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Phil. Trans. R. Soc. Lond. B 1985 **312**, 57-65
doi: 10.1098/rstb.1985.0177

Phil. Trans. R. Soc. Lond. B **312**, 57–65 (1985)

Printed in Great Britain

Analysis of clonal restriction of cell mingling in *Xenopus*

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[Plates 1 and 2]

Cell fates were traced by injecting horseradish peroxidase into single blastomeres of *Xenopus* embryos at 2- to 512-cell stages. At later stages the number, types and locations of all labelled progeny were observed. Progeny of a single labelled ancestral cell divided coherently until the 12th cell generation, the onset of gastrulation, and then dispersed and mingled with unlabelled cells. Cell mingling was restricted at mediolateral and anterior–posterior boundaries. These boundaries were always respected by progeny of any blastomere labelled at the 512-cell stage but they were frequently crossed by progeny of blastomeres labelled at the 256-cell or earlier stages. The boundaries defined seven morphological compartments each populated exclusively by a group of ancestral cells at the 512-cell stage. Each blastomere that contributed progeny to the nervous system also gave rise to a wide range of cell types in all three primary germ cell layers but the clone was restricted to a single compartment.

Analysis of clonal restriction of cell mingling was done *in vitro*. Twenty to thirty blastomeres were excised from one ancestral cell group at the 512-cell stage and combined *in vitro* with 20–30 blastomeres from another group. One group of blastomeres labelled with horseradish peroxidase was placed in contact with another group of unlabelled blastomeres, maintained *in vitro* for up to 2 days, and then processed histologically to show the distribution of labelled and unlabelled cells. Mingling was significantly greater in combinations of two of the same ancestral cell groups than in combinations of two different ancestral cell groups. A similar result was observed when a single labelled cell was combined with either the same or different ancestral cells. In all experiments the cells were significantly larger in combinations of different ancestral cell groups, indicating that they had undergone fewer divisions. These results are consistent with the hypothesis that boundaries observed *in vivo* are lines of clonal restriction formed by mutual inhibition of cell motility and cell division following contact between progeny of different ancestral cell groups.

Introduction

Introduction of horseradish peroxidase (HRP) as a stable intracellular lineage tracer has made it possible to trace the fates of individual blastomeres of *Xenopus* embryos. *Xenopus* embryo cells injected with HRP or biotinylated HRP divided and developed normally, the label was transferred to all the progeny and could be detected in them as many as 20 cell generations later, after they had differentiated (Jacobson & Hirose 1978, 1981; Hirose & Jacobson 1979; Jacobson 1981*a*, *b*, 1983; Jacobson & Moody 1984; Jacobson & Huang 1985). A single blastomere, labelled at the 2- to 512-cell stage, distributed its labelled progeny into a region called a clonal domain. There were systematic mapping relationships between the position of the labelled blastomere and the position of its clonal domain in the final morphological pattern. These regular relationships permit one to deduce a series of fate maps of embyos at successive

cell generations from the 2-cell (G1) to the 512-cell (G9) stage (reviewed in Jacobson 1985*a*, *b*). The purpose of constructing these fate maps was to find clonal restrictions. Clonal restrictions were shown when one or both daughter cells did not have the same fate as their maternal cells. Restrictions may lead to differences of fates that are either primarily histological or primarily morphological. In the former, different lineages lead to different types of cells; in the latter different clones are restricted to different parts of the morphological pattern (Jacobson 1985*a*, *b*). This paper deals with the latter type of clonal restriction which results in separation of lineages that occupy mutually exclusive morphological domains.

Cell dispersal and mingling did not start until the beginning of gastrulation (G12). Injection of a single blastomere at the 2-cell to 512-cell stage resulted in labelling of a coherent patch of progeny until G12. Cell dispersal and mingling occurred rapidly during the following three cell generations which corresponded with the period of gastrulation (Jacobson 1985*a*). Therefore, any restrictions of cell mingling would be expected to have an effect during and after gastrulation.

Restrictions of mingling occurred regularly at boundaries in the central nervous system and other tissues: an anterior–posterior boundary and a dorsal–ventral boundary (figure 1). The anterior–posterior boundary separated anterior structures from posterior structures, and the dorsal–ventral boundary separated dorsal structures from ventral structures. These boundaries defined four compartments on each side of the body, posterior-lateral (PL), posterior-medial (PM), anterior-lateral (AL) and anterior-medial (AM). The two anterior medial compartments fused in the ventral midline to form a single anterior-median (AM) compartment (Jacobson 1983, 1985*a*). There were no lineage relationships between the pattern of cell differentiation and the compartmental pattern. All blastomeres of the 2-cell to 512-cell embryo that gave rise to nerve cells also gave rise to a variety of types of mesodermal, ectodermal and endodermal cells (Jacobson & Hirose 1978, 1981; Jacobson 1982, 1983).

The evidence for clonal restriction of cell mingling in *Xenopus* embryos is essentially all of the same kind, namely the regular mapping relationships between the location of labelled progeny within certain invariant boundaries and their origins from discrete groups of blastomeres in the 512-cell embryo (Jacobson & Hirose 1981; Jacobson 1983). Blastomeres labelled at the 512-cell stage always contributed more than 95% of their progeny exclusively to one of the seven compartments. The progeny of all blastomeres belonging to the same ancestral group mingled in a single compartment but did not mingle with progeny of neighbouring ancestral cell groups (Jacobson 1983, 1985*a*). When individual blastomeres were injected with HRP at stages before the 512-cell stage, the clonal domain of a maternal cell was partitioned between its daughters, so that the line of partition frequently corresponded with a compartmental boundary (Jacobson & Hirose 1981; Jacobson 1983). When the maternal clonal domain was not completely partitioned but was unequally fractionated between the daughter cells, the inequalities appeared as abrupt differences in cell packing densities across compartmental boundaries (Jacobson & Hirose 1983). For example, such boundaries could be seen after labelling one blastomere at the 2-cell stage (figure 2*a*, plate 1). Because the first cleavage plane corresponded with the embryo's midline, and because cell mingling across the midline was restricted to the anterior-median compartment, only that compartment contained a mixture of labelled and unlabelled cells: all other parts of the embryo were either completely labelled or completely unlabelled. Exactly the same morphological compartment was populated by descendants of a single labelled blastomere located in the anterior-median ancestral cell

