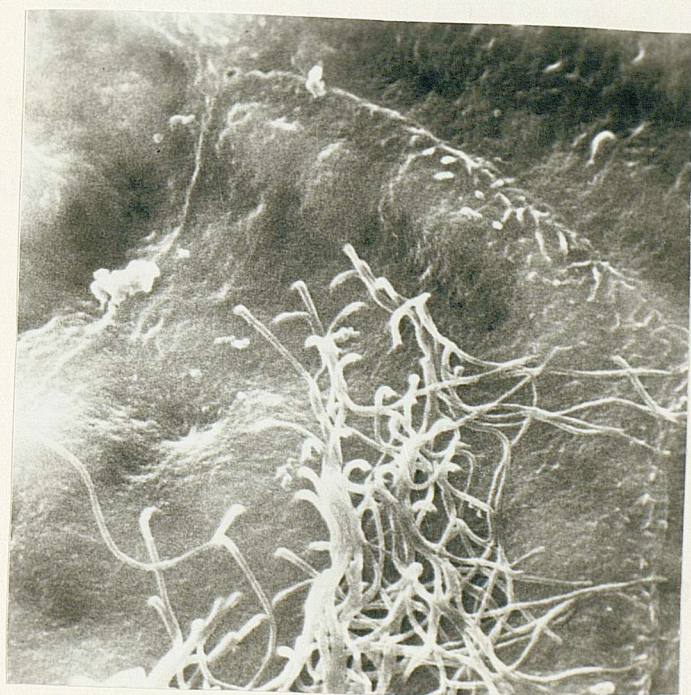
27°c EMBRYO.

Fig. 7.

This cell could perhaps be one that is just about to form a
patch of cilia in the darkened depression in the centre.
x 6000

Fig. 8.

These cells could presumably be the next stage on, where cilia
have formed from the patches, but the patches have not yet raised up
x 3000.

7
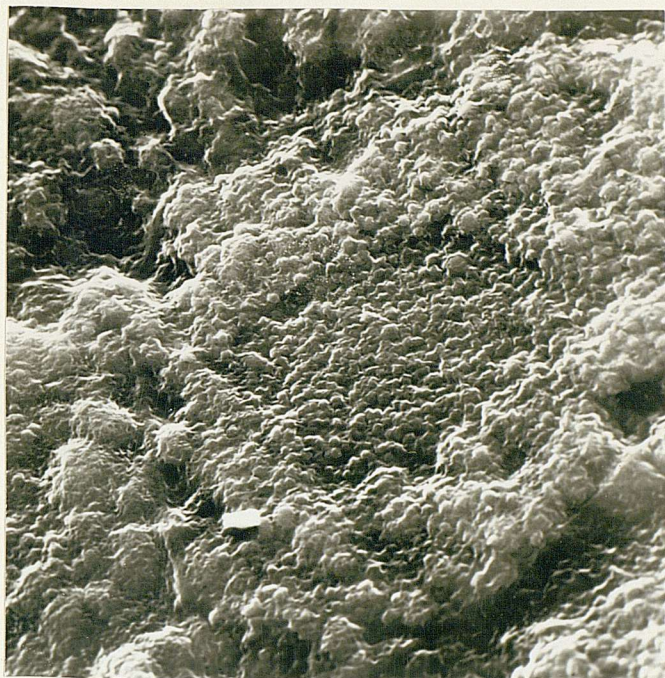
8

27°C EMBRYO.

Figs. 9 and 10.
_____

More patchy ciliated cells not fully raised up to the level of
the rest of the cells.

x 6ooo and 15ooo (respectively).

9

10

27°C EMBRYO.

Fig. 11.

Another illustration of a patchy ciliated cell which is not
completely raised up to the level of the neighbouring cells.
x 6000.
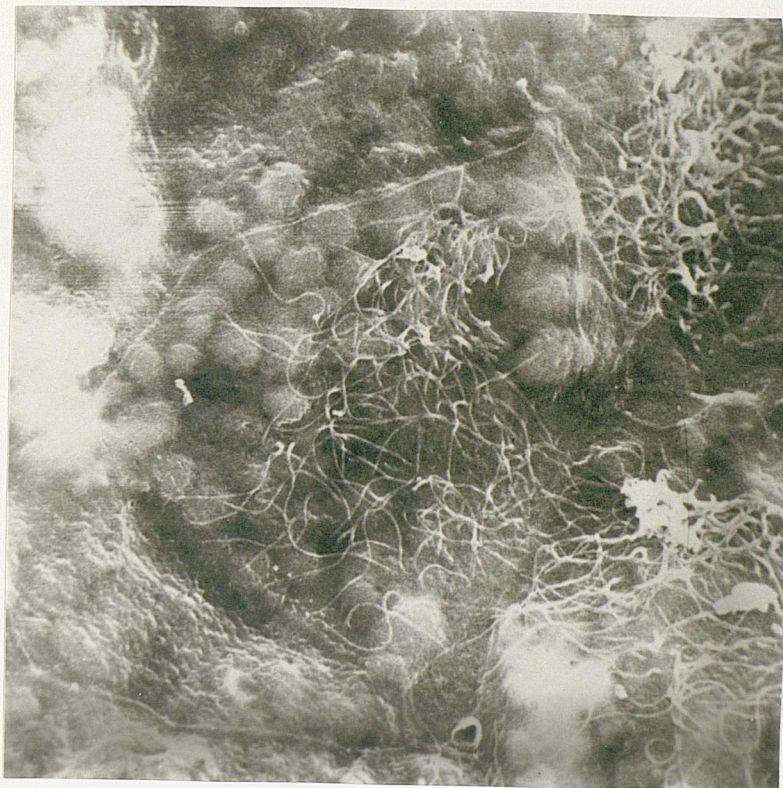
Transmission Electron Microscopy.

To check whether these patchy ciliated cells were in fact mucus cells with just patches of cilia, particular attention was paid to this work. Mature mucus vesicles plus cilia were looked for in the same cells. This situation was indeed often found. (See fig 21).

A clue to the formation of the cilia in these cells, is seen in other sections of the cells in the same embryo, where there occur an abnormal number of centrioles for functional mucus cells, figs 22-31. Normally in mucus cells, there are only two centrioles, and they occupy fairly deep positions in the cells. In these cells, however, there must be at least nine, because nine have been counted in one section alone. They are found associated with the nucleus, and higher up in the supra-nuclear cytoplasm, and finally it would appear that they form the basal bodies of cilia, fig 32. By this time, directly beneath these, numerous mitochondria have formed, as would be expected. also to be noticed, is the association in every case seen, of Golgi (33) apparatus with these centrioles. They usually seem to precede the centrioles to the outer cell surface. The centrioles themselves are associated and enshrouded in very fine, dense material, suggesting active synthesis.

To use the correct terminology, the centrioles described next to the nucleus, should have been called "procentrioles", as they are not quite fully formed. The amorphous dense material surrounding them provides the raw material for their growth into centrioles. Such groups of procentrioles as seen in these patchy cells, are called "generative clusters". The smaller spherical bodies seen, are the procentriole precursor bodies, and they have a diameter of about 800A.

27°C EMBRYO.

PATCHY CILIATED CELL.

Fig. 21.

This low magnification photograph shows cilia and their associated
mitochondria in one part of a cell and mucus secreting vesicles in
another. This is clear evidence for the patchy nature of some ciliated
cells.

x 11,200.
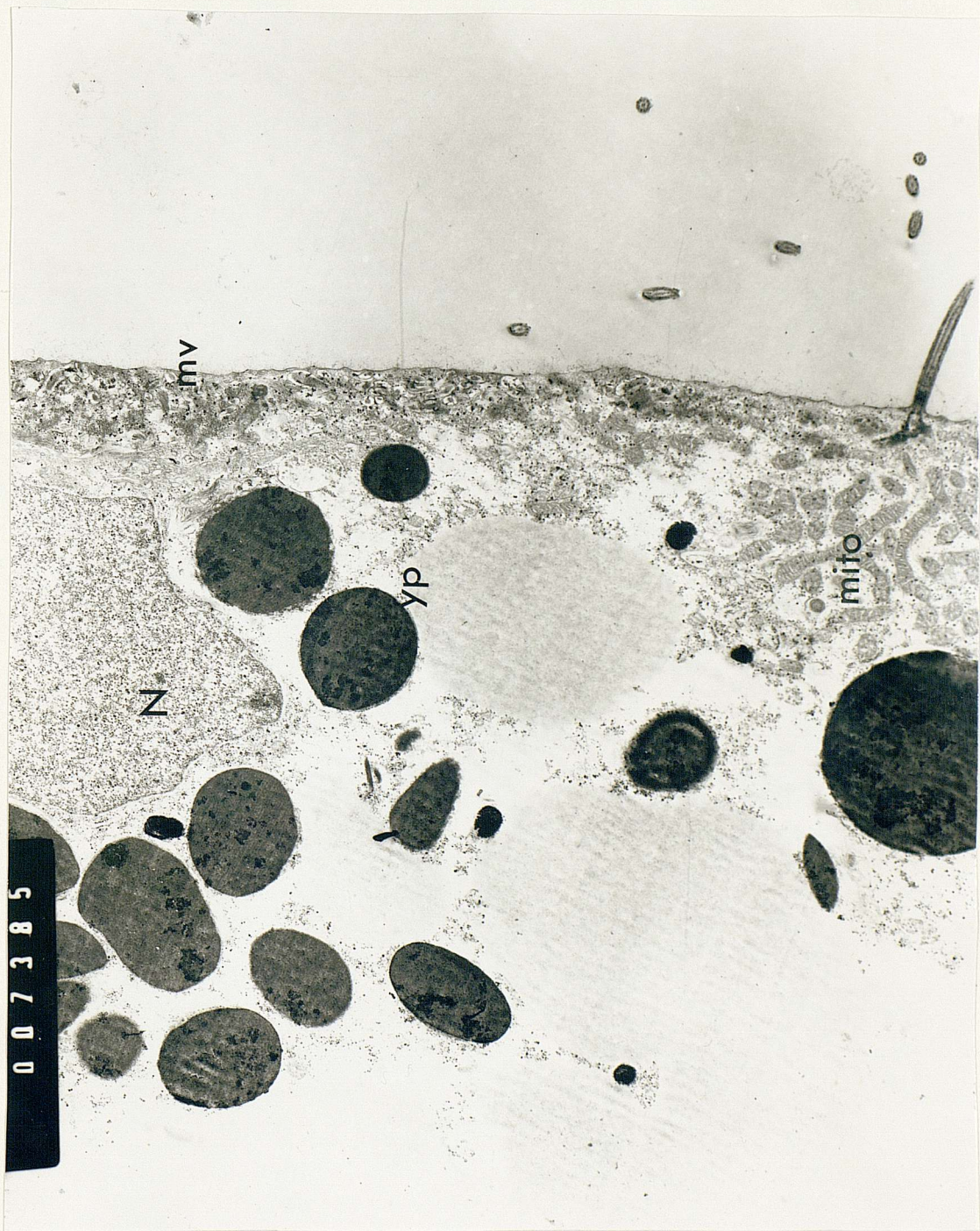
Figs. 22 - 31.
_____

Photographs 22 - 31 all show quite extensive centriolar activity.
As many as nine centrioles can be counted in one picture. These
centrioles appear to be replicating in otherwise mucus cells, as
mature mucus vesicles can be seen under the cell surface. In many
cases, the replicating centrioles appear to be preceeded to the outer
surface by fairly extensive Golgi apparatus. This is indicative of
membrane and vesicular formation, probably when the centrioles
reach the cell surface, as in fig. 32.

