The Distribution of Sulphur in the Differentiating Visceral Cartilage of Xenopus

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WITH ONE PLATE

INTRODUCTION

During embryonic development the differentiation of different tissues depends largely on the synthesis of specific substances characteristic of each tissue. From this viewpoint it is of interest to study the uptake of sulphur by the early embryo, especially since the incorporation and retention of this isotope in sulpho-mucopolysaccharides has now been well established by various authors working on fully differentiated tissues (see review by Dziewiatkowski, 1958).

So far some work has been done on the distribution of radiosulphate in early embryos (Amprino, 1955a, b; Friberg & Ringertz, 1956; Johnston & Comar, 1957), but for amphibians in particular no information is yet available. The present paper deals with the incorporation of radiosulphate in various embryonic tissues of Xenopus, in particular in the visceral cartilage of ectomesodermal (neural crest) origin.

MATERIAL AND METHODS

Embryos of X. laevis in stages 29–47 (Nieuwkoop & Faber, 1956) were used. Carrier-free sulphate-S35 (specific activity about 6 c./mg. S) was added to full-strength Holtfreter’s solution at 5 µc./c.c. The medium also contained 0·05 per cent. sulphadiazine (May & Baker). All cultures were maintained at room temperature.

Two types of experiments were performed:

(1) Anterior halves of Xenopus embryos in various stages of development, transected behind the gill-bud, were transferred into the radioactive medium for 4 or 6 hours. The epidermis was still unhealed at the end of culture.

(2) Small fragments consisting of the three germ-layers, removed from the

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gill-bud region of embryos in stage 29/30, were cultured as explants. The explants were cultured in radioactive medium immediately after excision or after 2, 4, or 6 days' explantation in non-radioactive medium. As the explants were completely covered by epithelial tissues (ecto- or endo-dermal) some time after explantation, they were split open just before being cultured in order to facilitate penetration by the tracer molecule. The explants had healed when removed from the radioactive medium, generally after 3 hours.

Fixation was carried out immediately after labelling or after a further culture for 2 or 4 days in non-radioactive medium. After thorough rinsing the specimens were fixed in Bouin, sectioned at 5 μ, and bleached when necessary with lithium carbonate. Autoradiographs were prepared with Kodak AR. 10 film. After exposing for 30 or 45 days and processing, the preparations were stained with methyl green-pyronin or toluidine blue. The latter stained either ortho- or metachromatically. The strength of labelling was assessed by subjective estimation, the range of autoradiographic densities being ample enough to justify this procedure. Under the resolution conditions of the preparations it is impracticable to distinguish between labelling of the peripheral and of the more central cytoplasm, so that 'labelled cytoplasm' may refer to one or both.

RESULTS

Observations on the whole head

Conspicuous differences exist in the uptake of sulphate by the various tissues in different stages of development. The main points will be given in the order of developmental stages.

Stage 29/30. Considerable uptake was observed at this stage. Although the distribution of tracer is more uniform than in later stages, mesodermal derivatives (undifferentiated mesenchyme of ecto- and endo-mesodermal origin, and heart) show an uptake a little higher than other tissues. As to the ectodermal derivatives, the white matter of brain and the ear showed fairly high uptake.

Stage 40. At this stage the various ectomesodermal elements later to give rise to visceral cartilage were distinguishable as mesenchymal condensations (precartilage). These future cartilage cells picked up particularly large amounts of sulphate, which was found in the cytoplasm and mainly in the intercellular space, while its presence in nuclei was doubtful. No metachromatic substance was detected in the intercellular space. The uptake in the other tissues was as mentioned for the earlier stage.

Some heads labelled at stage 40, and fixed after 2 or 4 days' culture in non-radioactive medium, were morphologically very much distorted and their development was delayed as compared with that of intact embryos, but the cells were still quite healthy. By the time of fixation the cartilage matrix was prominent. After 2 days, in spite of a general decrease of radioactivity in these heads, the cartilaginous tissue retained as much (or even more) tracer as the heads
fixed immediately after labelling. In this tissue the tracer was in the cytoplasm and the matrix. Especially in the matrix, which showed faint metachromasia, the amount of tracer was higher relative to that in the cytoplasm than in precartilages fixed immediately after labelling. This migration of tracer was still more pronounced after 4 days of culture.