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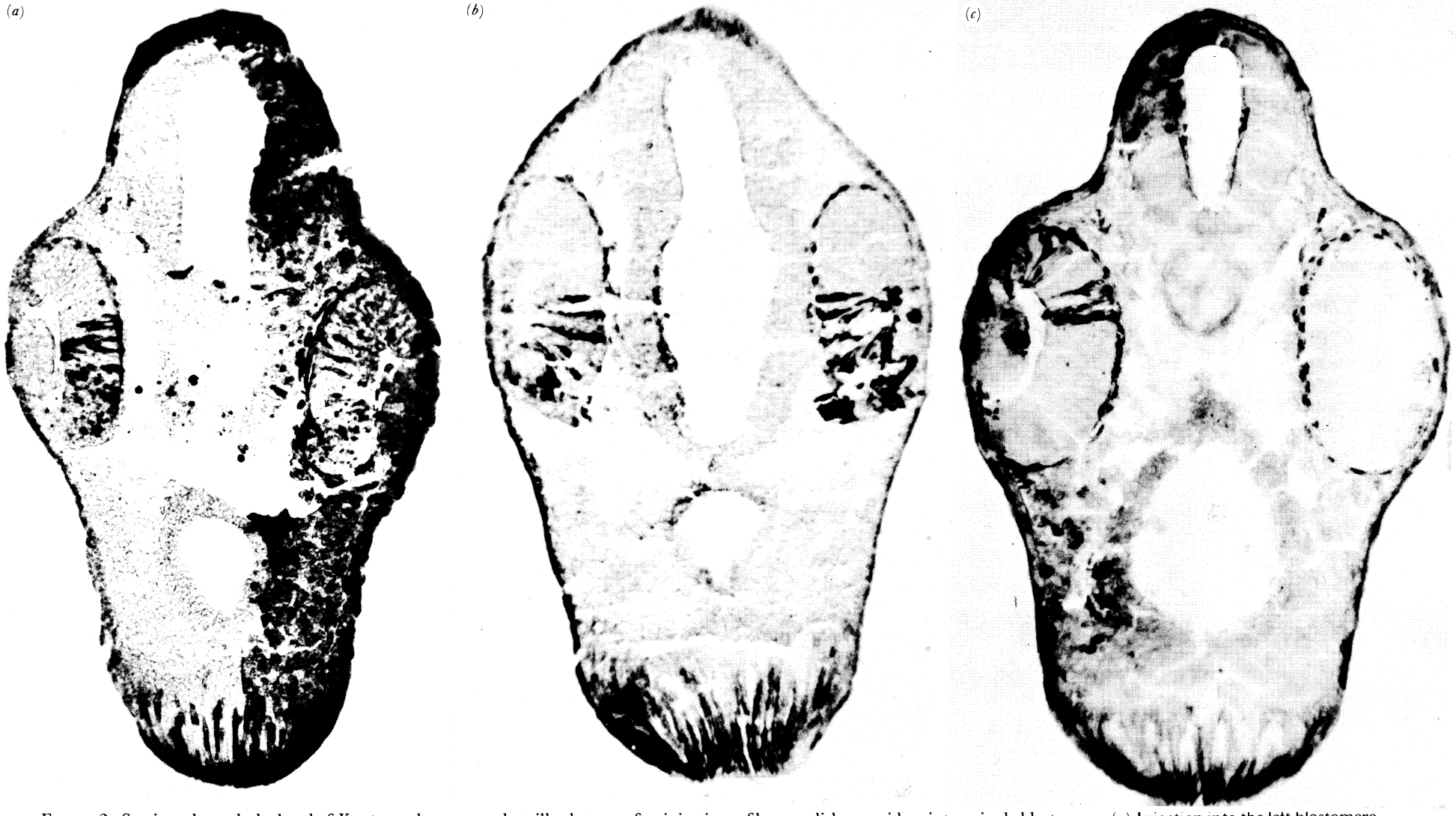

FIGURE 2. Sections through the head of *Xenopus* embryos at early tailbud stages after injections of horseradish peroxidase into a single blastomere. (*a*) Injection into the left blastomere at the 2-cell stage. (*b*) Injection into a single blastomere of the AM ancestral cell group at the 512-cell stage. (*c*) Injection into a single blastomere of the right AL ancestral cell group at the 512-cell stage (Magn. × 100.)