Fig. 22 = x11,200.	Fig. 23 = x20,600.
Fig. 24 = x41,000.	Fig. 25 = x50,000.
Fig. 26 = x80,000.	Fig. 27 = x50,000.
Fig. 28 = x50,000.	Fig. 29 = x32,000.
Fig. 30 = x16,000.	Fig. 31 = x20,600.

G. ves
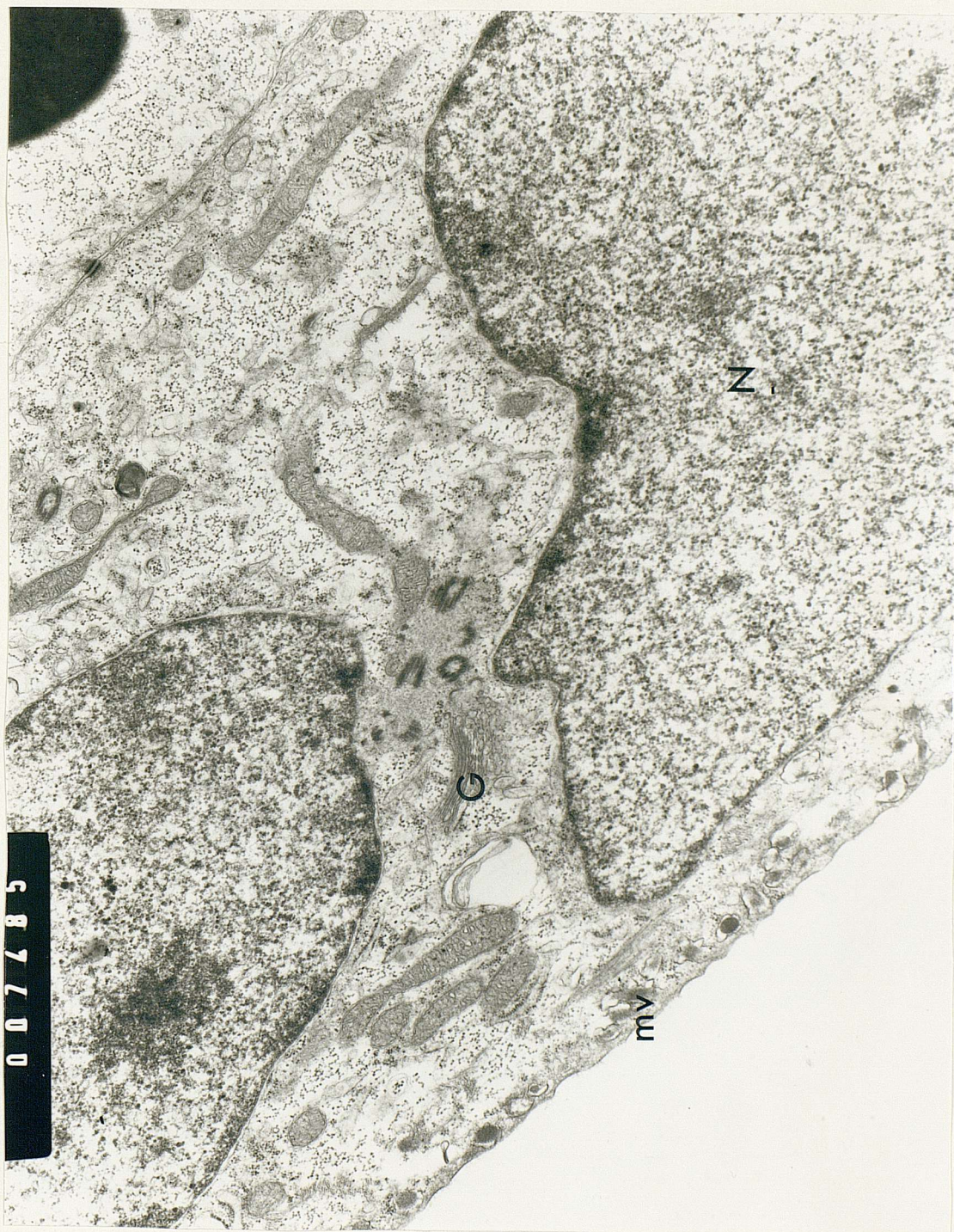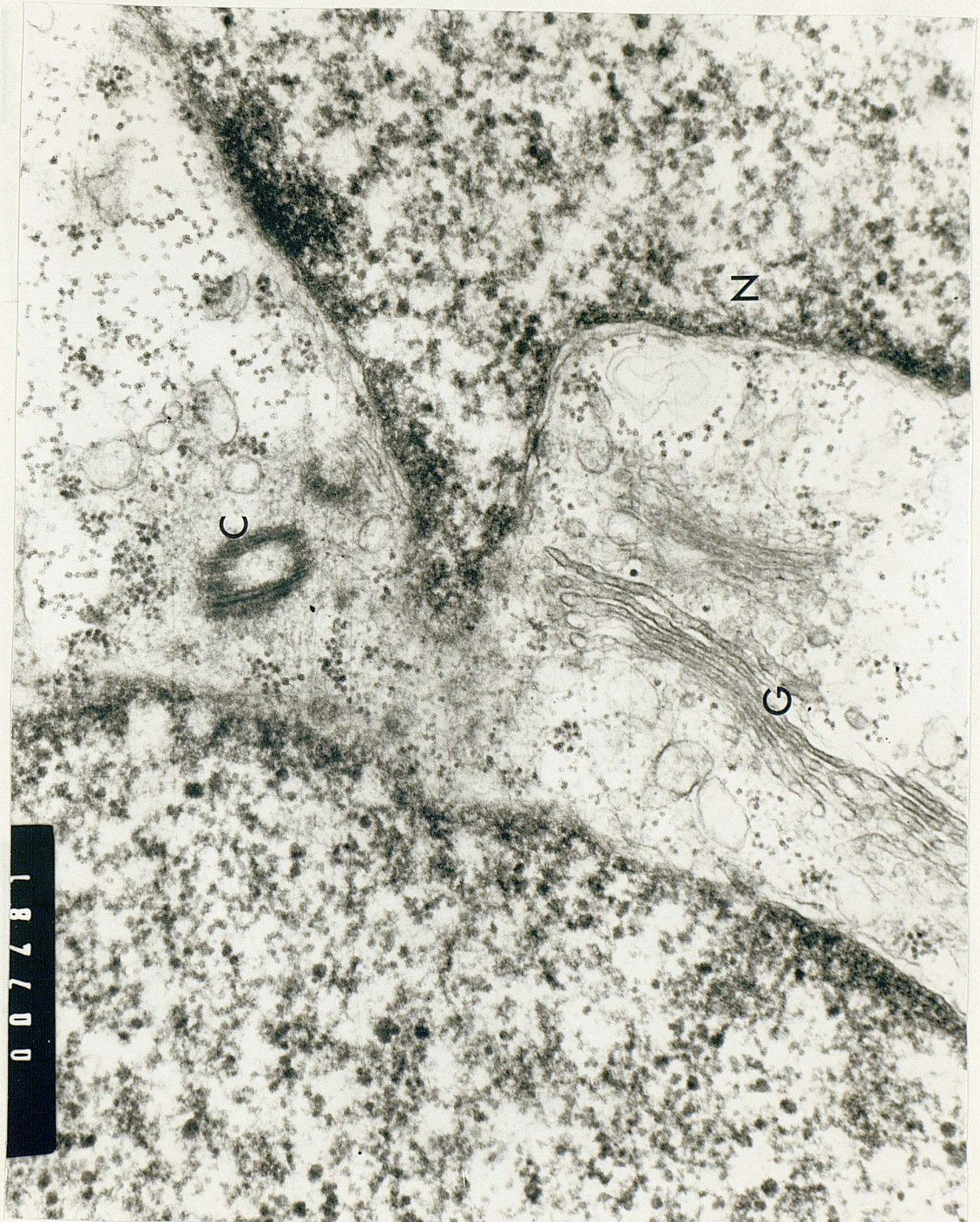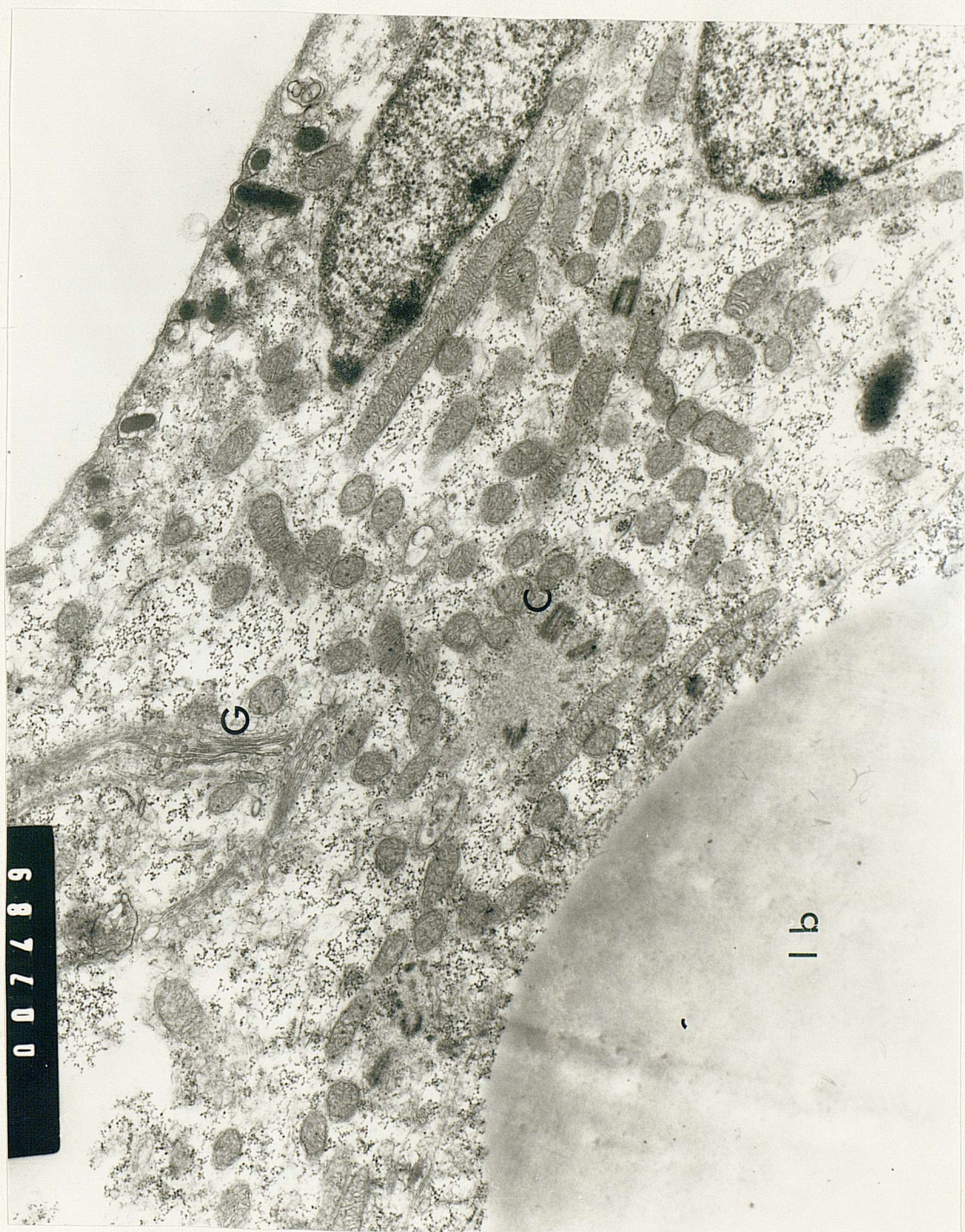
27^oC EMBRYO.

