Permeability alterations in amphibian embryos caused by salt solution and measured by tritiated thymidine uptake

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WITH ONE PLATE

In attempts to label the nuclei of intact amphibian embryos with tritiated thymidine we found, in agreement with other authors (Tencer, 1961; Quertier, 1962; Chibon, 1962), that when the nucleoside is dissolved in tap water or media of low salt concentration it fails to enter the embryo. However, when tritiated thymidine was either injected into the embryo or applied to isolated tissues immersed in salt solution, the marker diffused readily through the tissues. Evidently the outer surface of the amphibian embryo is furnished with a special permeability barrier which prevents or reduces the entrance of these molecules. This barrier corresponds most likely to what Holtfreter (1943a, 1943b) has described as the 'surface coat'. In order that the results of our present experiments may be better understood some of the pertinent properties of the coat will be reviewed.

Characteristics of the surface coat

A protective coat, while still absent in the ovarian egg, arises after the egg is deposited in water. All the cells subsequently found at the surface of the embryo are protected on the outside by a coat. During gastrulation, when the originally coated entoderm and mesoderm are carried inward, the cells of the archenteron wall retain the non-adhesiveness of a coated surface whereas the sheet of mesoderm cells breaks up to form new configurations involving apparently a dispersion of the cell coats. In a similar fashion, a piece of gastrula ectoderm grafted into the interior of an embryo, or larva, tends either to break up into single cells, or it forms an inverted vesicle with the coated side turned inward. These and other data indicate that the coat results from a reaction of the cell periphery to the

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surrounding water and that it cannot be maintained by cells inside the embryo except when they border on an inner lumen.

Possibly the integrity of the coat depends at least in part upon a calcium proteinate complex since it can be attacked and apparently dispersed not only by proteolytic enzymes (Townes, 1953), but also by chelating agents such as sodium citrate and versene, as well as by alkali. These agents have been used successfully to disaggregate the continuous sheet of peripheral cells of the amphibian embryo, a process which is associated with the release of a cloud of mucinous material. The dissociated cells remain fully viable, indicating that the cell membrane proper is not seriously damaged by these treatments. Previous studies have also shown that the coated surface has a semi-solid consistency, is capable of forming wrinkles, is non-adhesive, and that water, simple inorganic ions and vital dyes pass through this barrier at a reduced rate as compared with the non-coated cell surfaces of the interior.

There is still much uncertainty concerning the structural equivalent of the coat in sections observed under the light microscope and the electron microscope. Most likely the pigment granules which were thought to be present in the coat (Holtfreter, 1943a), actually belong to deeper strata of the cell (Dollander, 1953). It is also uncertain if the cortical structures observed by Wartenberg (1962) in the electron micrographs of sectioned oocytes of Triturus can be considered as precursors of the coat. Balinsky (1961) did not find evidence for an outer protective coat which covered adjoining cells. This author did find desmosomes near the periphery of the cells. These may account for the lateral adherence of the cells to each other but they cannot explain various physiological characteristics of the surface of the uncleaved amphibian egg and early embryo. It may well be that a special technique is required in order to demonstrate the evasive coat in fixed preparations. There have been difficulties in the detection of the coats of amoebae and the slime mold, Physarum. In these instances, numerous observations made on the live organisms pointed to the existence of a special protective layer peripheral to the cell membrane. Utilizing special techniques in conjunction with electron microscopy, Brandt & Pappas (1960) have determined many of the characteristics of the surface coat in amoeba, which in this case, consists largely of mucopolysaccharides.

The physiological significance of the coat is best illustrated by the fact that intact amphibian embryos can be raised in tap water which is, of course, a very hypotonic medium. When only a small number of the coated surface cells is surgically removed, the wound will not heal but becomes the point at which the cytolysing action of water begins, a process which spreads unchecked throughout the whole embryo. The non-coated cells of the interior when placed in water succumb within less than 10 min. For the purpose of providing a proper culture medium for non-coated embryonic amphibian cells, a balanced salt solution was devised by Holtfreter.

Previous work has shown that isolated embryonic cells can be cultured for
Alteration of permeability in amphibian embryos

long periods in this isosmotic medium. However—and this is of special interest in connection with the present studies—whole embryos of certain amphibian species raised in Holtfreter solution may suffer developmental abnormalities. The direction of the invagination movements of gastrulation may become reversed leading to the formation of exogastrulae, and the cells of the neural crest, instead of moving inward, may individually migrate out of the embryo (Holtfreter, 1933, 1943a). Both phenomena have been ascribed to a partial degradation or softening of the coat, an interpretation which is supported by the fact that if the salt concentration of the medium is still further increased, the surface becomes sticky and emigration of these cells is accentuated.

In view of the above observations, it came as no great surprise when we found that the permeability of the surface barrier of the amphibian embryo, from early cleavage through tail bud stages, can be increased by means of Holtfreter solution so that molecules larger than simple inorganic ions—tritiated thymidine in the present experiments—may enter the embryo.