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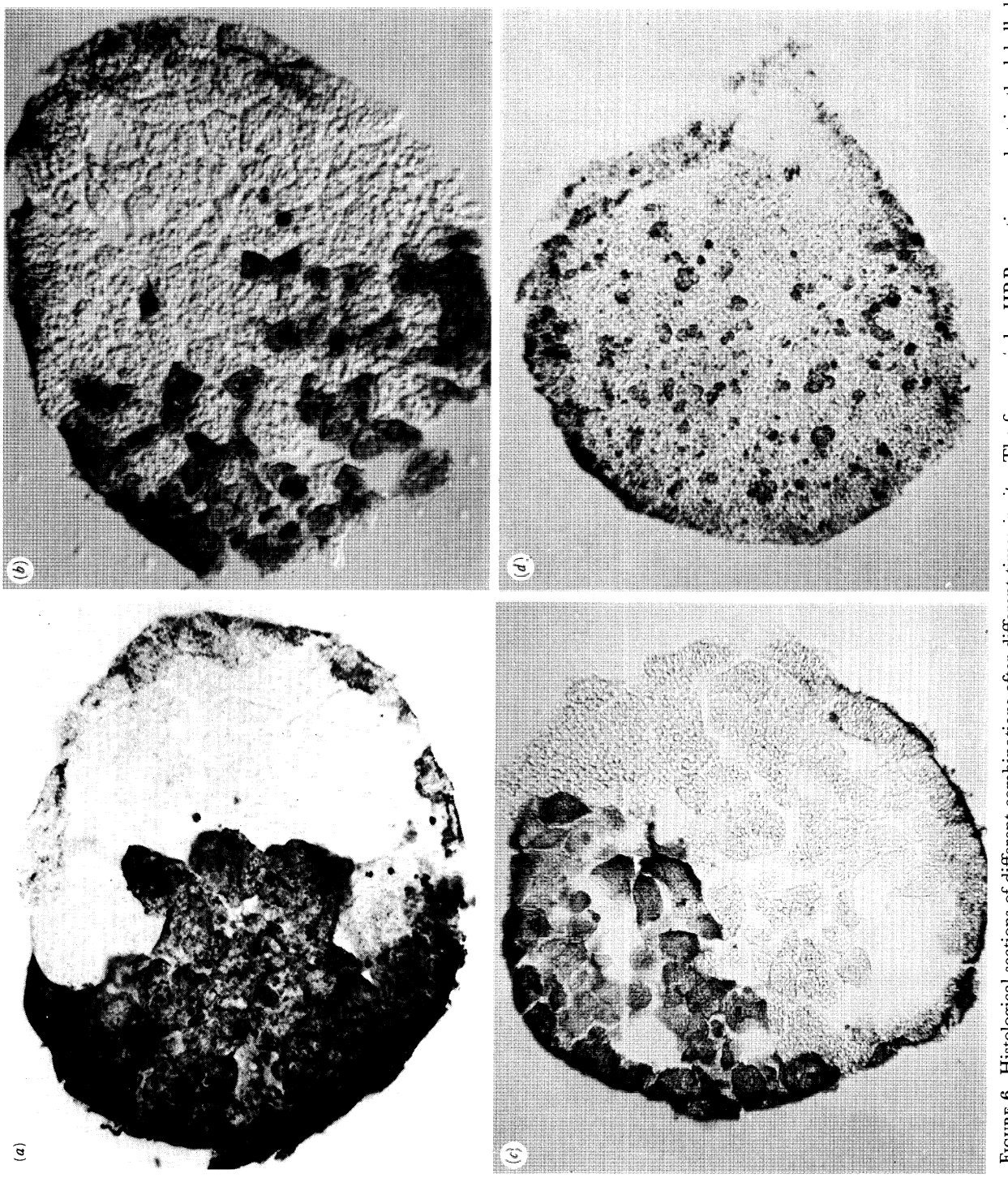

FIGURE 6. Histological sections of different combinations after different times *in vitro*. The fine granular HRP reaction product in the labelled cells should be distinguished from coarser pigment granules in some cells at the surface of the specimens. (Magn. × 180.) (*a*) AM + PL combination, 12 h; (*b*) AM + AM combination, 12 h; (*c*) AM + PL combination, 24 h; (*d*) AM + AM combination, 24 h.

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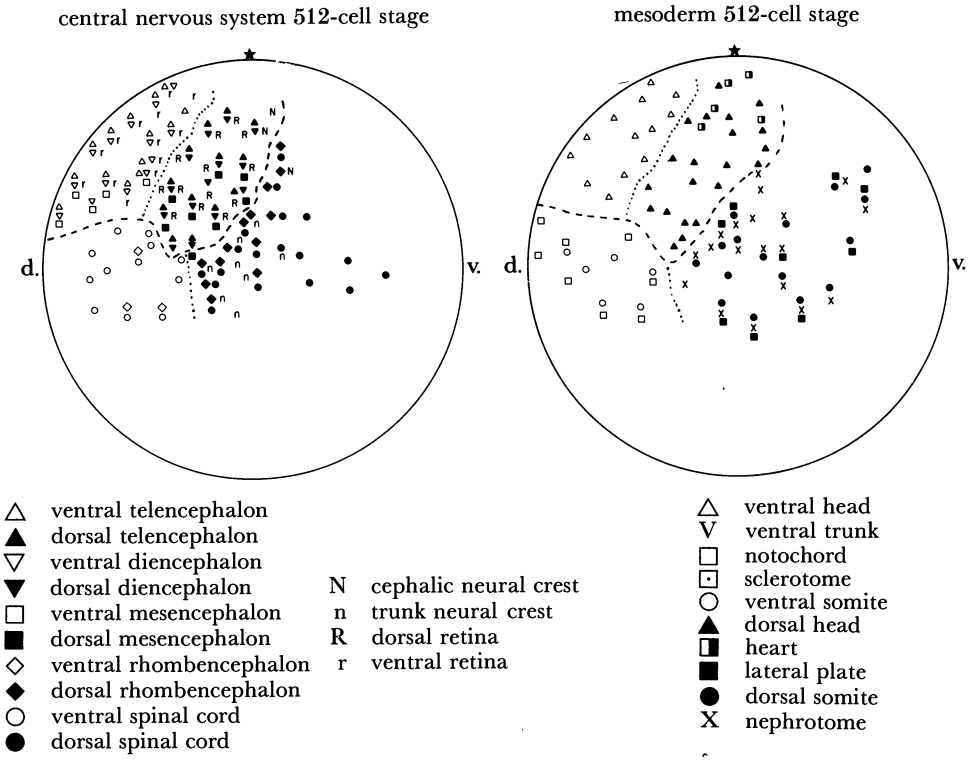


FIGURE 1. Fate maps of *Xenopus* embryo at the 512-cell stage. The right lateral aspect of the embryo is shown, the star is at the animal pole and the dorsal and ventral poles are indicated d. and v. Positions of symbols show the positions of single blastomeres that received an intracellular injection of horseradish peroxidase in a series of embryos at the 512-cell stage. The different symbols show the final positions of labelled progeny in the central nervous system and mesoderm. There were four groups of blastomeres, each of which yielded both mesodermal and neural progeny in a single morphological compartment. The distribution of labelled clones in different regions is shown by different symbols. The dashed lines show the boundary between anterior and posterior ancestral cell groups, the dotted lines separate the medial from lateral ancestral cell groups. The experiments outlined in figures 3 and 4 were designed to excise the anterior-median group (white triangles) and the posterior-lateral group (black dots). The possibility of accidental inclusion of parts of the anterior-lateral group (black triangles) or posterior-medial group (white circles) is evident. The diameter of the embryo is about 1.2 mm.