Fig. 32.
_____

This shows what happens when the centrioles do reach the cell

surface, i.e. cilia are formed via formation of basal bodies by the

centrioles. By this time, mitochondria are in close association

with the ciliary bases.

x 20,600.

mito

bb
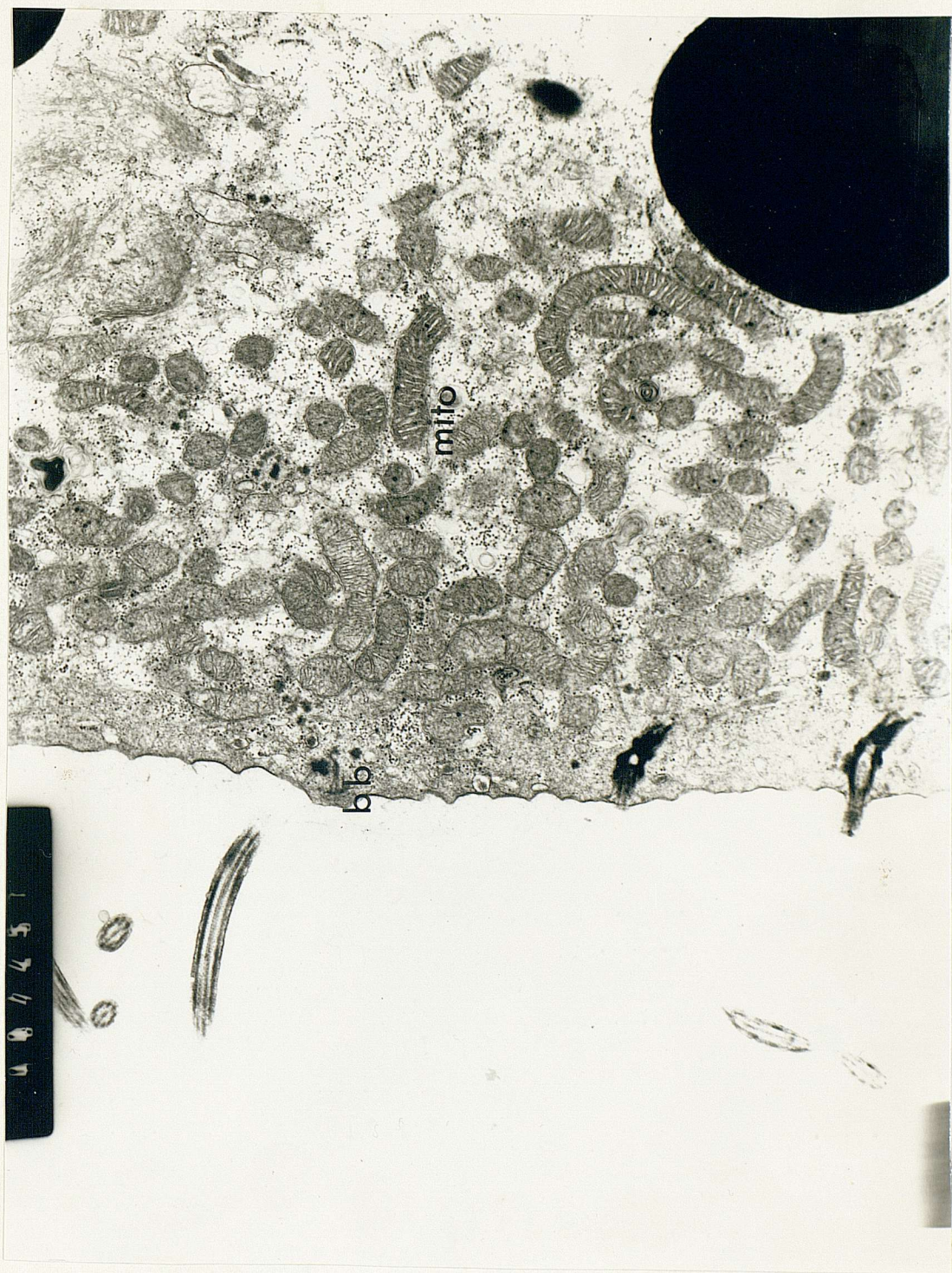
27°C EMBRYO.

Fig. 33.

This is an enlargement of some of the Golgi apparatus

preceeding the replicating centrioles to the cell surface. This

particular set of apparatus seems fairly active, see photo.

x 80,000.

ves

mito

G

27°C EMBRYO.

Fig. 34.

This is an enlargement of a couple of mucus vesicles to show how the mucus packet has shrunk away from the vesicle wall during fixation and processing.

x 80,000.
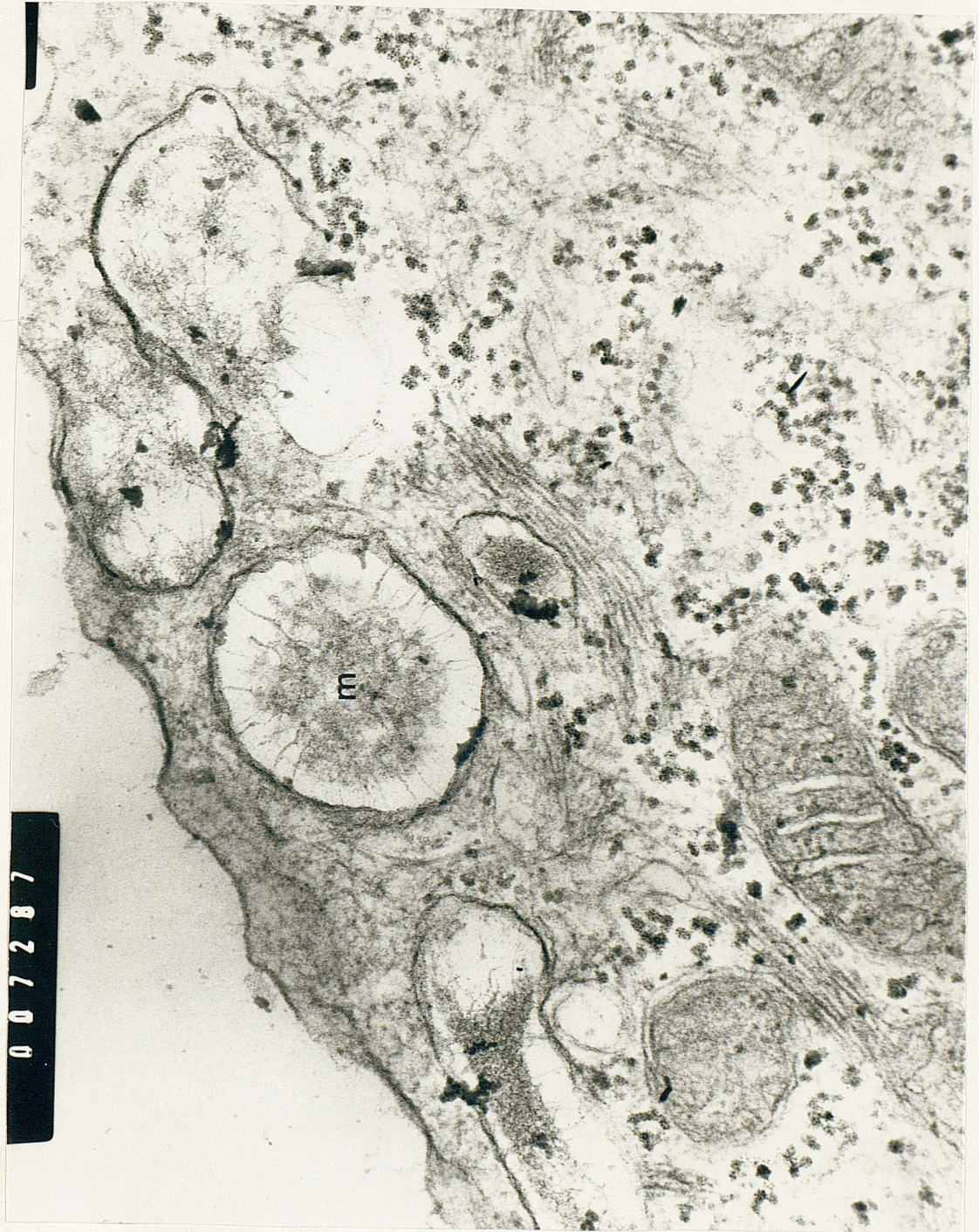
27°C EMBRYO.

Fig. 35.

This is a photograph of a normal mucus cell, showing clearly mucus
vesicles, a large mitochondrion with clearly defined cristae, and
a fairly extensive Golgi apparatus.

x 64,000.

20°C EMBRYO.

Fig. 35.

Large area of microvilli on two mucus cells. Note the desmosome between the two cells.

x 20,000.

Fig. 36.