MATERIAL AND METHODS

Four different urodele species were used in the experiments: *Amblystoma maculatum*, *A. tigrinum*, *Pleurodeles waltlii* and *Triturus alpestris*. The ages varied from early cleavage stages to swimming larvae. In the pre-neurula stages only the outer jelly layers were removed and the fertilization membranes kept intact. The older animals were subjected to treatment without any membranes. Care was taken to minimize contamination by micro-organisms by repeated washings of the embryos before exposure to labelled thymidine, and by the addition of antibiotics to the medium.

The culture medium routinely used in these experiments was Holtfreter's solution, the original formula of which he has revised (personal communication) by reducing the NaHCO₃ to one-tenth so as to lower the pH of the solution from 8.3 to the more neutral pH of 7.5. This salt solution is compounded as follows: **Solution A**: NaCl 3.5 g.; KCl 0.05 g.; CaCl₂ 0.1 g.; distilled water 900 c.c. **Solution B**: NaHCO₃ 0.02 g.; distilled water 100 c.c. The two solutions are autoclaved in separate containers and mixed together when they are cool.

In all experiments reported here the concentration of tritiated thymidine contained in the medium was 5 μc./ml., or 10 μc./ml. (specific activity 3 μc./mM; Schwarz BioResearch Inc., Mt. Vernon, N.Y.). Immediately following removal from the thymidine-containing media the specimens were washed several times prior to fixation in Smith's fixative, then sectioned at 7 or 12 μ and prepared for autoradiography according to a method developed by Aldridge (see Gerber et al., 1962). The deparaffinized sections were covered with a film of NTB-2 or NTB-3 bulk emulsion (Eastman Kodak, Rochester, N.Y.) and left in light-tight boxes at low humidity for 1 to 4 weeks. Subsequently, the autoradiographs were developed and stained faintly with customary nuclear dyes.
The use of urodeles, instead of anurans such as *Rana pipiens* and *Xenopus laevis*, has the distinct advantage that their cells contain fewer melanin granules and these, because of their brownish-black color, can be easily distinguished from the bluish-black silver grains in the emulsion. It is quite difficult to distinguish the black pigment granules of most Anura from the silver grains of the emulsion.

Since the β particles emitted by tritium have been estimated to travel an average distance of less than 1μ in tissue sections, the number of silver grains actually observed does not fully reflect the concentration of the labelled thymidine taken up by the nuclei. The values for grains per nucleus reported here have been corrected for variations in the length of the exposure period. Since the relative sensitivities of NTB-2 and NTB-3 emulsions are known, and since the number of grains per nucleus increases linearly in relation to exposure time (Kopriwa & Leblond, 1962) it has been possible to correct the recorded values to values equivalent to an exposure period of 2 weeks with NTB-3 (Text-fig. 1 and Table 2), or 18 days with NTB-3 (Table 1).

As expected, the density of grains appearing above the nuclei, varied considerably with the following parameters: (1) concentration of the salt solutions used, (2) differences of stage and of species employed, (3) exposure time of the specimens to labelled thymidine and the mitotic rate during this time, (4) exposure time of the emulsion to the sections, and (5) the number of mitoses that occurred between the time of thymidine uptake and fixation of the specimens.

Not all of these variables have been satisfactorily explored in the present study. We contented ourselves with obtaining semi-quantitative data by using the procedures outlined in Text-fig. 1 and Tables 1 and 2. The reported values for the number of grains per nucleus have been corrected for the background which did not exceed 6 grains per 100μ².

**RESULTS**

The effect of increasing degrees of salinity of the medium on the uptake of tritiated thymidine is indicated in Text-fig. 1. While the concentration of thymidine was kept constant (5 μc./ml.), the salt concentration of the medium was varied from that of tap water to that of 10, 25, 50 and 100 per cent. Holtfreter solution. After developing for periods of 20 to 40 hr. in these media the embryos were washed and fixed.

Thymidine dissolved in tap water failed to label any of the nuclei in the treated embryos of the three species used. With increasing tonicity of the solution beyond 10 per cent., the number of grains per nucleus increased in a roughly linear fashion. The greatest number of grains per nucleus was obtained when embryos of *T. alpestris* were raised in full strength Holtfreter solution plus thymidine from early gastrulae to late neurulae. The number of grains was slightly reduced when somewhat older embryos of *A. maculatum* were used, and considerably reduced
in the case of *A. tigrinum* treated at the neurula stage or later. Although it is possible that the differences in the slopes of the three curves reflect species-specific differences of susceptibility to the permeability enhancing action of the salt.

![Graphic presentation of data representing typical results from a series of experiments designed to show the effect of various salt concentrations upon uptake of tritiated thymidine from the media.](chart)

**TEXT-FIG. 1.** Graphic presentation of data representing typical results from a series of experiments designed to show the effect of various salt concentrations upon uptake of tritiated thymidine from the media.

- (Δ) *Triturus alpestris*: Five embryos raised in the media for 20 hr. from early gastrula to late neurula.
- (○) *Amblystoma maculatum*: Four embryos raised in the media for 40 hr. from late gastrula to early tail bud.
- (●) *Amblystoma tigrinum*: Four embryos raised in the media for 40 hr. from neurula to late tail bud.

