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Differential Oxidation of Dyes in the Developing Embryos of Amphibia

By

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Introduction

Child ('43. '49) has found that differential intracellular oxidation of reduced dyes took place in the developing embryos of urodeles, *Triturus torosus* and *T. rivularis*. As a rule, the pattern of oxidation represented a gradient. But the pattern was not always uniform, it varied according to the condition under which the experiments were performed. In an extreme case the pattern was found to be reversed in direction. Whereas in the aerated solution, oxidation of dye took place least rapidly in the dorsal or in the anterior region of embryos, in the completely reduced solution oxidation of dye occurred most rapidly in the dorsal or in the anterior region.

These phenomena can be considered as a clue to the physiological analysis of the embryonic differentiation. Therefore, this brings on the subject of the present study.

Before going further, I wish to express my sincere thanks to Prof. M. Ichikawa for his kind guidance and encouragement throughout the course of this work. I am also indebted to Dr. H. Takaya for his valuable criticism.

Material and Method.

Embryos of *Rhacophorus schlegelii* var. *arborea* were used. In the early course of development, embryos of this species lack completely dark pigment, so that they are quite adequate to the materials of a staining experiment. Embryos of *Triturus pyrrhogaster* were used when necessary. As redox-indicators toluidin blue and Janus green were employed. These dyes were diluted in Holtfreter's solution in the concentration 1 : 10,000 to 1 : 50,000. Of these concentrations 1 : 20,000 was found to be most available for the purpose of the present experiment.

Reduction of these dyes was done by adding sodium hydrosulphite into the solution. About 10 mg. of sodium hydrosulphite was sufficient to make

10 c. cm. of the toluidin blue solution colorless. In the case of Janus green, as Child has pointed out, coloration of embryos took place even in the reduced solution if excess of sodium hydrosulphite was not added. Therefore, to make colorless staining with this dye, sodium hydrosulphite was used twice as much as in the case of toluidin blue. One to three embryos with their vitelline membrane were immersed into the reduced-dye solution. In a few minutes the coloration of embryos began to appear. The coloration was at first found in a limited region, but spread gradually to the whole surface of the embryo. As a rule, 20 minutes after the removal to Holtreter's solution the whole surface of the emryos became uniformly colored. Therefore, the regional difference of the body of an embryo was easily distinguished by rapidity and depth of coloration.

By this method, however, it was uncertain whether the preliminary staining of embryos might be uniform. To avoid the error which might be brought about by the differential adsorption of dyes, data of the observation were collected merely from those embryos which showed uniform coloration within 30 minutes oxidation.

Toluidin blue was chiefly used because of its moderate rapidity of oxidation. But the dye often showed metachromasy. To correct the error which might be produced by such aberrant coloration, Janus green was also used because of its lack of metachromasy.

Observation.

i) Cleavage and blastula stages ..... The cap of the animal pole was always the region in which coloration occurred most rapidly. Subsequently the coloration progressed from this region towards the vegetal pole. Rarely however, there were cases in which the coloration began in the region other than the animal pole. (cf. Table 1.) In these cases spreading of the coloration was always irregular, no common pattern of the spreading being found.

Table 1 Results of the embryos in cleavage and blastula stages showing region where the coloration began.

region	animal pole	other regions than animal pole	total
cases	58	7	65
%	89%	11%	

ii) Early gastrula stage..... In addition to the coloration of the animal pole the dorsal border of the blastopore showed rapid oxidation. From animal

pole spreading of the coloration towards the vegetal pole took place quite similarly as that found in the blastula. As soon as the invagination groove was formed, its dorsal border showed rapid coloration. In this case the coloration generally began in the center of the dorsal region and then spread sideways along the border. But there were few cases in which the coloration first appeared at either side of the blastopore and spread afterwards to the other side. (cf. Table 2.) Thenceforth coloration further spread from the dorsal border to the anterior and lateral direction. The ventral part of the blastopore and the vegetal pole of embryo was the region least rapidly oxidized. As above enumerated, it was common throughout the whole observations that the region of an embryo, which presented the coloration more rapidly, became more deeply colored, while the region which showed the coloration less rapidly became less deeply colored: From this fact, it seemed apparent that the region which took coloration most rapidly showed to be the region of the highest activity of oxidation of dyes.

Table 2 Results of the embryos in early gastrula stage showing region where the coloration began.

region	a. p. alone	a. p. and d. b.	a. p. and either side of d. b.	d. b. alone	other region than d. b. and a. p.	total
cases	5	14	15	2	9	45
%	11%	31%	33%	5%	20%	
cases in which anim-veg. gradient was observed	75%					
cases in which dorsi-ventral gradient was observed		69%				

a. p.....animal pole.
d. b.....dorsal border of the blastopore.
Striped portion did not show the gradient.

Of the two gradient patterns demonstrated in gastrulae, the one of the animal pole was a little more frequently observed than the one of the dorsal border of the blastopore. The first was found, as shown in Table 2, in 75% of the observed specimens, and the second was in 69 % of the cases. Presumably these different frequencies observed may be regarded as indicatives of the characteristics of the respective gradients.

iii) Yolk plug stage.....The oxidation of dyes began simultaneously at the dorsal and lateral borders of the blastopore. The coloration spread gradually in anterior and lateral direction. Also at the animal pole was found the coloration which spread gradually towards the vegetal pole.