This is an enlargement of three desmosomes showing a clear line (probably protein) between each half, and long tonofibrils (keratin) extending into the cells. They may serve an adhesive or contractile function for the cells.

x 80,000.
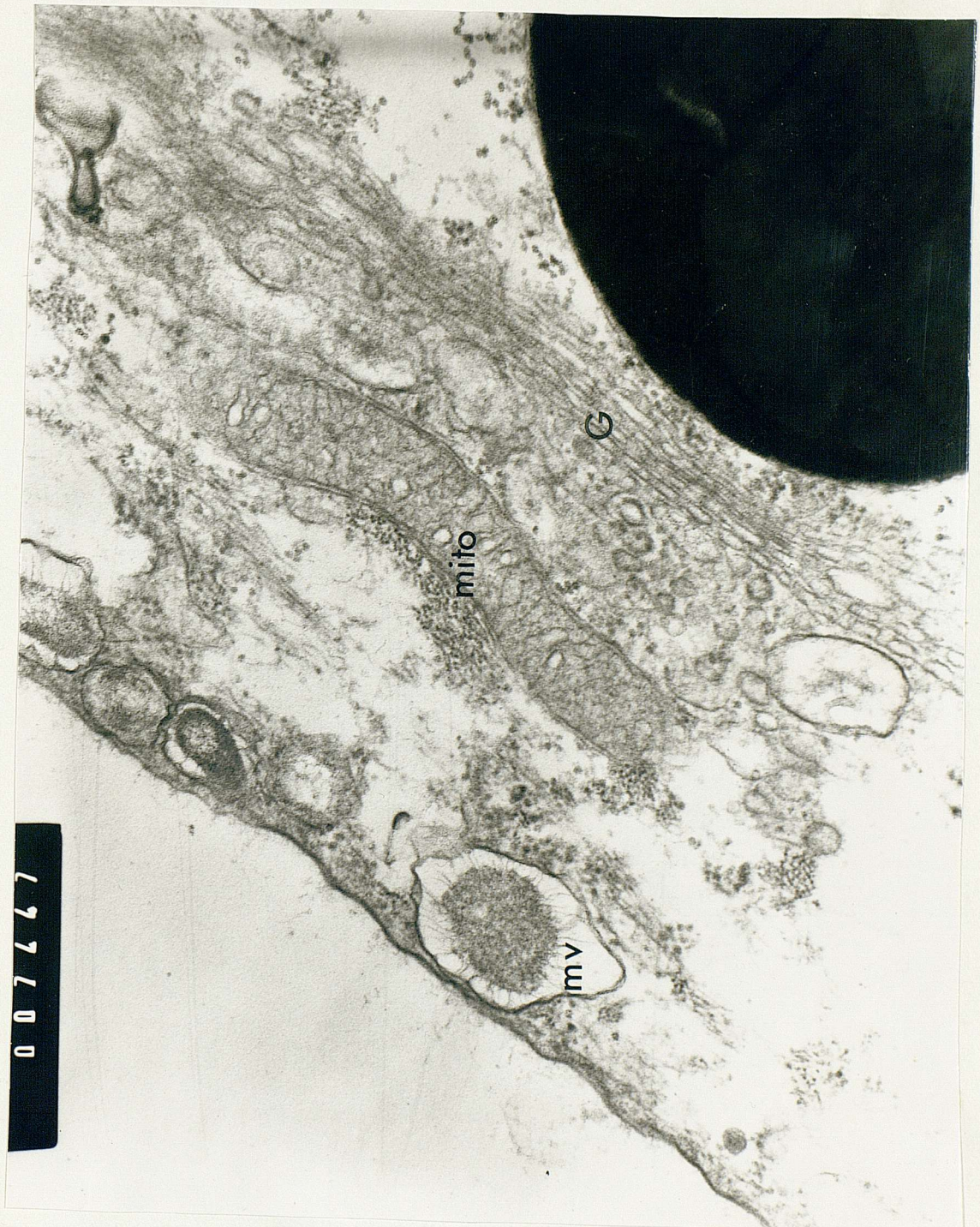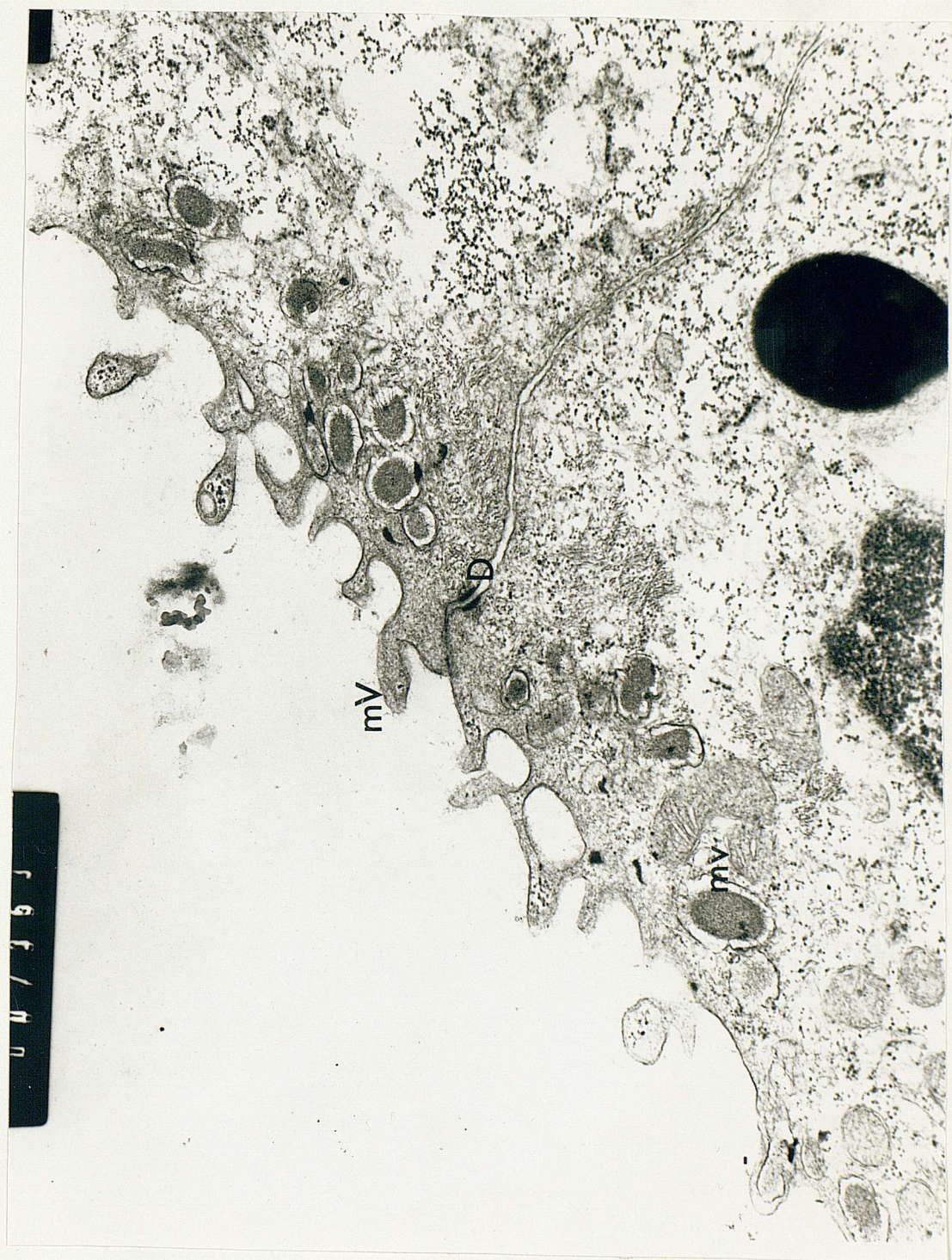
COMPARISONS OF THE MORPHOLOGY OF THE EPIDERMAL CELLS AS SEEN FROM THE

lu TRANSVERSE SECTIONS OF THE EMBRYOS.

These were araldite sections, stained with toluidine blue.

These sections, as well as providing an easy cell counting method, also gave a system in which to see, at a glance, if the cells of embryos developed at $27^{o}C$ differed noticeably in their morphology from the cells of the other embryos.

A general overall picture of both mucus and ciliated cells was also gained. What was noticed, was the confirmation of the patchy ciliated cells of the T.E.M. and S.E.M. photographs of the $27^{o}C$ embryos, and the general picture of distribution of organelles within both the ciliated and mucus cells, as previously mentioned. Drawings of some of these epidermal cells are given overleaf, not to scale.

A CILIATED CELL FROM AN EMBRYO DEVELOPED AT 7°C.

The zoning in this ciliated cell again consists of the lipid and yolk bodies being in the lower part of the cell, as with the mucus cells. Here, however, in the supra-nuclear region, are many many mitochondria, with cilia all along the free cell surface.

A CILIATED CELL FROM AN EMBRYO DEVELOPED AT 20°C.

The same applies as for the above cell, with cilia again all along the free cell surface.
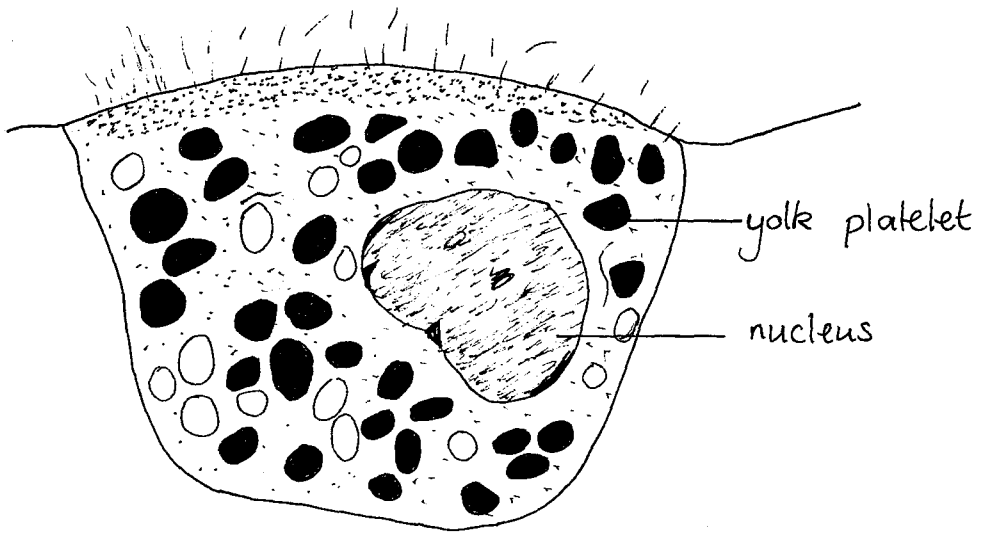
A ciliated cell from an embryo developed at 7°C.

yolk platelet

nucleus

A ciliated cell from an embryo developed at 20°C.

cilia

mitochondria.

lipid body
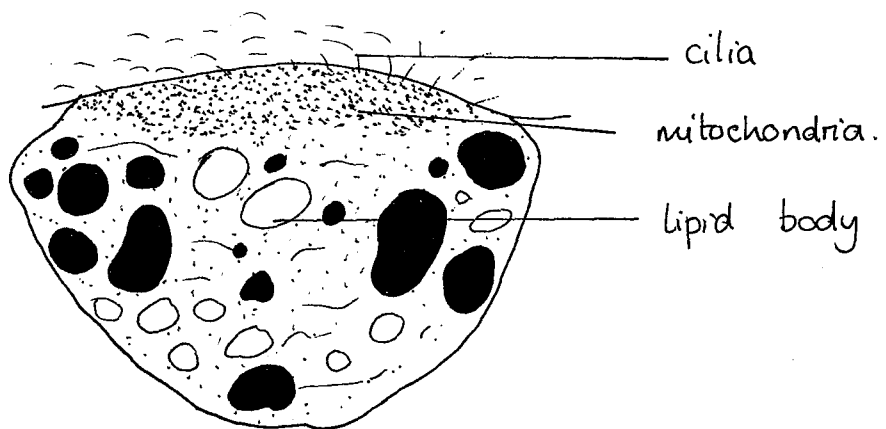
PATCHY CILIATED CELLS FROM EMBRYOS DEVELOPED AT 27°C.