Each point together with the vertical line represents the mean value and standard error of the mean for the number of grain counts over thirty separate nuclei taken from one embryo (ten nuclei were randomly selected from each of the three germ layers or their derivatives) which was raised at the indicated level of salt concentration.
solution, there is good reason to believe that they are mostly due to the differences in the stages employed. To clarify this question the following experiments were performed.

Embryos of different ages from two species were immersed for 5 hr. in Holtfreter solution containing tritiated thymidine (Table 1). In the earliest stages tested all nuclei of both species became heavily labelled. From the neurula stage on, a marked decrease in the percentage of labelled nuclei and in the number of grains per nucleus was observed until, in advanced tail bud and larval stages no, or hardly any, nuclear labelling could be obtained in either species. In between, there was an unexpected refractory period around the time of gastrulation when the uptake of labelled molecules was reduced to near background levels. This period commenced somewhat earlier in *Pleurodeles* than it did in *A. maculatum*.

During the neurulation period, there was a renewed intensification of labelling in both *Pleurodeles* and *Amblystoma*. In *Pleurodeles*, the permeability of the surface epithelium became confined to the caudal region at the early tail bud stage. Finally, while neither of the species could be labelled at the late tail bud stage, there was again a difference at the much later stage of the swimming larva: no labelling in *Pleurodeles*, but in *Amblystoma* a rather dense cloud of silver grains appearing in 30–40 per cent. of the nuclei of the pharyngeal gill epithelium and the adjacent tissues. This latter difference can probably be related to the fact that only in the larvae of *Amblystoma* used in this experiment had the gill clefts already opened, thus giving the thymidine free access to the pharynx. This implies that while the epidermis of advanced stages becomes completely impermeable to thymidine, even when it is administered dissolved in full-strength Holtfreter solution, the epithelium of the pharynx does not represent a permeability barrier.

Altogether, the similarities in the behavior of the two species used in this experiment were much more striking than the differences mentioned above. These data seem to justify the conclusion that the difference in the slopes of the three curves in Text-fig. 1 reflect differences in the reactivity of the stages rather than the three species employed.

As can be seen from Table 1, there was not a strict parallelism between the percentage of nuclei per embryo labelled and the intensity of their labelling, although, as a rule, both increased or decreased simultaneously.

An increase in the exposure period from 5 to 10 hr. (*A. maculatum*) was found roughly to double the number of grains per nucleus at all stages except blastula, when there was a less pronounced increase in the number of grains. In embryos beyond the mid-neurula stage, the percentage of labelled nuclei and the extent of the embryonic regions containing labelled nuclei were also increased as compared with the data obtained with a shorter treatment.

In another series of experiments (Table 2), various stages of three species of amphibia were exposed for still longer periods (20–40 hr.) to Holtfreter solution containing labelled thymidine. Under the conditions of this long-term treatment,
### Table 1

The effect of age on the uptake of tritiated thymidine of two urodele species raised in 100 per cent. Holtfreter solution for 5 hours at 23° ± 1°C.

| Stage* and species tested | Regions of embryo containing labelled nuclei | Percentage of total embryos containing labelled nuclei | In region where labelled nuclei appear | Estimated total ³H-thymidine incorporated per embryo
<table>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Percentage of nuclei labelled</td>
<td>Grains per nucleus†</td>
<td></td>
</tr>
<tr>
<td>Pleurodeles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cleavage</td>
<td>Whole embryo</td>
<td>100</td>
<td>100†</td>
<td>+ + + + §§</td>
</tr>
<tr>
<td>Blastula</td>
<td>Whole embryo</td>
<td>100</td>
<td>100†</td>
<td>+ + + + §§</td>
</tr>
<tr>
<td>Early gastrula</td>
<td>Whole embryo</td>
<td>100</td>
<td>5†</td>
<td>+ + + + §§</td>
</tr>
<tr>
<td>Early neurula</td>
<td>Whole embryo</td>
<td>100</td>
<td>10†</td>
<td>+ + + + §§</td>
</tr>
<tr>
<td>Late neurula</td>
<td>Neural tube and caudal mesoderm</td>
<td>30–40</td>
<td>20–40</td>
<td>+ + + + §§</td>
</tr>
<tr>
<td>Early tail bud</td>
<td>Caudal, neural and mesoderm tissue</td>
<td>20–30</td>
<td>5–20</td>
<td>+ + + + §§</td>
</tr>
<tr>
<td>Late tail bud</td>
<td>No labelled nuclei</td>
<td>0</td>
<td>0</td>
<td>O</td>
</tr>
<tr>
<td>Swimming larva (ca. 8–9 mm.)</td>
<td>No labelled nuclei</td>
<td>0</td>
<td>0</td>
<td>O</td>
</tr>
<tr>
<td>A. maculatum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blastula</td>
<td>Whole embryo</td>
<td>100</td>
<td>100</td>
<td>+ + + + §§</td>
</tr>
<tr>
<td>Early gastrula</td>
<td>Whole embryo</td>
<td>100</td>
<td>20–30</td>
<td>+ + + + §§</td>
</tr>
<tr>
<td>Late gastrula</td>
<td>Whole embryo</td>
<td>100</td>
<td>5†</td>
<td>+ + + + §§</td>
</tr>
<tr>
<td>Early neurula</td>
<td>Whole embryo</td>
<td>100</td>
<td>15†</td>
<td>+ + + + §§</td>
</tr>
<tr>
<td>Early tail bud</td>
<td>Whole neural tube and somites</td>
<td>30</td>
<td>10–15</td>
<td>+ + + + §§</td>
</tr>
<tr>
<td>Late tail bud</td>
<td>No labelled nuclei</td>
<td>0</td>
<td>0</td>
<td>O</td>
</tr>
<tr>
<td>Swimming larva (ca. 13–14 mm.)</td>
<td>Endoderm and underlying connective tissue including muscle of the pharyngeal region</td>
<td>&lt;1</td>
<td>30–40</td>
<td>+ + + + §§</td>
</tr>
</tbody>
</table>