But the coloration was not so deep, and the spreading was not so marked as was observed in early gastrulae.

iv) Neurula stage ..... In neurulae with apparent neural folds, the intracellular oxidation was the most rapid in the anterior and the posterior regions of the neural fold. Then the coloration progressed along the fold posteriorly from the anterior region and anteriorly from the posterior region at nearly the same rate. But the oxidation scarcely occurred in the neural plate, inside of the neural fold. Outside of the neural fold, i. e., inthe flank of emdryo, the coloration was faint, but progressed from the anterior region towards the posterior.

Discussion.

It is known that the intracellular redox-potentials of egg-cells are approximately 19–20 in rH value under aerobic condition, and 6–9 in rH value under anaerobic one. With amphibian embryos, Dorfman ('38) showed electrometrically that both in animal and in vegetal poles of unfertilized eggs of *Rana esculenta*, the rH value is approximately 20; i. e., no regional difference is found in so far as the rH value is concerned. Although no measurement of the rH value has been made under anaerobic condition, it seems reasonable to state that the more reduced is the solution in which embryos are immersed, the lower becomes the rH value of embryos, and inversely the more the reduced solution is oxidized, the higher becomes the rH value of embryos. In the present experiments the embryos were immersed in reduced-dye solution, and the oxidation of dyes carried out under aerobic condition, that is, in aerated Holtfreter's solution. Hence, it follows that the intracellular redox-potential of embryos which was lower than the standard redox-potential of the dye employed, has become to be elevated by removing to the aerated Holtfreter's solution. If this is the case, further it may be inferred that the region of an embryo, which presents the coloration first, corresponds to the region in which change of the rH value occurred most promptly. The present experiments verify the cap of animal pole, the dorsal border of the blastopore and the neural fold as such regions.

Fischer and Hartwig ('37), Piepho ('38) and Child ('43) have found that with embryos of urodeles the reduction of dyes took place in gradient pattern. The regions of embryo which, as pointed out by these authors, reduce the dye most rapidly coincide perfectly with the region which, as evidenced by the present experiments, oxidize the dye most rapidly. From this comparison, it may be stated that it is the same region that manifests the highest activity of reduction in the anaerobic condition and the highest activity of oxidation in the aerobic condition. In such region, therefore, change of the redox-potential must be very rapid in accordance with the change of the external

conditions.

Child ('43. '49.) found that at the animal pole of embryos the oxidation of dyes was most rapid in the reduced solution of Janus green, while the oxidation was least rapid in the same region if the oxygen content was gradually increased. Child interpreted this fact as follows: up-take of oxygen is highest at the animal pole in the aerated solution, whereas activity of oxidase is highest at the animal pole in the reduced solution. In the present experiments, it was found that at the animal pole rapid oxidation of dyes occurred in the aerated solution. Thus, when this fact is compared with those found by Child, we find a remarkable discrepancy between the condition of the solution and the process of oxidation. In so far as the present experiments are concerned, neither oxygen nor oxidase activity alone cannot be consulted to be the cause, because oxygen is present in the ambient solution. Under these considerations, it appears quite difficult to find separate reasons for different pattern which occurred under different conditions. Presumably the same speculation hold true for the dye-oxidation in the dorsal border of the blastopore and the neural fold.

The fact should be pointed out here that in early gastrulae the center of the oxidation did not always coincide with the center of the dorsal border, but very often was found at either side of the blastopore. From this fact, we are inclined to assume that the center of the oxidation does not necessarily coincide with the center of the presumptive chorda region, i. e., the organisation-center. In yolk plug stage, the dorsal part of embryo oxidizes the dye more rapidly than ventral part, while there is no distinct difference at least in rates of oxidation between the presumptive neural plate and the presumptive epidermis. The difference of the two regions can be recognized only when the neural fold is formed. In neurulae, the difference is also discernible between neural fold and neural plate. From these facts in consideration, it seems that the activity of oxidation in a given region of embryo is not necessary a representation of the developmental activity of that region as is postulated by the gradient-theory of Child. However, it must be pointed out that in such region as the dorsal border of the blastopore and the neural fold where the oxidation of dyes occurred most rapidly, the conspicuous formative movements of gastrulation or neurulation took place intensively. In this line of thought, it seems likely that the activity of oxidation bears a direct relation to the formative movements of the embryonic tissues.

Summary

1. In developing embryos of *Rhacophorus schlegelii* var. *arborea*, differential intracellular oxidation of reduced dyes was observed

2. It was evidenced that in such regions as the cap of animal pole,

the dorsal border of the blastopore and the neural fold, dye-oxidation proceeded most rapidly

3. It was discussed that the region of embryo in which the oxidation of dye proceeded most rapidly corresponded to the region in which the change of the rH value occurred most promptly.

4. The activity of dye-oxidation seemed to bear a direct relation to the formative movements of embryonic tissues.

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