All these cells, contrary to the other ciliated cells developed at different temperatures, have only patches of cilia along their free cell surface instead of all along it. The mitochondria are also confined to the area where the cilia occur.

The lipid and yolk bodies are still zoned to the lower portions of the cells.

"Patchy" ciliated cells from embryos developed at 27°C.

lipid body

yolk platelet

cell membrane

cilia

nucleus

outer cell membrane

mitochondria

mucus secreting part of the cell.
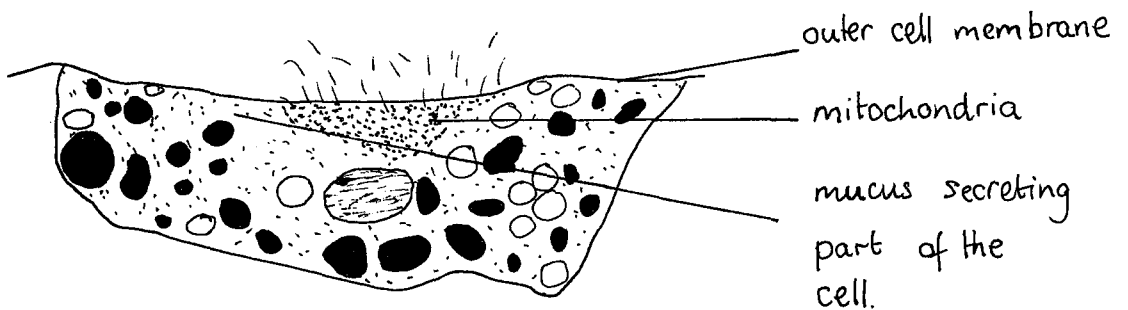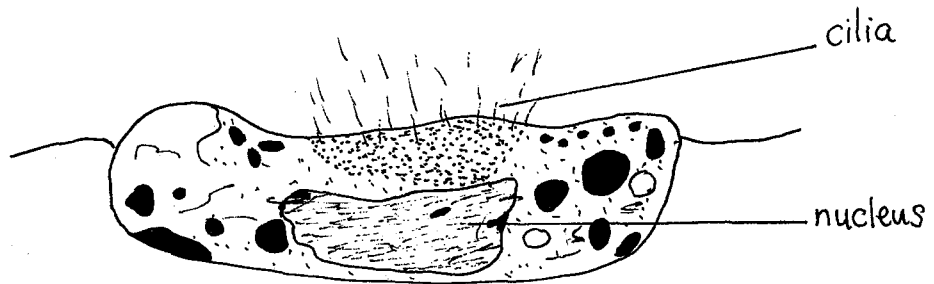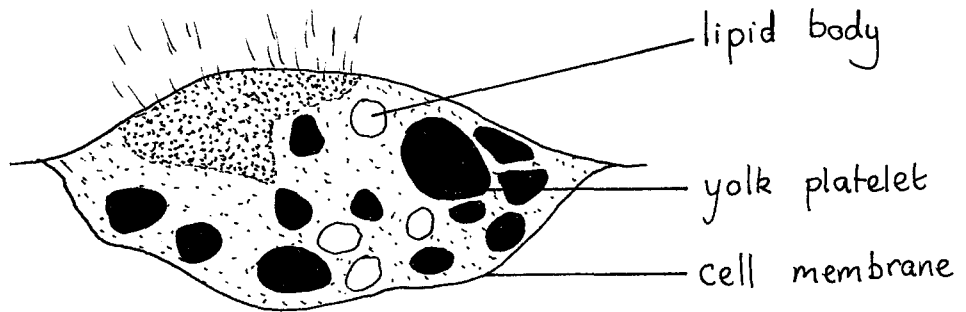
MORE "PATCHY" CILIATED CELLS FROM EMBRYOS DEVELOPED AT 27°C.

Again, cilia are confined to only a part of the free cell surface.

TYPICAL MUCUS CELL OF A 27°C EMBRYO.

This illustrates that although the ciliated cells of embryos developed at 27°C differ from other ciliated cells, the mucus cells appear the same.

More "patchy" ciliated cells from embryos developed at 27°C.
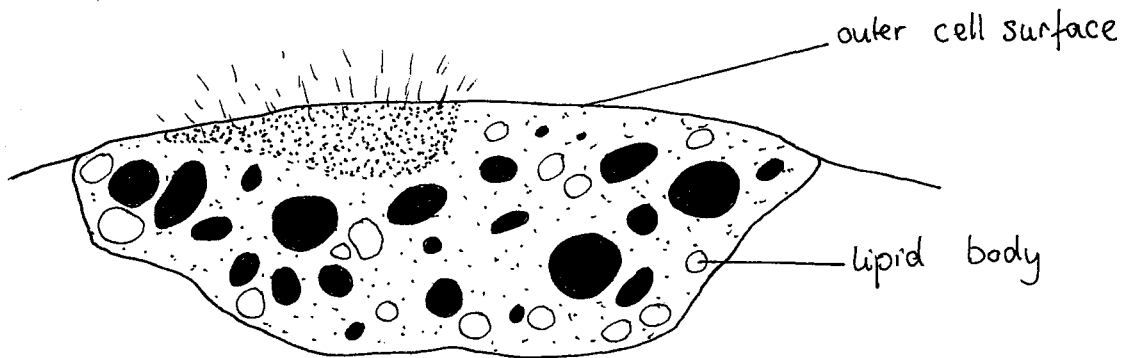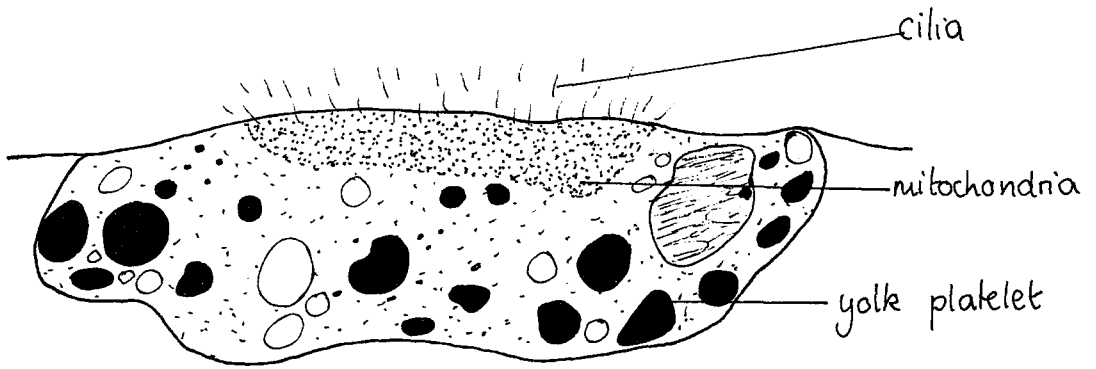
cilia

mitochondria

yolk platelet

outer cell surface

lipid body

Typical mucus cell of a 27°C embryo.

layer of mucus vesicles below surface.

nucleus
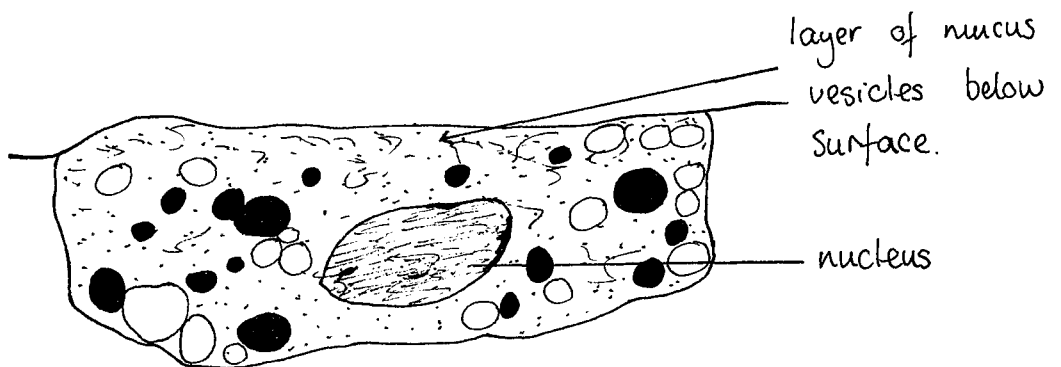
TYPICAL MUCUS CELL FROM AN EMBRYO DEVELOPED AT 12°C.

This is a typical mature mucus cell, and illustrates the zoning of the larger yolk platelets and lipid bodies in the bottom half of the cell, and the layer of mucus vesicles in the top, supra- nuclear region.

TYPICAL MUCUS CELL FROM AN EMBRYO DEVELOPED AT 7°C.

The same applies as for the above.

Typical mucus cell from an embryo developed at 12°C.

layer of mucus
vesicles

nucleus

yolk platelet

Typical mucus cell from an embryo developed at 7°C.

lipid body

cell membrane.
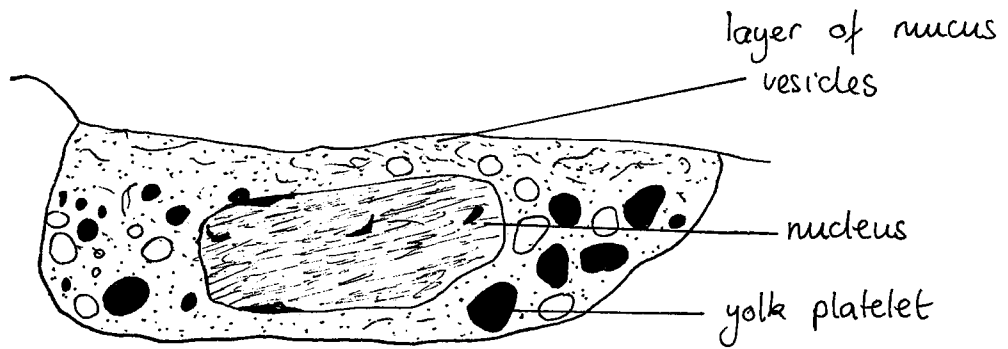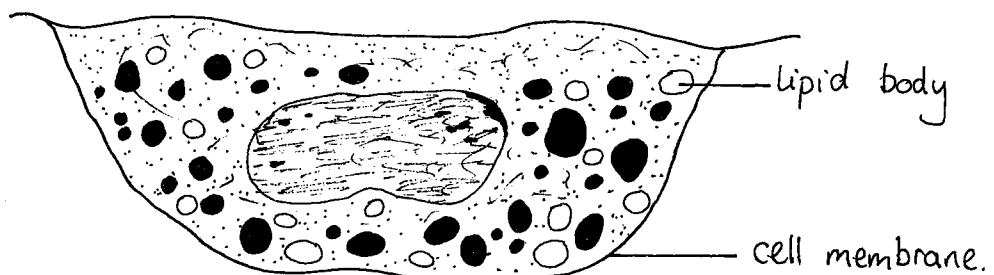
N.B. Drawings not to scale.