group of the 512-cell embryo (figure 2*b*). By labelling single cells in different ancestral cell groups at the 512-cell stage, the progeny could be seen to approach the same boundaries from opposite directions (figure 2*c*). The accumulated evidence of more than a thousand single cell injections is consistent with the hypothesis that the boundaries are lines of clonal restriction of cell mingling. These boundaries appeared at regular positions. However it could be argued that they resulted from a combination of small clone size and limited cell dispersal in the intact embryo. In fact, after injection of a single blastomere the dispersal and mingling of the progeny were rapid and extensive within compartments and the compartment boundaries were the only places at which restrictions of mingling were regularly seen (Jacobson & Hirose 1981; Jacobson 1983). Nevertheless, we do not regard this as conclusive evidence that compartmentalization is the result of clonal restriction of cell mingling. Therefore we have tried to obtain more direct evidence that the observed boundaries are really lines of clonal restriction of cell mingling.

It is not difficult to think of experiments that might test objectively the validity of lines of clonal restriction but most of these are not feasible for technical reasons. For example, the

feasibility of injection of different intracellular labels into two neighbouring cells on opposite sides of a line of clonal restriction depends on knowing the precise positions of those lines or on being able to find such pairs by trial and error. However, there is a low probability of finding pairs of cells that satisfy the requirements of the test. Therefore, we have used another approach: observing the amount of mingling between isolated cells excised from opposite sides of a presumptive line of clonal restriction. This experiment was designed to meet the objection that the observed restrictions of cell mingling at certain places in the embryo were not true lines of clonal restriction but were only secondary effects of gastrulation movements which constrained cells to move within narrow streams. The main purpose of this paper is to present evidence showing that clonal restriction of cell mingling expressed *in vitro* is like that observed in the intact embryo: progeny of ancestral cells belonging to the same group expressed a preference for mingling with each other, while progeny of ancestral cells belonging to different groups were restricted from mingling with each other. The fact that clonal restriction of cell mingling was expressed in cells isolated from the embryo showed that it was based on intrinsic differences between cells removed from different regions of the 512-cell embryo.

Analysis of cell mingling *in vitro*

These experiments were aimed at showing whether clonal restriction of cell mingling can occur *in vitro* in cells isolated from gastrulation movements and from other activities in the embryo or whether restriction of mingling is contingent on conditions that exist only in the intact embryo. Two types of experiments were done (figures 3 and 4). The principle was to combine cells from two ancestral cell group, one of which was labelled with HRP, to identify their progeny at later times. The variables were whether the combinations were made from the same or different ancestral cell groups, the time *in vitro*, and whether the cells were maintained throughout as coherent groups or were disaggregated before they were recombined *in vitro*.

Methods

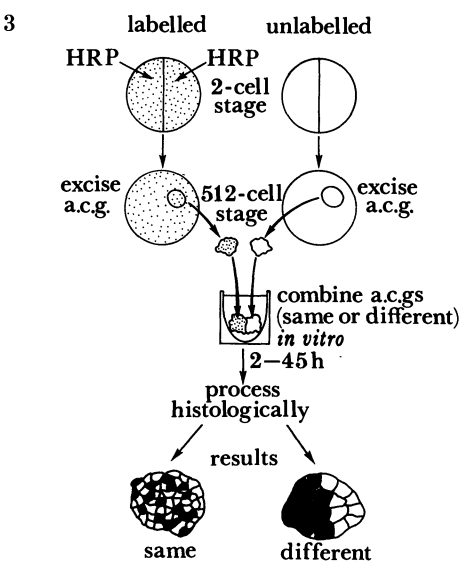
Fertilized *Xenopus* laevis eggs were obtained and dejellied chemically as previously described (Jacobson 1983). Embryos were selected at the 2-cell stage with well marked pigment symmetry about the first cleavage furrow, which passed through the point of sperm entry. To label all the cells of the embryo, an injection of horseradish peroxidase (Sigma, Type IX, 5% in sterile water, 1–2 nl) was given into both blastomeres by means of a glass micropipette. Unlabelled embryos were selected to match the labelled ones. Embryos were raised in 100% Steinberg's medium containing gentamicin (40 mg l^{-1}) until they reached the 256-cell or 512-cell stage. All instruments, containers and solutions were sterilized.

Experiment 1

The aim of this experiment was to observe cell mingling between progeny of two groups of blastomeres combined *in vitro* and to determine whether the amount of cell mingling was dependent on differences in the clonal origins of the two pieces forming the combinations.

At the 512-cell stage, a small piece containing about 20–30 cells was excised from anterior-medial and posterior-lateral ancestral cell groups. The locations of those ancestral cell groups have been described by Jacobson (1983) and are shown in figure 1. The pieces extended from the surface to two or three cell layers below the surface. Similar pieces were excised from

both labelled and unlabelled embryos. One unlabelled and one labelled piece were placed in contact at their internal surfaces in a well of a 96-well culture dish, coated with 5% agar, containing 0.3 ml aggregation medium (Nakatsuji & Johnson 1982; constituents in grams per litre: NaCl, 4.34; KCl, 0.18; Na_2HPO_4, 0.089; KH_2PO_4, 0.019; Na_2SO_4, 0.27; $CaCl_2$, 0.22; $MgCl_26H_2O$, 0.263; bovine serum albumin fraction V, 5.0; gentamicin sulphate, 0.04; HEPES, 1.19). Four different combinations were made (in each case the underlined ancestral cell group was labelled); AM + AM, PL + PL (these are called homotypic), AM + PL, PL + AM (these are called heterotypic). In all cases the fragments adhered within seconds and cells could be seen dividing. The combinations were cultured at 20 °C for different times, from 2 to 45 h, and then processed histologically.