Stages 45–46. The uptake was very high in the visceral cartilages, which were in an early stage of differentiation and with metachromatic intercellular matrix. The tracer was in both cytoplasm and matrix, whilst none or little was in the nucleus and nuclear membrane (Plate, fig. A). The labelling of matrix relative to that of cytoplasm increased from the 4th to the 6th hour of stay in tracer. The matrix was still too thin for internal differences in tracer content to be resolved by the autoradiographs. The future cartilage of the auditory capsule and the pre-chordalia were still at the stage of condensed precartilage and their uptake was less than that in visceral cartilages, except for a restricted area of the auditory capsule, assumed to be the centre of chondrification, which showed a high uptake.

No difference from the earlier stages was generally detected in tissues other than cartilage. One exception was the pharyngeal mucosa, which showed a fairly high uptake and metachromasia. Metachromatic substance appearing in the intercellular space of mesenchyme was accompanied by a little uptake.

Stage 47. In this stage much cartilaginous ground substance was deposited in the visceral skeleton, and the uptake by this tissue is very conspicuous (Plate, fig. B). The distribution of tracer was very characteristic in that it was especially abundant in the matrix newly deposited at the periphery of the chondrocytes. Other parts of the matrix and cytoplasm incorporated much less sulphur and none was detectable in the nucleus (Plate, fig. C). The amount of sulphur in the matrix, relative to that in the cytoplasm, was greater after 6 than after 4 hours of labelling.

Observations on explants

The differentiation of the explants was quite similar to what it would have been *in vivo* for the explanted tissues, i.e. they developed pharyngeal endoderm, cartilage, mesenchyme, muscle-cells, and epidermis. As the development was very much delayed in the explants, cartilaginous tissue in its early stage of differentiation did not appear before the 6th day of culture. When the labelling took place during this late stage of differentiation, uptake of tracer was very much higher in this tissue than in others and it occurred mainly in the matrix and cytoplasm. When labelling took place during the earlier stages of differentiation, the distribution of sulphate was rather uniform, though perhaps the amount in the undifferentiated mesodermal elements was a little greater than elsewhere.

Two explants labelled during the 4th day of explantation were fixed after a further 2 days’ culture in non-radioactive medium. The uptake in newly differentiated cartilages was higher than in the undifferentiated mesodermal cells in the
explants fixed immediately after labelling, whilst other tissues had lost the tracer, thus increasing the contrast in tracer content between chondral and non-chondral elements. The intercellular matrix, which had not yet appeared at the time of culture in tracer, was well labelled.

**DISCUSSION**

So far the distribution of radiosulphate in early embryos has been recorded for the chick (Amprino, 1955 *a, b*; Johnston & Comar, 1957) and the rat (Friberg & Ringertz, 1956). Particularly high uptake by cartilaginous tissue has usually been demonstrated. The present results extend these observations to the developing amphibian embryo. In the explanted tissue fragments, as well as in toto, a higher uptake in mesodermal (ecto- and endo-mesodermal) mesenchyme is already recognizable in the undifferentiated stage, to become very conspicuous in the differentiating cartilages.

In early stages, when no matrix has yet been deposited in the intercellular space, the tracer is mainly in the cytoplasm of precartilage cells. At the onset of matrix deposition the intercellular space shows metachromasia and both cytoplasm and matrix become strongly labelled (see Plate, fig. A). In the fully differentiated cartilages the tracer is found mainly in the matrix and especially in the immediate periphery of the chondrocytes (see Plate, figs. B, C). Our observations on the shift of relative concentrations of tracer from the chondrocytes to the matrix in (a) the cartilage of stage 40, kept from 2 to 4 days in normal medium after labelling, (b) the cartilage of stages 45-46 and 47, from the 4th to the 6th hour of labelling, and (c) the explants cultured for 2 days after labelling, all indicate that sulphate is first stored in the chondrocytes before being released in the matrix. Other authors have reached similar conclusions on fully differentiated cartilage (Pelc & Glücksmann, 1955; Fell, Mellanby, & Pelc, 1956; Mancini, Núñez & Lustig, 1956; Johnston & Comar, 1957). Nearly the same observations were also obtained in the cartilage of vertebrae and extremities (both of endomesodermal origin) of chick and mouse embryos differentiating in organ culture (Okada, 1959, and unpublished data). It can then be concluded that the differential localization of sulphur in the different stages of cartilage histogenesis is general to both the ecto- and endo-mesodermal cartilages of the vertebrate embryo.