* Each group represents three separate embryos, except for the early neurula of each species which is represented by one embryo.
† Mean value of at least ten nuclei from each of the three primary germ layers or their derivatives minus background. Adjusted time of exposure: 18 days NTB-3.
‡ Levels of labelling too close to background for accurate assessment of mitotic activity. These values should be considered as minimum estimates.
§ + + + + 100 or more grains per nucleus; + + + 31–99 grains per nucleus; + + 11–30 grains per nucleus; + 5–10 grains per nucleus; + ± 1–4 grains per nucleus; O background.
∥ These values are estimated by adjusting the values of the previous column according to the percentage of the embryo's tissues represented by labelled nuclei.

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the refractory period observed with the shorter term treatment around the time of gastrulation (Table 1) did not become apparent. Also, as compared with the data of Table 1, there was a more widely spread and more intense nuclear labelling when treatment was begun at the late gastrula and neurula stages. But, as regards the labelling of the more advanced stages (tail bud and larvae), the prolonged treatment failed to be more successful than the short-period treatment.

We wish to stress the point that throughout these experiments no morphological or histological abnormalities occurred to a greater extent than in the control embryos raised in water, except for the occasional persistence of a small yolk plug in some of the species used. *Pleurodeles* embryos suffered no morphological abnormalities even when raised in 100 per cent. Holtfreter solution from early cleavage to late tail bud. The failure of early embryos to exogastrulate, or of early tail bud stages to release neural crest cells into the isosmotic medium, can be mainly ascribed to the fact that these embryos were immersed in Holtfreter solution with their supporting egg membranes left intact. Within the period of post-operational observation (up to 40 days in some cases), we found no evidence that the radioactive thymidine employed at the present concentrations and for the maximum duration of 40 hr., caused sufficient damage to the nuclei to be detectible microscopically. Nor could abnormalities of cell differentiation be observed.

The photomicrographs of the Plate, Figs. A to C, may serve to illustrate the thoroughness of the nuclear labelling obtained in the cases of most intensive thymidine incorporation. Fig. A shows, at high magnification, a cloud of discrete silver grains associated with the nuclei. In the sections through an early gastrula (Figs. B and C), the nuclei of both the peripheral and internal cells stand out as dark bodies. This is due, not so much to the faint nuclear stain, as to the accumulation of silver grains which are practically absent in the cytoplasmic regions.

Figure D illustrates the result of a method by which the present labelling technique may be extended to the study of other embryological problems. In this case an intact embryo of *A. maculatum* had been heavily labelled with tritiated thymidine. Subsequently a cephalic part of its neural crest was grafted into the neural crest region of an unlabelled neurula. The grafted cells have migrated ventral into the first branchial arch. Their labelled nuclei contrast sharply with the unlabelled nuclei of the host cells, thus demonstrating the usefulness of this labelling technique for ascertaining the morphogenetic fate of embryonic grafts.

**DISCUSSION**

The foregoing experiments have shown, in confirmation of the findings of previous workers (Tencer, 1961; Quertier, 1962; Chibon, 1962), that tritiated thymidine fails to enter intact amphibian embryos when it is added to immersion media of low salt concentration. The embryos were, however, made permeable to thymidine by using a physiological salt solution of rather low salinity—the
Fig. A. Labelled nuclei (× 210) of a *Pleurodeles* blastula raised for 6.5 hr. (from early cleavage) in 100 per cent. Holtfreter solution containing tritiated thymidine. Sections were exposed to NTB-3 for 26 days.

Figs. B and C. Low (× 42) and medium (× 165) power photomicrographs of *Triturus alpestris* gastrula, treated for 20 hr. with 100 per cent. Holtfreter solution and tritiated thymidine from cleavage to gastrula, exposed to NTB-3 for 5 days.

Fig. D. Labelled ectomesenchymal cells surrounding the mesodermal muscle plate of the first branchial arch of *Amblystoma maculatum*. These cells migrated from a cranial neural crest graft labelled with tritiated thymidine. The pharyngeal endoderm lies to the left of the labelled cells (× 165).