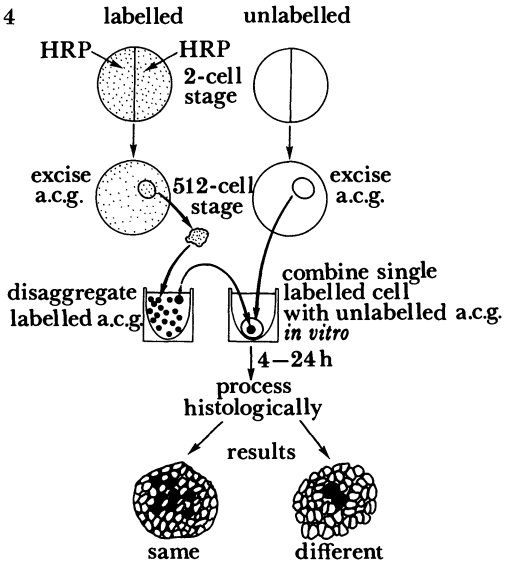


FIGURE 3. Design of experiment 1. a.c.g., Ancestral cell group.
FIGURE 4. Design of experiment 2. a.c.g., Ancestral cell group.

Experiment 2

The aims of this experiment were to study cell division and mingling of the progeny of a single labelled blastomere, derived from an identified ancestral group of a totally labelled 512-cell embryo, after the labelled cell was combined with 20–30 blastomeres excised from the same or different ancestral cell group of an unlabelled 512-cell embryo.

At the 256-cell stage, labelled embryos were transferred to disaggregation medium (aggregation medium without calcium, magnesium, or bovine serum albumin, with added sodium citrate at 5.88 g l^{-1}). After about 40 min, when the labelled embryos reached the 512-cell stage, a single ancestral cell group was excised and gently dissociated by drawing the cells repeated into a fine, fire-polished glass pipette, with an internal tip diameter of about 200 μm. This resulted in separation of most, but not all cells. The cells were transferred to a large volume of aggregation medium. A group of about 20–30 cells was excised from one ancestral cell group of an unlabelled embryo and transferred to a culture well containing aggregation medium. A single labelled cell was transferred into the same culture well and inserted into the unlabelled group of cells. In some cases the labelled cell was seen to divide at this time. After 2–24 h the specimens were fixed in the culture wells and then removed for histological processing.

Histological processing

After the culture period, aggregates were fixed in mixed aldehydes (1% (by mass) paraformaldehyde, 2.5% (by volume) glutaraldehyde) for 6–8 h in their culture wells, washed in 0.1 M phosphate buffer with 5% (by mass) sucrose for at least 6 h, and then in phosphate buffer with 15% (by mass) sucrose for 1 h. The specimens were removed from their wells, soaked in OCT embedding compound (Tissue Tek Inc.) for several minutes, embedded in OCT, and frozen. Frozen sections were cut at 24–28 μm and mounted on gelatin-coated slides. An effort was made to cut sections perpendicular to the plane between the two fragments, which was sometimes indicated by differences in pigmentation of the superficial cells. However, in most cases there were no external features by which to orient the specimens so that the plane of section was random with respect to possible lines of restriction of mingling between labelled and unlabelled cells. Sections were soaked in 200 ml 0.0125% diaminobenzidine tetrahydrochloride in 0.1 M phosphate buffer (pH 7.4) for 5–10 min, after which 1 ml of 0.15% H_2O_2 was added and the reaction was monitored under the microscope at intervals for 5–10 min until a dark brown intracellular reaction product appeared.

Analysis of histological preparations

All slides were evaluated independently by both authors: slides were coded numerically so that observers did not know whether the specimens were formed from the same or different ancestral cell groups. Each section was compared with a series of diagrams showing seven progressive stages of mingling of black and white discs in a single plane. Each section was assigned a 'mingling index' which corresponded to whichever of the seven schematic stages of mingling it most closely resembled. A mingling index of 0 indicated total segregation, while an index of 7 indicated complete intermingling. The average diameter of the labelled cells was measured using a microscope fitted with a filar-micrometer eyepiece. Measurements were made of the longest and shortest diameter of labelled cells that had clearly defined boundaries. Unlabelled cells were not measured because their boundaries were unclear.

Results and discussion

Experiment 1

All combinations of two pieces excised from the same ancestral cell group (homotypic) were segregated (mingling index 0–3) when cultured for less than 12 h ($n = 34$). Mingling was first seen (index 3 or 4) at 12 h in 16 cases while the remaining 16 showed no mingling (index 0–3) after 12 h. Complete or almost complete mingling (index 5–7) was first observed after 16 h and the majority of homotopic combinations were partly mingled (index 4 or 5) after 16 h in culture. Therefore, all combinations, heterotypic ($n = 54$) and homotypic ($n = 64$) cultured for 16 h or longer were compared (figure 5). These results were highly significant ($p < 0.005$). They show that there was a marked preference for intermingling between cells that belonged to the same ancestral cell group and a marked tendency for cells to segregate if they belonged to different ancestral cell groups.

The most likely reason for discrepancies in the results was accidental inclusion of cells from more than once ancestral cell group in pieces designed to contain cells of one group only. The position of the AL group between AM and PL groups makes it highly probable that a few AL

cells were accidently included in many cases (figure 1). The combination intended to have been AM + AM, PL + PL or AM + PL could actually have been AM + AM + AL, PL + PL + AL and AM + PL + AL. This could have resulted in the observed skewing of the mingling index of the homotypic cases to the left, and of the heterotypic cases to the right, in figure 5. Another possible cause of error was that in some heterotypic specimens that were well segregated, the plane of section was tangential to the boundary between labelled and unlabelled cells. Cells appeared to be mingled in sections passing obliquely or tangentially through the boundary region.

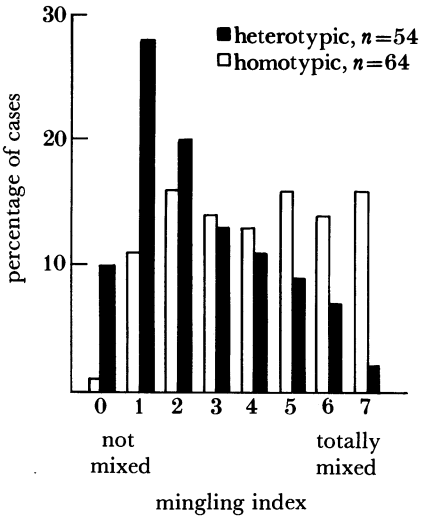


FIGURE 5. Numbers of cases of homotypic cell combinations (white bars) and heterotypic cell combinations (black bars) showing the index of mingling in each case, ranging from 0 (totally segregated) to 7 (totally mingled).