COMPARISONS OF THE NUMBERS OF CILIATED TO NON-CILIATED CELLS IN

EMBRYOS INCUBATED AT DIFFERENT TEMPERATURES.

The counting of the number of ciliated to non-ciliated cells for embryos incubated at different temperatures, was carried out to see if this ratio remained constant, or altered in any way according to the temperature. The comparisons of the ratios obtained from the cell counts, were of embryos of approximately equivalent stage developmentally.

To count the cell types, 1u sections of the embryos stained with toluidine blue, were simply examined at x400 under a Nikon microscope. The total number of outer epidermal cells was noted, and the number of ciliated cells within this outer layer. Tables were made out using the total number of epidermal cells (i.e. mucus and ciliated), the number of these that were ciliated, and finally the resulting ratio of ciliated:non-ciliated cells.

To test whether the ratio difference found between the different temperatures was statistically sound, the X^2 test was applied to the data. The Null hypothesis in this case, was that there was no difference in ratio between ciliated:non-ciliated cells at the four different temperatures of incubation.

At least ten different sections were examined to compile each set of results.

TABLES OF THE COUNTS OF THE CILIATED : NON-CILIATED CELLS.

FROM THE 1u ARALDITE SECTIONS.

7°C EMBRYOS.

Total no. epidermal cells	No. of ciliated cells	Ratio.
33	3	1 : 12
59	5	1 : 12.8
84	7	1 : 13
63	6	1 : 11.5
74	7	1 : 11.6
79	8	1 : 10.9
82	8	1 : 11.3
76	6	1 : 13.7
51	5	1 : 11.2
90	8	1 ; 12.3

12°C EMBRYOS.

64	5	1 : 13.8
71	8	1 : 9.9
59	5	1 : 12.8
49	6	1 : 9.2
74	7	1 : 11.6
80	8	1 : 11.0
68	7	1 : 10.7
102	9	1 : 12.3
93	10	1 : 10.3
56	7	1 : 9.0

20°C EMBRYOS.

Total no. epidermal cells.	No. of ciliated cells.	Ratio.
125	14	1 : 9.9
92	7	1 : 14.1
136	17	1 : 9.0
102	8	1 : 13.8
141	10	1 : 15.1
90	11	1 : 9.2
72	9	1 : 9.0
103	10	1 : 11.3
92	8	1 : 12.5
78	9	1 : 9.0

27°C EMBRYOS.

49	10	1 : 5.9
37	5	1 : 8.4
32	6	1 : 6.3
36	7	1 : 6.1
46	6	1 : 8.7
60	10	1 : 7.0
30	6	1 : 7.2
33	5	1 : 7.6
38	9	1 : 5.2
75	10	1 : 8.5
55	12	1 : 5.6
67	8	1 : 9.4
52	7	1 : 8.4
34	7	1 : 5.9

Comparison of the range of Ratios at the different temperatures.

At 7°C the range is from 10.9 - 13.7

At 12°C " " " " 9.0 - 13.8

At 20°C " " " " 9.0 - 15.1

At 27°C " " " " 5.2 - 9.4

To see if the big difference of range for the 27°C embryos was statistically significant, the X^2 test was applied to the ratios individually thus:-

NULL HYPOTHESIS: There is no difference in the ratios of the 27°C
embryos, 20°C, 12°C or 7°C embryos.

The average ratio of embryos incubated at 7°, 12°, and 20°C, is 11.5

27°C ratios.	Average (expected) ratio	X^2
5.9	11.5	2.73
8.4	"	0.84
6.3	"	2.35
6.1	"	2.54
8.7	"	0.68
7.0	"	1.76
7.2	"	1.61
7.6	"	1.32
5.2	"	3.45
8.5	"	0.78
5.6	"	3.03
9.4	"	0.38
8.4	"	0.84
5.9	"	2.73

Total of the X^2 figures.

2.73

0.84

2.35

2.54

0.68

1.76

1.61

1.32

3.45

0.78

3.03

0.38

0.84

2.73

Total = 25.04

With thirteen degrees of freedom, the probability is 0.01 - 0.001, i.e. highly significant, and the Null hypothesis is disproved, and therefore the ratio at 27°C is statistically different.

COMPARISONS OF THE CELL SIZES OF EMBRYOS DEVELOPED AT DIFFERENT

TEMPERATURES BUT OF THE SAME DEVELOPMENTAL STAGE.

These comparisons were done to see if embryos developed at this wide range of temperature all underwent the same number of mitoses or not. If, for example, some embryos had smaller cells than the others, this would mean that these embryos had more cells and hence had undergone more mitoses (assuming the actual size of the embryos themselves was the same).

It seemed quite obvious from the beginning of taking the S.E.M. photographs of the $27^{\circ}C$ embryos, that their cells were larger than those of the 7°, 12° and $20^{\circ}C$ embryos. The whole matter of cell size and hence number of mitoses was therefore approached by measurements taken from these photographs, all at known magnification.

To make any comparison between embryos of different incubation temperatures valid, embryos of as near the same developmental stage as possible were used. All the embryos measured were of approximately the 10 somite stage.

Method of cell measurement.
_____

As the magnification factor in each case was known, direct
comparisons rather than actual cell sizes were used. The cells were
accurately traced around from the photographs at x1K and x2K. The
different areas covered by the tracings was then worked out by placing
the tracing on mm graph paper and counting the number of mm squares
encompassed by the cell outline. Thus the figures given are of a
comparison of the number of mm squares, not of the actual cell sizes.
At least three tracings were made for each averaged figure which is
presented.

Areas of the different cells.

	At x1K	At x2K	
7°C	228	794	mm squares
12°C	535	1934	" "
20°C	496	1866	" "
27°C	874	2962	" "

Magnification factors of the above table. (1)

7°C	*	x3.5	*
12°C	*	x3.6	*
20°C	*	x3.8	*
27°C	*	x3.4	*

Magnification factors (2).

	At x1K	At x2K
7°C	*	*
	x2.4	x2.4
12°C	*	*
	x 0	x 0
20°C	*	*
	x1.7	x1.6
27°C	*	*

N.B.   It must be remembered in reading the figures, that those for x2K are not twice those of the x1K. This is because the magnification of the object is magnified twice about both the horizontal and vertical axis, i.e. in actual fact, x4. The figures given approximate an average of x3.6 magnification. The margin of error is presumably from error in counting these large numbers of mm squares, and the magnification printing error of the photographs.

From the three tables shown, it does appear that, at the same developmental stage, embryos incubated at 7°C have smaller cells than those incubated at higher temperatures. Those developed at 12°C and 20°C have approximately the same cell size, whereas those developed at 27°C have considerably larger cells.

From this, it would seem that at 7°C, because the embryos have smaller cells, there would be more cells per embryo. Likewise, there would seem to be fewer cells per embryo for those developed at 27°C, as

the cells are larger. However, the actual size of the embryos them-
selves was measured, and it was found that the 27°C embryos were
actually larger than the 7°C ones. This does not mean that there are
the same number of cells per embryo, and that the 27°C ones are just
larger, however, because the embryo size at 27°C is 2.2x greater than
those at 7°C, but that the cell size at 27°C is 3.8x greater. This
means that although the embryos are bigger at 27°C, there are still less
cells per embryo at 27°C, compared with the 7°C embryos. Hence it
would appear that embryos at 27°C undergo less mitoses.

The different sizes of the whole embryos was calculated, as before,
by tracing around the embryonic outline, and counting the number of
mm squares that the outlines encompassed. The results are as follows:-

At x50

	7°C	27°C
Averaged results	3,788	8,584

i.e., the 27°C embryos are 2.2x bigger than the 7°C ones.

Some copies of the tracings done are given on the following page.

COPIES OF TRACINGS DONE OF THE EPIDERMAL CELLS.

27°C CELLS.

These five cells are examples of the size of 27°C cells at x2K.
The cell divided up into squares shows how the total number of mm
squares for each cell was arrived at. Each square with 100 marked
in it has an area of 100 mm squares. The figures in the incomplete
squares are the number of mm squares covered by the area.

The range of size of these 27°C cells covered here is from 2781-
3901 mm squares.

20°C CELLS.

The range of size here is from 1848-1535 (in these two examples).
These cells are therefore smaller than those developed at 27°C.

Copies of Tracings done of the Epidermal Cells.

27°C cell × 2K

9	44	50	30			
58	100	100	100	100	70	26
14	100	100	100	100	100	100
13	100	100	100	100	100	100
70	100	100	100	100	100	100
50	100	100	100	100	76	
69	100	100	100	69		
	20	29				

Total = 3,855
mm squares

27°C cell at ×2K

3,901

3,085

2,835

2,781

20°C cells × 2K.