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TABLE 2

Permeability alterations as measured by autoradiography in some urodele embryos raised in 100 per cent. Holtfreter solution plus tritiated thymidine at 23° ± 1°C. for extended time periods.

Stage* at which the specimens were

<table>
<thead>
<tr>
<th>Species tested</th>
<th>Immersed in radioactive thymidine</th>
<th>Removed from radioactive thymidine and fixed</th>
<th>Hours exposed to radioactive thymidine</th>
<th>Percentage of nuclei labelled†</th>
<th>Grains per nucleus‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. alpestris</td>
<td>Cleavage</td>
<td>Early gastrula</td>
<td>20</td>
<td>100</td>
<td>+ + + $</td>
</tr>
<tr>
<td>T. alpestris</td>
<td>Early gastrula</td>
<td>Early tail bud</td>
<td>40</td>
<td>100</td>
<td>+ +</td>
</tr>
<tr>
<td>A. maculatum</td>
<td>Late gastrula</td>
<td>Early tail bud</td>
<td>40</td>
<td>100</td>
<td>+ +</td>
</tr>
<tr>
<td>A. tigrinum</td>
<td>Late neurula</td>
<td>Late tail bud</td>
<td>40</td>
<td>0</td>
<td>○</td>
</tr>
<tr>
<td>A. tigrinum</td>
<td>Mid-tail bud</td>
<td>Larva, ca. 10 mm.</td>
<td>40</td>
<td>20–40</td>
<td>↓</td>
</tr>
</tbody>
</table>

* Each group represents at least five separate embryos, except for the last two which contained three and two embryos respectively.
† Based on at least 100 randomly selected areas of the embryo studied with oil immersion objective.
‡ Mean value of at least ten nuclei from the three primary germ layers or their derivatives, minus background. Adjusted time of exposure: 2 weeks NTB-3.
$ + + + 100 or more grains per nucleus;
++ 21–99 grains per nucleus;
+ 5–20 grains per nucleus;
↓ 1–4 grains per nucleus;
〇 background.
highest salt concentration used (full strength Holtfreter solution) being con-
sidered isosmotic to the cells of the embryo. The amount of radioactive thy-
midine that had entered the embryo and become localized in the nuclei was
estimated by counting the silver grains in the emulsion of the radioauto-
graphic sections.

It was found that the intensity of nuclear labelling and the regional distribution
of labelled nuclei within the embryo varied with the following parameters: de-
velopmental age of the treated specimen, salt concentration of the immersion
fluid containing the thymidine, duration of the treatment, and, to some extent,
the kind of amphibian species employed. It became evident that it is essentially
a combination of the natural and the artificially induced permeability properties
of the surface of the embryo which can explain the whole gamut of the results
obtained. The extent of nuclear labelling can therefore be used as an indication
of the degree of permeability of the surface of the experimental material. Let us
discuss some of the findings in more detail.

Age-conditioned changes of permeability of the surface of the embryos

In an amphibian egg the material composition of the peripheral cell layer
changes considerably in the course of development, particularly in consequence
of the inward movement of large areas of the peripheral cells during the period
of gastrulation-neurulation. By way of these movements, all of the material of
the prospective mesoderm, entoderm and neural plate become transported into
the interior, and the surface layer becomes then exclusively made up of epidermis.
One might suspect that these thorough morphogenetic changes are associated with
changes in the permeability of the successive surface layers. Our experiments
provide, in a roundabout way, evidence for it. What they show is a stage-depen-
dent susceptibility of the embryonic surface to a permeability-increasing effect of
salt solution. Broadly speaking it was found that this effect, while being con-
spicuous during the early developmental stages up to the end of neurulation,
fell off rapidly thereafter and that at the stages of late tail bud and swimming
larva the surface, now consisting entirely of epidermis, could no longer be made
permeable to the entrance of tritiated thymidine. Evidently this age-dependent
increasing resistance to the effect of salt solution upon permeability reflects a
naturally occurring tightening up of some surface structure in the course of
development.

This general trend—decrease with age of the susceptibility (or permeability)
of the embryonic surface—could be observed in all four of the amphibian species
studied. This broad statement requires, however, a few corrections and ampli-
fications.

It was observed that around the period of gastrulation the amount of incor-
porated thymidine dropped sharply from the preceding high values during the
cleavage stages, but that this ‘depression period’ was only temporary, being
followed during the time of neurulation, by a short period, when again an extensive labelling could be obtained. Thereafter, the final and irrevocable downward trend with respect to thymidine uptake (permeability) set in (Table 1).

We cannot offer any explanation for the early depression period. It is interesting, however, that while working with the uptake of water by intact amphibian embryos of various stages, Lovtrup (1960) has likewise encountered a markedly reduced permeability during the gastrulation period, followed by an increased permeation of water at subsequent stages. Since the embryos were raised in a physiological salt solution (Lehmann solution), it is possible that in this instance, too, the salts have altered the natural permeability of the embryos. On the other hand, considering the difference in size between the molecules of water and thymidine, it is also possible that water would pass into embryos raised in a relatively salt free medium, e.g. tap water. Apparently such an experiment has not been done with amphibians.