In all cases, the cells that remained segregated were larger than those that intermingled (figure 6, plate 2). After 12 h the mean diameter of the segregated cells was 36 μm (s.e., 15), and that of the mingled cells 18 μm (s.e., 6). After 24 h the mean diameter was 25 μm (s.e., 7) for segregated cells and 9 μm (s.e., 6) for intermingled cells. Because *Xenopus* blastomeres divide without any growth, the mean diameter of the blastomeres is a good measure of the cell generation (Klein 1984). The mean diameters of these blastomeres in normal *Xenopus* embryos were 42.5 μm at the 11th cell generation (G11), 33 μm at G12, 27 μm at G13, 21 μm at G14 and 17 μm at G15. Therefore, at 12 h the mingled cells were at G13 or G14 while the segregated cells were still at G11 or G12. After 24 h the mingled cells had reached G16 while the segregated cells were at G13 or G14. Cell counts are less reliable in these experiments because the exact number of cells at the start was not known. Therefore, the number of cells that died or separated from the combinations could not be determined.

The differences in cell size indicate that the cells divided fewer times in combinations of different ancestral cell groups than in combinations made from the same ancestral cell groups. From their size it appeared that the segregated cells had progressed beyond the 12th generation, when cell mingling normally begins. Therefore, it seems unlikely that the failure to mingle was a direct result of the larger sizes of the cells. Rather, failure to mingle and failure to divide both appear to result from interactions between cells originating from different ancestral cell groups. Apparently all the cells were affected, not only those at the interface in the heterotypic combinations (figure 6). Therefore, the inhibition of mitosis must have been transmitted across

a distance of about 150–200 μm, through the entire population, in the heterotypic combinations.

Experiment 2

The number of labelled single cells that combined with the unlabelled fragment was low: 184 of the 254 cases did not contain labelled cells. Nineteen homotypic and 32 heterotypic cases were analysed. The results are shown as a scatterplot (figure 7). In the homotypic combinations the number of labelled cells increased with time whereas the number did not increase or increased less in the heterotypic combinations. After 16 h *in vitro*, 100% of the homotypic

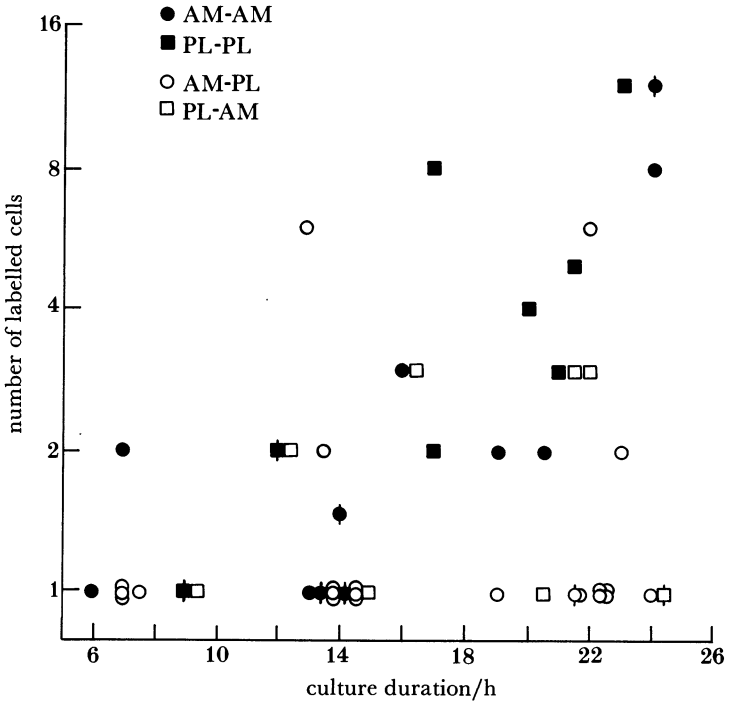


FIGURE 7. Semilogarithmic scatterplot showing the number of labelled progeny of a single labelled cell implanted in an unlabelled ancestral cell group *in vitro*, as functions of the origins of the cells forming the combinations and the time *in vitro*. Black symbols show homotypic, (AM + AM, PL + PL). White symbols show heterotypic combinations (AM + PL, PL + AM). The labelled group is underlined. Symbols with vertical bars show cases in which two labelled cells were implanted. In these cases the number of labelled progeny was twice the number shown.

combinations and 33% of the heterotypic combinations contained two or more labelled cells. In the homotypic combinations all the labelled single cells had divided at least once after 16 h whereas in two-thirds of the heterotypic cases the labelled cell had apparently failed to divide. That these failures were actually due to lack of division rather than loss of progeny of the labelled cell was shown by measurements of the cell diameters.

The average cell diameter in cases that contained a single labelled cell was 42 μm, indicating that the cell was at the 11th generation. The diameter was 23 μm in cases containing two or three labelled cells, 17 μm in cases containing four to seven labelled cells, and 12 μm in cases containing eight to twelve labelled cells. Therefore, these cells had reached the 14th, 15th and 16th generations. The diameters of the unlabelled cells could not be measured. Labelled cells were dispersed and mingled with unlabelled cells in all specimens containing more than two

labelled cells. Failure of division of labelled cells was strongly correlated with their combination with cells derived from a different ancestral cell group. This indicates that mutual contact inhibition of cell division between progeny of different ancestral cell groups is one of the possible mechanisms of formation of lines of clonal restriction in *Xenopus* embryos. The non-proliferating cells could form a physical barrier to mingling and the effectiveness of the barrier could be increased by inhibition of cell motility as well as cell division.