The intercellular accumulation of sulphur revealed by the autoradiographs shows parallelism with the appearance of metachromasia in these sites. According to the evidence presented by other authors (Dziewiatkowski, 1951; Boström, 1952; Boström & Mänsson, 1952), it is valid to consider that the intercellular deposition of sulphur is in the form of chondroitin-sulphate. On the other hand, the precise chemical form in which the sulphate is found in the cytoplasm of precartilaginous cells is not yet known. It was suggested, however, that in precartilaginous cells of the chick embryo the future mucopolysaccharide component of the intercellular matrix is already demonstrable as PAS-positive
granules inside the cells (Moscona & Moscona, 1952). At any rate, it is now quite certain that these future cartilaginous cells establish their specificity in sulphate metabolism prior to their visible differentiation (present observations; and for the chick embryo those of Amprino, 1955b; Johnston & Comar, 1957; and Okada, 1959).

The question necessarily arises whether the specificity revealed by sulphate metabolism foreruns the so-called 'determination' of the future cells of visceral cartilage. It is known that the determination of visceral cartilage cells does not occur in the neural crest of the neurula, and that the cartilage differentiation of this material can be realized only under the inductive influence from endoderm and sometimes from notochord (Newth, 1954; Wilde, 1955; E. W. Okada & Ichikawa, 1956). But explantation experiments on *Rana japonica* indicate that the ecto- and endo-mesodermal layers of the gill region taken from tail-bud stages (approx. stage 29/30 in *Xenopus*) can differentiate into cartilage in the absence of both endoderm and notochord (E. W. Okada & Ichikawa, unpublished). In contrast, according to our results the precartilaginous cells first indicate their specificity by stage 40. Comparing the conclusions from these two different experimental approaches it is clear that the 'determination' detected by purely embryological tests precedes the establishment of metabolic specificity in these cells, but the latter foreruns the visible differentiation of chondroblasts.

**SUMMARY**

1. The incorporation of sulphate into tissues of *Xenopus* embryos in various developmental stages was studied by means of autoradiography.

2. Particularly high uptake was demonstrated in the precartilages as well as in the differentiated cartilages.

3. In the earlier stages, when no intercellular matrix has yet been deposited, the tracer is mainly in the cytoplasm of precartilaginous cells, while it is mainly in the matrix and especially in the immediate periphery of the chondrocytes of the differentiated cartilaginous tissue.

4. The movement of tracer stored in the cytoplasm to the intercellular matrix accompanies the histogenesis of cartilage.

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**REFERENCES**


T. S. OKADA and J. L. SIRLIN


EXPLANATION OF PLATE

Fig. A. Basibranchial cartilage of a stages 45–46 embryo. The strength of autoradiographs is about equal in cytoplasm and cartilage matrix. Cell-bodies (retracted from matrix) indicated by arrows. Five arrows with heel indicate cells in which the autoradiograph is seen to originate in the cytoplasm and not in the nucleus. $\times 800$.

Fig. B. Stage 47 embryo. Strong autoradiographs on visceral (branchial) cartilages (A) and presumed centre of chondrification in the auditory capsule (B). Weaker autoradiographs on cartilage of the auditory capsule (c) and chorda (d); in the latter the photographic contrast is due to stain and not to an autoradiograph. Inset magnified in fig. C. $\times 60$.

Fig. C. Inset from fig. B. magnified. Intense autoradiographs on parts of the cartilage matrix in the immediate vicinity of the chondrocytes, and no autoradiographs on the more distant matrix. Much weaker autoradiographs on the chondrocytes. $\times 900$.

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