Another observation of interest was the fact that at the early tail bud stage regional differences in permeability appeared. By now, the whole surface had become refractory to our treatment, with the exception of the dorsal line of fusion of the neural folds, or the more caudal portion of it, where thymidine could still enter and become incorporated in the neighboring tissues. This observation can be brought in line with the findings of Holtfreter (1943a) that in tail bud stages of *T. torosus* which were reared in physiological salt solution, neural crest cells slip through the epidermis along the very same dorsal line. Although such cell migration was not observed in the species here employed, our data nevertheless point in the same direction. Evidently the surface of the epidermis (coat) of the neural folds is 'softened' by salt solution more readily than is that of the rest of the epidermis. Perhaps it is this local peculiarity which permits the neural folds to fuse with each other at the end of neurulation.

Finally, in the oldest stages here employed, the larvae of *A. maculatum* of 13–14 mm. length, we encountered the strange situation that while the whole epidermal surface had become impermeable to thymidine, even when thymidine had been administered together with Holtfreter solution for 10 hr., it was now the epithelium of the pharynx and subjacent tissues which became labelled. When this occurred, the gill slits were found to have opened, thus giving the marker direct access to the pharynx. One may wonder whether the remainder of the intestinal tract is likewise permeable to thymidine, perhaps even without the simultaneous application of the salt solution.

**Considerations concerning permeability alterations of the amphibian embryo**

The data leave little doubt that the permeability barrier is represented by a special surface structure the permeability of which can be altered in direct proportion to the tonicity of the surrounding salt solution. We do not know what kind of alterations of the cell surface occur in connection with its induced per-
meability to larger molecules, but evidently, full strength Holtfreter solution (pH 7.5), even when applied for several days, does not affect the integrity of the embryo surface as seriously as do the various disaggregating agents mentioned above. Whereas the disaggregated (and decoated?) cells are immediately cytolysed when transferred from Holtfreter solution to water, the embryos treated with Holtfreter solution alone (or with thymidine dissolved in it), suffer in no way when transferred to water.

It would be of interest to know whether the permeability alterations in this study can be ascribed to a partial neutralization of charges in the surface layer by the salt solution. As previously mentioned there is evidence for the release of a mucinaceous material from the embryo when it is treated with disaggregating agents. Also there is some evidence that molecular configurations of mucopolysaccharides are altered by increasing ionic strength. These data suggest that the effect of the increasing salt concentration in our experiments may increase permeability of the embryo by weakening a mucopo-protein binding substance, possibly the surface coat of Holtfreter. In this manner, the barrier to the diffusion of thymidine, either into or between the surface cells, may be altered.

It is also conceivable that thymidine gains entrance into the embryo by an active transport mechanism such as pinocytic activity of the surface cells. The same salts which are contained in our medium have been shown to induce pinocytosis in amoeba (Brandt, 1958). Furthermore, invagination processes resembling pinocytosis have been observed to occur in ectoderm cells of T. pyrrhogaster exposed to either Holtfreter solution or to a solution containing proteins (Yamada, 1962).

The question of what makes the surface of the outer cell layer (epidermis) in embryos of advanced ages refractory to treatment with salt solution cannot be definitely answered. There are, however, the following observations which may be relevant. It has been shown that it is at the early tail bud stage that the epidermal cells acquire a tightly packed layer of small vacuoles directly beneath the outer surface, a structure which seems to operate, in part, as a new permeability barrier (Holtfreter, 1946). There is also the observation of Holtfreter (personal communication), that the disaggregating agents, citrate, alkali and versene are fully effective only when applied to early embryos. In order to disaggregate the epidermis or older stages, the action of the above agents must be supplemented by trypsin.

While we cannot satisfactorily characterize the mechanisms underlying the permeability changes brought about by a relatively low salinity of the medium, there is no doubt that it is some peculiarity confined to the cell surface bordering the outer medium which controls the results obtained here. Additional experimental data lend further support to this conclusion. Instead of applying tritiated thymidine to the immersion fluid we have also injected small amounts of it into the interior of embryos of different ages which were cultured in 10 per cent. Holtfreter solution. The marker spreads through large portions of the embryo label-
ling a high percentage of the nuclei in a gradient decreasing from the point of injection. This confirms the foregoing data according to which thymidine, once it has entered the embryo, readily spreads through the body, although we do not know whether it is passed on from cell to cell or travels through the intercellular spaces. Of more immediate interest is the observation that by the technique of injection, the inner tissues of a larva can be labelled, as well as the nuclei of the epidermis. This, as we have seen, cannot be done when thymidine is applied externally to larval stages. It is then evident that it is not the ordinary cell membrane present in all cells of all stages which interferes with the passage of thymidine. The difference in the results between external and internal application of the marker must be ascribed to some structural-physiological feature which is uniquely present in the outermost surface of the peripheral cell layer. It does not seem unreasonable to identify this feature with the structurally elusive surface coat of Holtfreter.