The mutual inhibition of mitosis that we have observed when cells of different clonal origin were brought into contact *in vitro* may be similar to that reported at an intercompartmental boundary in *Drosophila* (O'Brochta & Bryant 1985). We have not been able to see any significant increase in sizes of cells at compartmental boundaries in *Xenopus* embryos that might indicate slower rates of mitosis at or close to the boundaries that we have observed in specimens labelled with HRP. However, it might be significant that in the central nervous system, both the anterior–posterior bondary at the isthmus, and the dorsal–ventral boundary, corresponding with the sulcus limitans, are regions of low cell density in all vertebrates.

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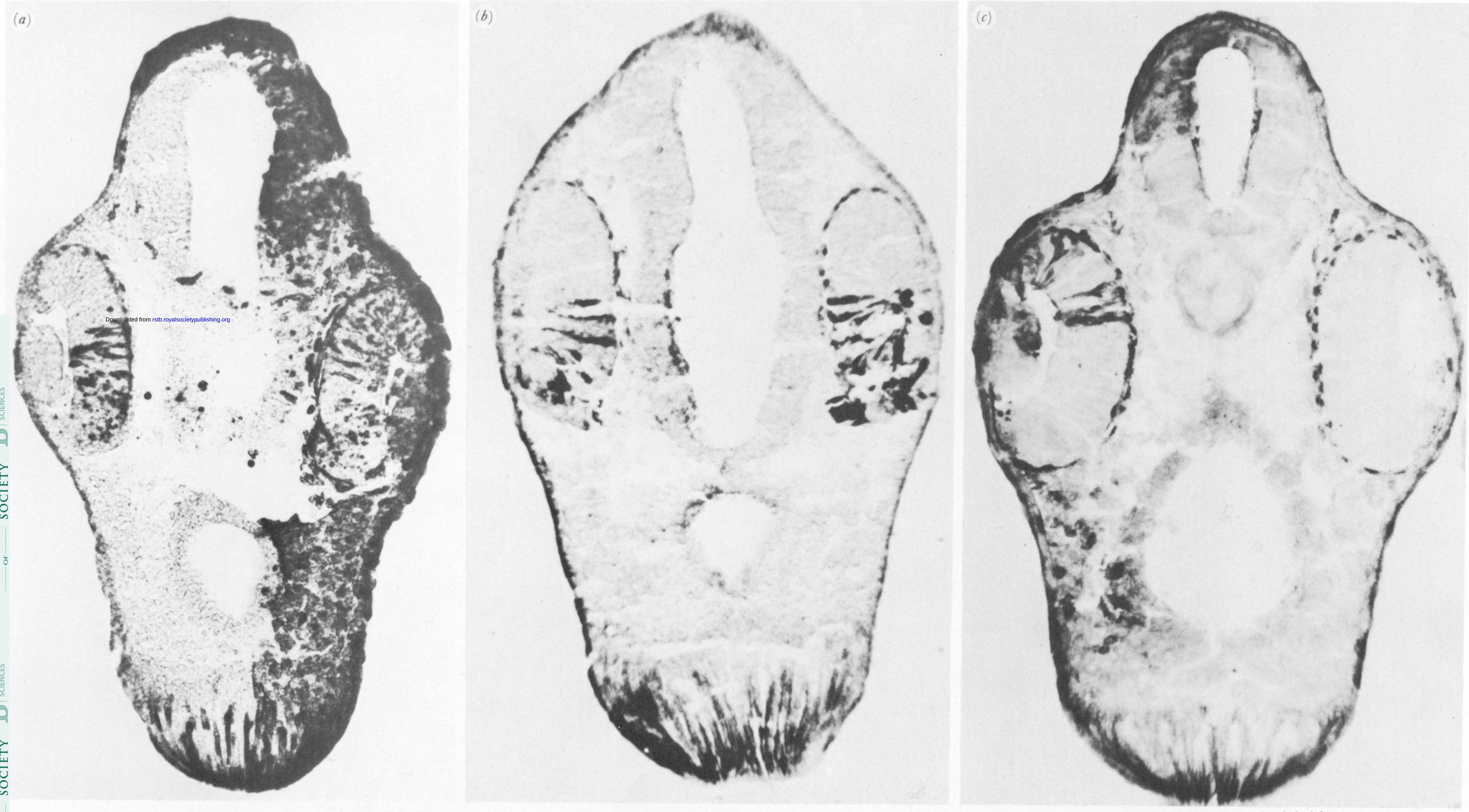