1,848

1,535
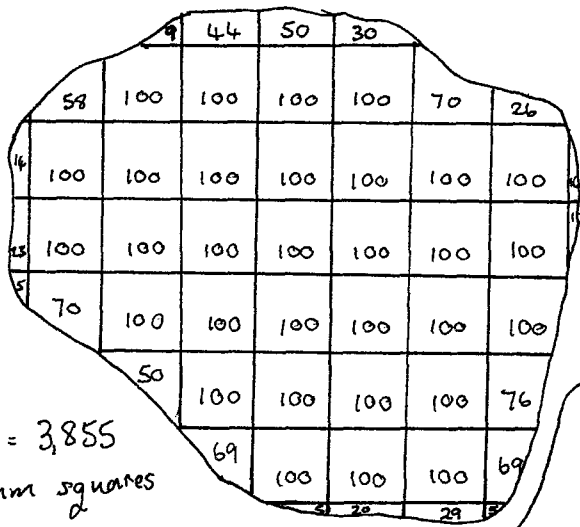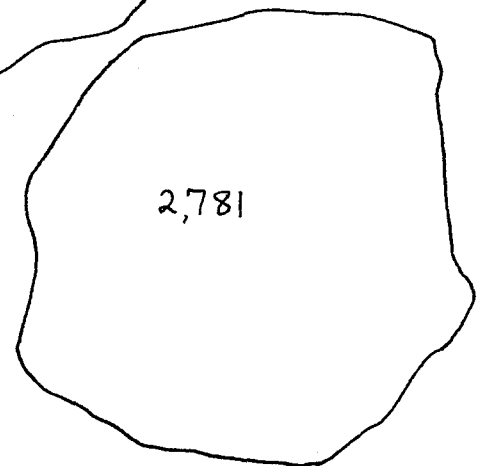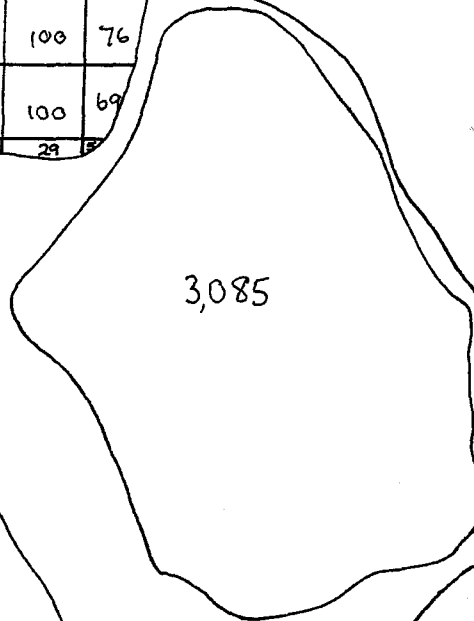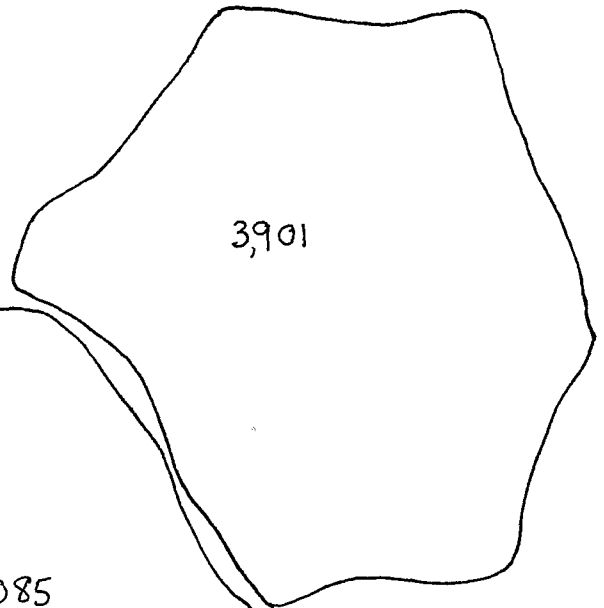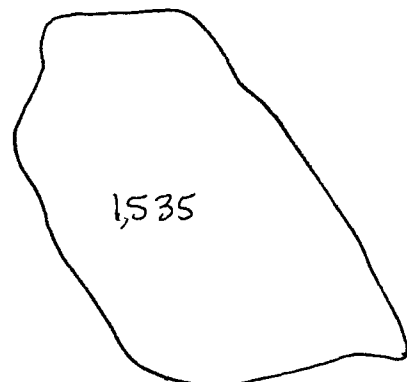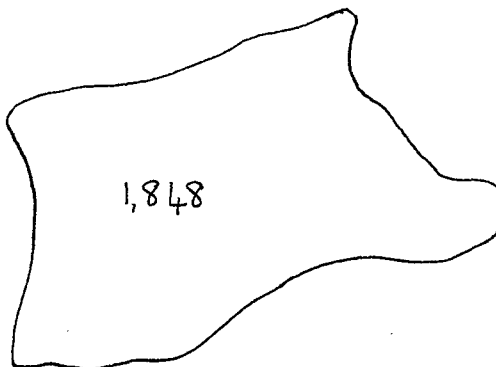
12°C CELLS x2K.

The range for these two examples is from 1877-2102 mm squares.

7°C CELLS x2K.

The range here is from 718-898 mm squares. These are therefore definitely smaller than any of the ciliated cells developed at other temperatures.

CELLS AT x1K.

Here one example of a cell at each development temperature x1K is given. The largest is the one developed at 27°C. The smallest is the one developed at 7°C.

12°C cells at ×2K
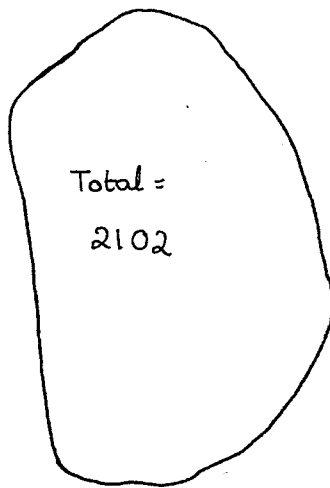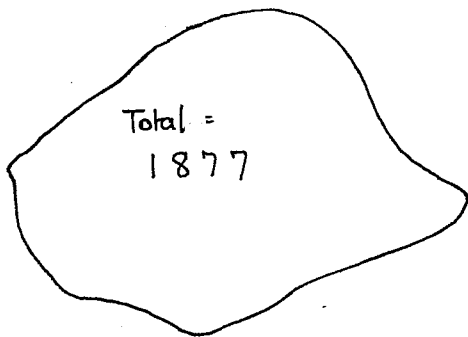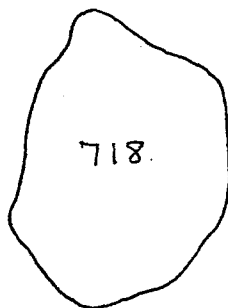
Total = 1877

Total = 2102

7°C cells at ×2K

898

718

876

12°C cell ×1K

555

20°C cell ×1K

516

27°C cell ×1K

905

7°C cell ×1K

233
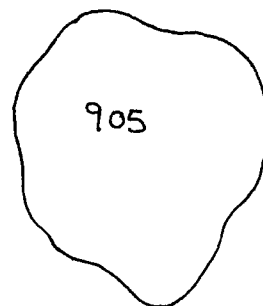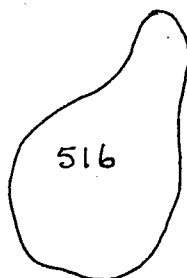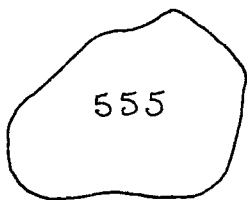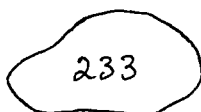
Whole Embryo Developed at 7°C ×50

Total = 3,865.
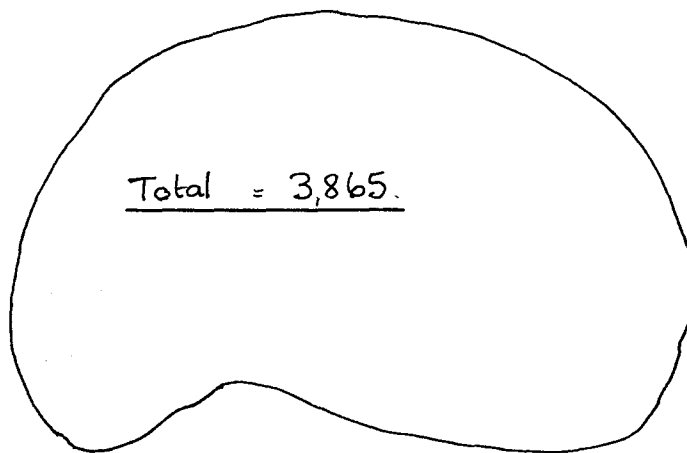
Whole Embryo Developed at 27°C ×50

Total = 8,450.
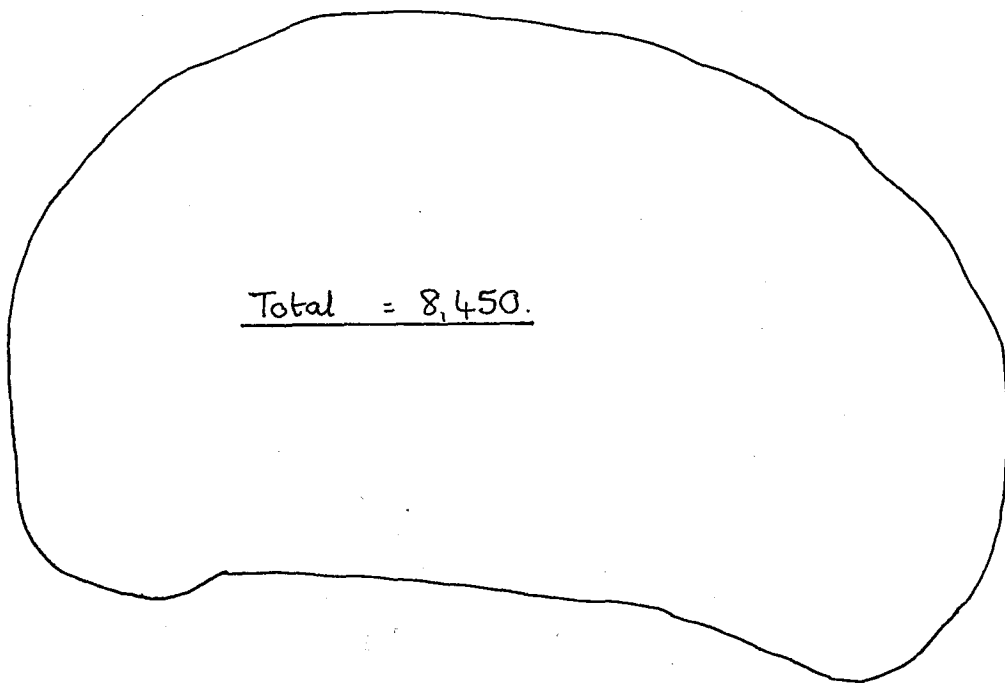
OBSERVATIONS ON THE CILIATION OF XENOPUS LAEVIS EMBRYONIC EPIDERMIS.

The following observations are of the preliminary work on <u>Xenopus</u> <u>laevis</u> embryos. It served as a way of developing techniques and provided material with which to compare the axolotl observations. These embryos were just developed at room temperature, and not the range of temperature the axolotl embryos were subjected to.

CILIATION OF XENOPUS LAEVIS EMBRYONIC EPIDERMIS.

The embryos were developed at room temperature and fixed in Karnovsky's formaldehyde/glutaraldehyde mixture, as for the axolotl embryos. They were then processed for S.E.M. and coated with gold-palladium.

Observations.

A very similar pattern of embryonic ciliation to that of the axolotls was seen. The earliest cilia were seen at about stage 20 (Niewkoop and Faber's tables), and fully ciliated embryos were seen at about stage 22-24. Only one incubation run was done, so these results were not checked.

From the photographs, it would appear that these ciliated cells may come up from beneath the outer epidermal layer, as they often occured in slight depressions. The spaced out pattern of occurence of the ciliated cells amongst the mucus cells was of the same pattern as the axolotls. The ciliated cells themselves seemed smaller in comparison with the mucus cells than the axolotl ciliated cells did. The cilia also were fewer, but longer. Apart from these slight differences, the ciliation of the two species was very similar.

DISCUSSION.

Development of cilia at different temperatures.

Regardless of the temperature of development, it seems that cilia
first appear just after the closure of the neural folds, between 1-3
somites. The very first cilia erupt in the anterio-dorsal area, and
thereafter the whole surface quite suddenly becomes ciliated. In this
work, it was found that ciliation seemed dependent on the morphological
state of the embryo, and prior closure of the neural folds seems
necessary before ciliation begins.

                                                     is contrary

This independence of temperature ^to Twitty's observations, who
postulated that because morphological development at low temperatures
was inhibited, ciliary determination would proceed at a greater rate.
However, the observation methods that Twitty used were much more
primitive than those used today. He had to rely on light microscope
observations of the direction of beat of the cilia, which in itself
is a very difficult procedure. Being able to accurately turn the
small pieces of ectoderm and re-orientate them successfully is in
itself no easy feat. His results do show too, that at the earlier
stage (18), a percentage of the room temperature embryos showed ciliary
determination, $1/8$ th in fact, as against 88% of the low temperature
ones, so the results were not completely clear cut. By the next stage,
all the low temperature ones and practically all the room temperature
ones were determined anyway.