There seems to be no reason to involve the external protective layers—fertilization membrane and egg capsule—in this discussion. Both these layers appear to be readily permeable to thymidine and there is little reason for assuming that their permeability varies with the tonicity of the outer medium. Our data do not support the claim of Steinert (1951) that nucleosides are unable to pass through the fertilization membrane of amphibian embryos immersed in Holtfreter solution. In all our experiments with stages prior to the tail bud, the fertilization membrane as well as the tough jelly capsule had not been removed. Their presence did not seem to interfere with the effectiveness of nuclear labelling. On the other hand, in the older stages which had been deprived of their external membranes, the labelled thymidine failed to pass through the epidermis. The results are thus contrary to what one might have expected if it were assumed that the permeability properties of the outer membranes played a significant role in these studies.

Some remarks concerning DNA synthesis and the usefulness of the present method to introduce metabolites into the intact embryo

We do not wish to enter the controversy regarding the utilization of 'cytoplasmic DNA' (Sze, 1953; Hoff-Jørgensen, 1954) as opposed to de novo and/or 'partial' DNA synthesis from nucleosides, nucleotides, or more simple molecules (Grant, 1958a, b), but we do wish to point out, as did Moore (1959), that when exogenous tritiated thymidine is made available to the early embryo, the DNA precursors present within the embryo, whatever their molecular form, may be bypassed as the exclusive source for newly synthesized DNA.

Since the embryo can be made permeable to tritiated thymidine, it is likely that other biologically active molecules might also be made available to the developing embryo by altering the surface permeability. Other nucleosides, as well as amino acids and specific metabolic inhibitors or analogs, might behave similarly.
Application of the present labelling method to problems of morphogenesis

It is worthwhile to point out that the method described here of transporting a radioactive substance into live tissues by way of experimentally increased surface permeability, is in several ways superior to the previously used methods of injecting the substance into the embryo, or of exposing isolated embryonic fragments. Our method has the merits of being very simple, effective and applicable to intact embryos within their fertilization membranes. Normal development of the treated specimens is not significantly interfered with, and the degree of labelling can, in general, be more precisely controlled than with the injection method.

The special usefulness of this labelling method to the embryologist, lies in the fact that it can be readily combined with subsequent transplantations of the labelled tissues into unlabelled hosts. Marking the graft with tritiated thymidine has several advantages over other methods in which either natural or artificial markers have been used in order to distinguish the transplanted from the host tissues. The use of naturally marked hetero- or xenoplastic grafts may involve the risk of tissue incompatibility and incongruities of growth and differentiation between graft and host. These potential shortcomings can be minimized in the case of thymidine-labelled grafts in which the donor can be chosen from the same species and, preferably, even from the same clutch of eggs. As contrasted with the use of some other radioactive compounds as cell markers, tritiated thymidine has the advantage that it becomes specifically incorporated into the nuclear DNA and does not secondarily diffuse into neighboring cells. Finally, as compared with the cell marking method by means of vital dyes, the advantage of the present method resides in the fact that thymidine markers can be applied to the innermost cells of the intact embryo and do not ‘fade out’ within a short time as do vital stains. There is, however, the danger of applying radioactive isotopes at such high doses that excessive cell death ensues. This danger can be readily minimized in the method presented here in which the amount of the isotope entering the tissues can be controlled.

The method of first labelling the nuclei of an entire normal amphibian embryo with thymidine and then transplanting certain parts of it into an unlabelled host is presently being employed for the study of a number of morphogenetic phenomena. Thus, one of the authors (M.C.J.) has found this method particularly useful in tracing the migration of grafted parts of the neural crest and their derivatives. Equally rewarding (C.A.L.) has been the application of this method to problems connected with in situ myogenesis in the somites of amphibian embryos, particularly for the study of the rate of mitosis in differentiating myoblasts and the subsequent attainment of multinuclearity in myotubes.

SUMMARY

1. A simple method has been devised by which a nucleoside, tritiated thymidine, can be made to enter intact amphibian embryos during early embryonic
Alteration of permeability in amphibian embryos

development and become distributed throughout the entire embryo. The final localization of the radioactive thymidine within the nuclei was determined by the conventional technique of autoradiography.

2. The procedure consisted of increasing the surface permeability by means of a physiological salt solution (Holtfreter solution). The concentrations of tritiated thymidine in these experiments were 5 μc./ml and 10 μc./ml.

3. In one series of experiments, the embryos of three urodele species were raised in media which varied between tap water and increasingly higher concentrations of salt solution. It was found that whereas tritiated thymidine externally administered in tap water or 10 per cent. Holtfreter solution failed to enter the embryo, the amount of thymidine incorporated into the DNA of the nuclei increased in roughly linear fashion with further increase in salt concentration of the outer medium.