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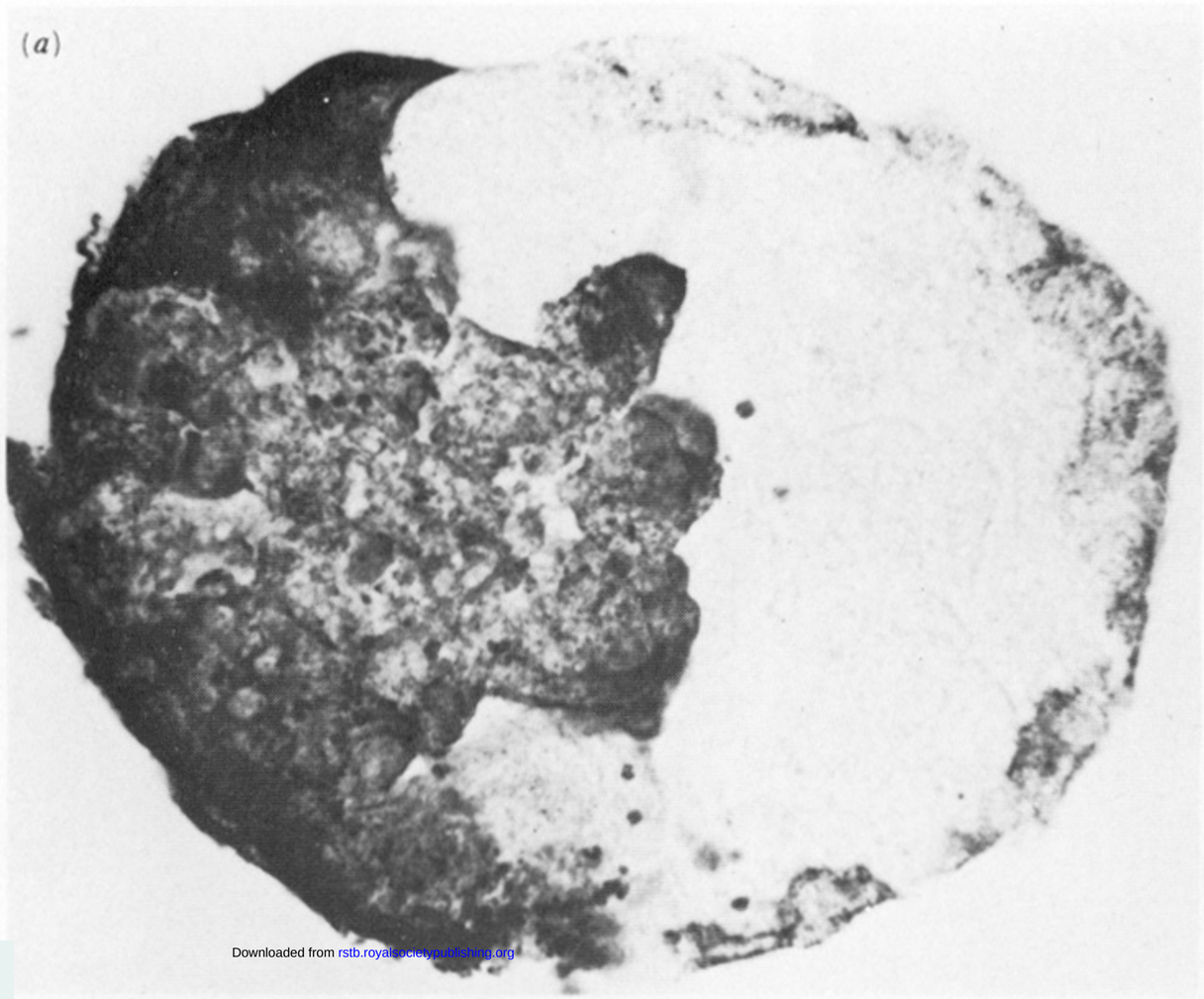

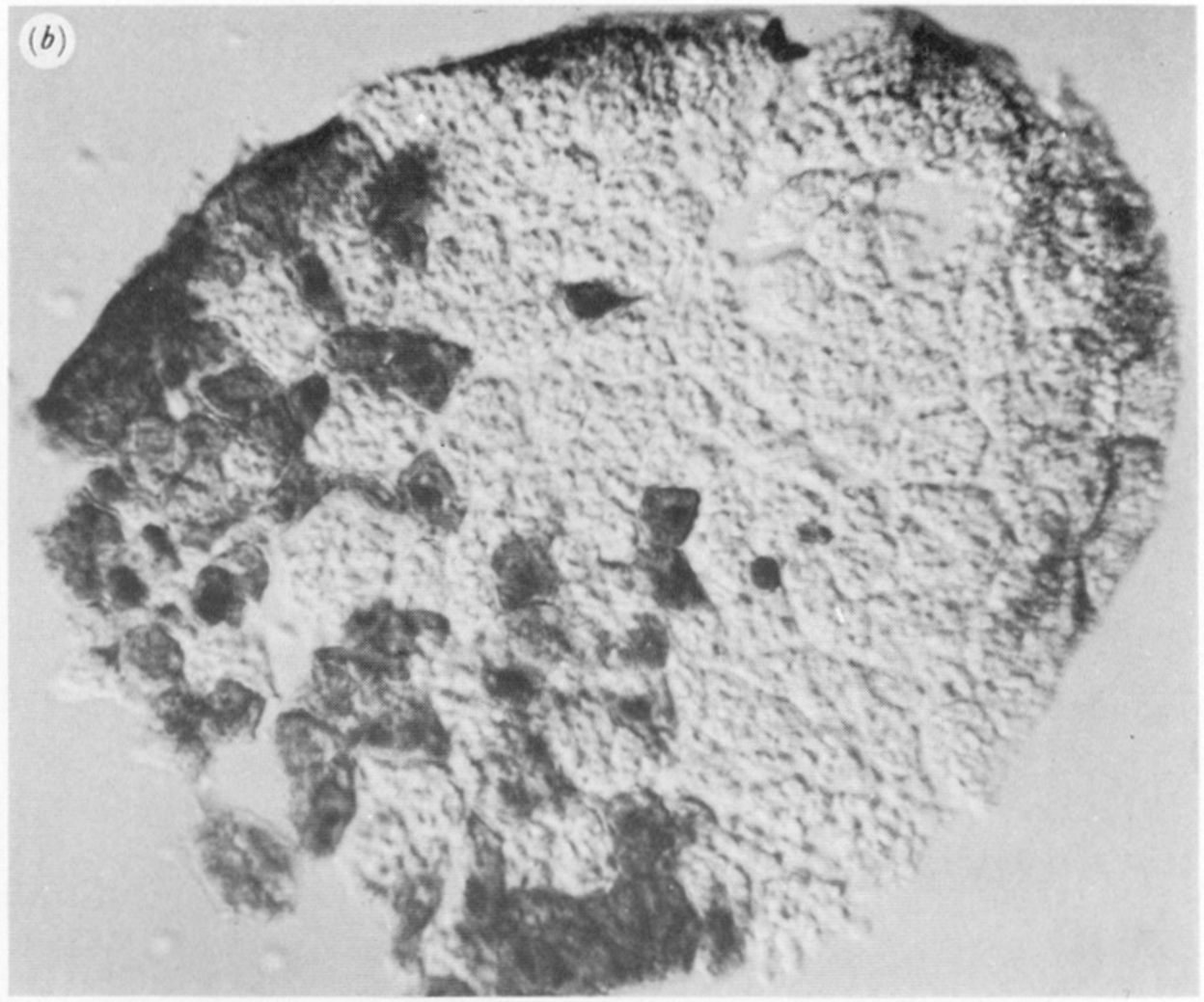

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