Flickinger (1971) supported Twitty's view of earlier determination at low temperatures and attempted to explain it in terms of R.N.A. production, i.e. at lower temperatures R.N.A. synthesis is retarded less than cell division and development. With a lengthening of the cell cycle at lower temperatures, Flickinger argues that this would allow more time for greater production of R.N.A. He carried out experiments on tail-bud stage amphibian embryos raised at $13^{o}C$ and $23^{o}C$, and determined that they had similar amounts of D.N.A. and hence had undergone the same number of cell divisions, but that those raised at the lower temperature had more R.N.A.

In the results given here, p.50 it can be seen that not very much difference in cell size and hence cell division does occur between embryos raised at $12^{o}G$ and $20^{o}C$, which are similar to the temperature Flickinger examined. However, he did not examine animals raised at the extremes of $7^{o}C$ and $27^{o}C$, as was done for this thesis. Such extremes of temperature may cause substantially different results, and may cause the number of cell divisions/embryo etc. to change, as seems to be the case here, see p.51.

More recent work by P. Chibon (1972) on embryonic newts (Pleurodeles walthii Michah) rather gives evidence for the opposite idea, that at lower temperatures, tissue differentiation should be later in comparison with cellular proliferation. He incubated embryos at 12°, 17°, 23° and $26^{o}C$, and an analysis of the cell cycle times found that the G_1 phase is non-existent at $12^{o}C$ and below, but rapidly lengthens until at $26^{o}C$ it occupies a quarter of the generation time. S phase was found to lengthen at low temperatures and is supposed to be a consequence of the decrease in the production of enzymes and precursors necessary to D.N.A. synthesis, as these are elaborated during G_1 phase, and no G_1 means a longer S phase.

Lengthening of the G_1 phase is now commomly regarded as related to differentiation itself. For example, meristematic plant cells, tumorous cells and very early embryonic cells generally have a very short G_1 phase or none at all. It is as development and differentiation proceed that G_1 appears and lengthens, when the various R.N.As are produced. The high sensitivity of G_1 to temperature suggests its duration is directly related to cellular metabolic activity. Hence in these embryos at low temperatures, with no G_1, differentiation should be later, not earlier, if anything.

Elizabeth Deuchar ('66) also disagrees with the idea that low temperatures cause early differentiation, and with regard to the reported early lens differentiation in amphibian embryos reared at low temperatures, she feels that the explanation of such chemodifferentiation continuing independently of morphological development is not at all likely. Morphological processes must still always depend on bio-chemical ones, whatever the temperature. As an alternative explanation Deuchar proposes that the earlier reaction could be as a result of a more rapid diffusion of the lens inducing agent through weakened cell membranes. It is noticeable that chilled amphibian embryos are very friable, especially on handling.

I feel too, that it is unlikely that in such drastically chilled ($7^{\circ}C$ embryos), with a considerable lowering of metabolic activity and the probable absence of a G_1 phase, that there is a premature development of cilia. Early ciliation means early replication of the centrioles. Centrioles contain D.N.A., so there must be early replication of the centriolar D.N.A. and at this low temperature there would have to be

premature D.N.A. synthesis. Such activity seems unlikely at a low metabolic rate with probably no G_1 phase. A point to note, is that Lovtrup (1959) found, in a study of the utilisation of energy sources during amphibian embryogenesis at low temperatures, that utilisation of fat is impeded at temperatures of $10^{\circ}C$ and below. This in turn causes proteins, which might otherwise have been utilised for synthetic purposes, to be mobilised for the supply of energy.

On the contrary, I would have expected perhaps, the opposite to earlier determination, i.e. later development of the cilia at the low temperatures. However, at $7^{\circ}C$ the cilia appear at just the same time as those at 12°, 20° and $27^{\circ}C$. The ratio of ciliated/non-ciliated cells is also the same at 7°, 12° and $20^{\circ}C$.

It does appear therefore, that whatever the temperature of development, the differentiation of cilia is stage dependent, and that the degree of morphological development and differentiation cannot easily be disrupted by temperature. This finding does rather nullify Twitty's long ago proposed theory.

DEVELOPMENT OF EMBRYOS AT 27°C.

The other major finding of this work, is the occurence of patches of cilia on otherwise differentiated mucus cells. The function of these patches is not immediately obvious and it can only be tentatively suggested that the high temperature triggers off some synthetic process which sets up a chain reaction ending with the formation of cilia.

From the T.E.M. photographs, it would appear that the process "sparked off", is the replication of the pair of centrioles associated with the nucleus. As centrioles contain D.N.A. , enough to code for 2-3 proteins, it would seem that perhaps it is the D.N.A. synthetic process that is initiated. It could be considered as a possibility perhaps, that D.N.A. synthesis is in some way channelled from mitosis into centriolar replication. This is because the 27°C embryonic cells have been found to be larger than the 7°,12° and 20°C embryonic cells, indicating that there are less cells/embryo (of the same stage) i.e. have undergone less mitoses.

The whole embryo sizes were checked and the 27°C ones were found to be larger than the 7°, 12° and 20°C ones, the 7°C ones being the smallest. Even so, the 27°C cells were still larger in comparison. Decker and Kollros (The effects of cold on hind limb growth and lateral motor column development in Rana pipiens,1969) substantiate this finding of the effect of temperature on the size of amphibian embryos, as they found that by developing Rana at 6°C, the results were animals whose total length at a given stage was less than those animals maintained at higher temperatures.

If the 27°C embryos do indeed undergo less mitosis, and D.N.A. synthesis is channelled into the centrioles, which in turn form cilia, this would be very similar to the case reported by Stubblefield and Brinkley (1966). Previous work by Stubblefield and Klevecz (1965) involved obtaining synchronised cell populations of Chinese hamster cells, using colcemid, the mitotic inhibitor. In 1966, whilst studying the effects of colcemid on the actual structure of the mitotic cells, Stubblefield and Brinkley found that there was a rather strange effect on some interphase cells, which was the rapid differentiation of centrioles into basal bodies, with the eventual formation of cilia. Centrioles have also been found capable of multiplication in artificially activated non-nucleate egg fragments, which is further evidence for their capability of autonomous differentiation and replication. Stubblefield and Brinkley suggest that all cells are capable of cilia production, but that the process occurs only during a specific period in their growth cycle.

Alternatively, D.N.A. synthesis might not be channelled into the centrioles, but synthesis is enhanced by the higher temperature and the centrioles replicate. The replication of centrioles is clearly seen from the photographs, and begins adjacent to the nucleus. From here they appear to move up into the supranuclear cytoplasm and finally form the ciliary bases. Where cilia form on the surface, mitochondria also congregate just beneath, to provide the cilia with the necessary energy to beat. Either side of the ciliated patches can usually be seen the already existing mucus packets.

A completely different idea, is that perhaps at 27°C, cell division is in some way inhibited, so that the patchy cells have not actually divided, yet have started differentiation. This fits in with the finding that the cells are larger than normal. What it really means therefore, is that these patchy cells should really have divided to form one mucus and one ciliated cell, but that the cell failed to divide. This idea would be more acceptable if the patch of cilia appeared on one side or half of the cell, but as seen, the patches of cilia can occur anywhere on the cell surface. Division of cells would also have to be inhibited just at this stage of development and not before. The embryos continue development to the formation of gill buds, but further development was not recorded at the time. It would be interesting to compare later larvae and adults of embryos and larvae developed at 7°,12°,20° and 27°C to see just what form they took.

One point to try and explain, is why the 27°C embryos are slightly bigger than the 12° and 20°C embryos. It must be assumed that all the embryos, when laid, have similar yolk reserves. How is it then, that the 27°C ones are able to grow larger before they start to feed for themselves? One explanation could be that they take up water, perhaps due to weakened membranes brought on by the high temperature of their immediate environment. This explanation, of course, destroys the proposal that 27°C embryos, because they have larger cells, necessarily have undergone less mitoses. The only way to settle this point of how many cells there are, would be to count all the embryonic cells as proposed before, or by D.N.A. estimations. An alternative explanation could perhaps be that at higher temperatures, the reserves are able to be utilised quicker and the synthetic machinery gets under way sooner, with the production of more R.N.As and the synthesis of large protein molecules.

Another unexplained point, is why only some of the already differentiated mucus cells form these patches of cilia. Mucus cells are differentiated before ciliated cells and it seems that the ones that develop these patches of cilia must do so at about the same time as the ciliated cells mature and appear on the epidermal surface. This is because the ciliated patches and fully ciliated cells appear to be of the same stage. The distribution of the patchy cells is fairly random, they do not appear to occur in any one specific area.

Lastly, it was very often noticed, that the ciliated cells of embryos developed at 27°C lacked microvilli. In particular contrast, the ciliated cells of embryos developed at 7°C showed very well developed microvilli. Microvilli serve to provide a greater area for absorption at the cell surface, so why they should be practically non-existent at 27°C is rather obscure.

SUGGESTIONS FOR FUTURE EXPERIMENTS.

To check the whole question of the number of cell divisions embryos have undergone, and of D.N.A replication, experiments involving D.N.A. estimations and labelling should be decisive.

Estimations of the total amount of D.N.A/embryo could be carried out by Feulgen photometry. By determining the amount of D.N.A of embryos developed at different temperatures, but of the same stage, one could check, for instance, if the $7^{o}C$ embryos had more D.N.A or not than the $27^{o}C$ embryos. More D.N.A should mean more cells. The actual number of cells per embryo could actually be checked by Cooke's method, as suggested in Nature 242 55.

D.N.A labelling could be done with H^3 thymidine to see where the D.N.A. was replicating. It would be interesting to see if the centrioles were labelled in the $27^{o}C$ patchy cells, but the chromosomes were not. This would give an idea as to whether these cells had stopped dividing and just the centrioles were replicating.

R.N.A estimations could also be done, comparing the R.N.A content of the 7° and $27^{o}C$ embryos. Embryos could also be developed between 7° and $12^{o}C$, and between 20° and $27^{o}C$, to see at what actual temperature the patches of cilia develop on the mucus cells.

It would also be interesting to see if chemicals could produce this same effect as high temperature. An obvious experiment, is to treat embryos with colcemid, as Stubblefield and Brinkley did, and see if this resulted in centriole replication and the formation of basal bodies.

SUMMARY.

The ciliation of the embryonic axolotl epidermis was observed over a range of incubation temperatures from 7°C to 27°C. Observations were made by light, scanning electron and transmission electron microscopy. It was found that ciliation occured at the same stage at all temperatures, i.e. between the 1-3 somite stage. This finding is contrary to the observation of Twitty (1928), who claimed that those embryos developed at 7°C became ciliated earlier.

At the other end of the temperature range studied, i.e. 27°C, a rather strange effect of temperature on ciliation was noticed. At this temperature, extra ciliation occured, in the form of patches of cilia on already differentiated mucus cells. From T.E.M. photographs, this extra ciliation was seen to be caused by replication of the mucus cells' pair of centrioles, and the eventual formation of ciliary bases and cilia. An explanation of this phenomenon is forwarded in terms of activated centriole replication.

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