4. Short term labelling experiments with two different urodele species immersed in 100 per cent. Holtfreter solution, demonstrated a period of intense thymidine uptake in cleavage stages during which all nuclei became labelled. This was followed by a refractory period around gastrulation during which the salt solution was much less effective in enhancing the labelling intensity of the nuclei. This refractory period was followed by a period of increased nuclear labelling during neurulation, after which a general decline in the ability to label nuclei began. At early tail bud stages permeability to thymidine was limited to certain regions of the embryo and was reduced to zero by mid tail bud. With the opening of the larval gill clefts some of the pharyngeal endoderm and subjacent tissue became labelled.

5. In a separate series of experiments, embryos of three urodele species were made permeable to tritiated thymidine at different stages of development by immersion in 100 per cent. Holtfreter solution for extended time periods. This procedure resulted in the labelling of all nuclei of the developing embryo when immersed at stages prior to early tail bud.

6. While the precise mechanism(s) concerned with the uptake of tritiated thymidine in these experiments was not determined, a striking correlation was noted between the presence of labelled nuclei and the behavior of the surface of the embryo at the different embryonic ages investigated. On the basis of these comparisons, the surface coat is postulated to be implicated in these alterations, thus permitting the entrance of the labelled molecule into the intact organism.

7. This method of labelling the nuclei of an intact embryo without interfering significantly with its normal development has advantages over other methods of introducing tritiated thymidine into the embryo. In combination with subsequent transplantations of labelled tissues into unlabelled hosts, this convenient method of producing relatively stable markers is a valuable tool for the study of various problems of morphogenesis and nuclear behavior.
Altérations de la perméabilité des embryons d'Amphibiens provoquées par une solution saline et évaluées par absorption de thymidine tritiée

1. On a mis au point une méthode simple par laquelle on peut faire pénétrer un nucléoside, la thymidine tritiée, dans des embryons intacts d'Amphibiens au cours des premiers stades de leur développement, la thymidine se trouvant répartie dans tout l'embryon. La localisation finale de la thymidine radioactive à l'intérieur des noyaux a été déterminée par la technique classique d'autoradiographie.

2. Le procédé consiste à accroître la perméabilité de surface au moyen d'une solution physiologique saline (solution de Holtfreter). La concentration de thymidine tritiée dans ces expériences était de 5 µC./ml et 10 µC./ml.

3. Dans une série expérimentale, on a élevé les embryons de trois espèces d'Urodèles dans des milieux variés, allant de l'eau courante à des solutions salines de plus en plus concentrées. Tandis que la thymidine tritiée administrée extérieurement dans l'eau ordinaire ou le Holtfreter à 10% n'a pas pu pénétrer dans l'embryon, la quantité de thymidine incorporée dans l'ADN nucléaire s'est accrue de manière grossièrement linéaire avec l'augmentation de la concentration en sels du milieu extérieur.

4. Des expériences de marquage à court terme avec deux espèces différentes d'Urodèles immergées dans le Holtfreter à 100% ont mis en évidence une période d'absorption intense de la thymidine pendant les stades du clivage, au cours desquels tous les noyaux sont devenus marqués. Ceci a été suivi d'une période réfractaire aux environs de la gastrulation pendant laquelle la solution saline stimulait beaucoup moins efficacement l'intensité du marquage des noyaux. Cette période réfractaire a été suivie par une période de marquage nucléaire accru pendant la neurulation, après laquelle a commencé un déclin général de l'aptitude au marquage des noyaux. La méthode qui consiste à rendre perméable à la thymidine la surface entière de l'embryon intact devient inefficace au début du bourgeon caudal, moment auquel seules certaines régions de l'embryon deviennent marquées.

5. Dans une série spéciale d'expériences, des embryons de trois espèces d'Urodèles ont été rendus perméables à la thymidine tritiée à différents stades du développement par immersion dans une solution de Holtfreter à 100% pendant de longues durées. Ce procédé a marqué tous les noyaux des embryons immergés à des stades antérieurs au jeune bourgeon caudal.

6. Bien qu'on n'ait pas déterminé le mécanisme précis en jeu dans l'absorption de la thymidine tritiée dans ces expériences, on a noté une corrélation frappante entre la présence de noyaux marqués et le comportement de la surface de l'embryon aux différents stades de développement examinés. Sur la base de ces comparaisons, on postule que le 'manteau' superficiel est impliqué dans ces altérations, permettant ainsi l'entrée des molécules marquées dans l'organisme intact.
7. Cette méthode de marquage des noyaux d'un embryon intact sans interférence importante avec son développement normal présente des avantages sur d'autres méthodes d'introduction de la thymidine tritiée ou d'autres composés, dans l'embryon. Combinée avec des transplantations ultérieures de tissus marqués dans des hôtes non marqués, cette méthode de production de marqueurs relativement stables est un outil de valeur pour étudier divers problèmes de morphogenèse et de comportement nucléaire.

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REFERENCES


*Note added in proof:*

A paper has recently appeared (Berntsson, K-E., B. Haglund and S. Løvtrop 1964, Water permeation at different tonicities in the amphibian egg, *J. exp. Zool.* **155**, 317–24) reporting that an increase in the salt concentration in the ambient medium results in a corresponding increase in water exchange in *Amblystoma mexicanum* at stage 10.

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