Osmotic Homeostasis in Development of the Salamander, *Hynobius retardatus*[1)]

By

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Osmoregulation, in the development of the eggs of fresh water animals, has aroused the interest of biologists for ages. In 1912, Backmann and Runnström attacked the problem of the osmotic variations in the course of development of the frog, *Rana temporaria*, and discovered remarkable facts. Particularly, that at the time of it's fertilization in pond water, the osmotic pressure of the frog egg is markedly reduced, almost to a tenth of that of ovarial eggs, which is isotonic with adult serum. After that, it continuously increases with the progress of development, and finally, the initial osmotic pressure is resumed in the young larva, about twenty-five days after fertilization. This interesting work has been received with considerable attention by many investigators. In the frog and triton, the peculiar change in osmotic pressure, as associated with embryonic development, has been generally confirmed (Backmann and Sundberg, 1912; Białaszewicz, 1912; Przyłęçki, 1917; Voss, 1926), although, in some recent investigations, it has been found that the osmotic lowering in the egg, at the time of fertilization, is not so markedly distinct as stated by Backmann and Runnström (Krogh, Schmidt-Nielsen and Zeuthen, 1938; Picken and Rothschild, 1948). Thus, while the frog egg develops poikilosmotically in fresh water, the reverse has been found by Svetlov (1929) and Gray (1932) in the trout, *Salmo fario*, which is nearly homoiosmotic in fresh water during the whole course of development.

The present investgation has been undertaken, to determine the type of osmotic homeostasis in egg, embryo and larva of *Hynobius retardatus*, the common salamander, found in Hokkaido district. This article has been made possible by collaboration and assistance of Dr. K. Aoki, and by Dr. Y. Watanabe's editing and arrangement of the format.

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Material and Methods

The egg sac and capsule of the fertilized salamander eggs, collected from the pond, were carefully taken off; then the eggs were reared in tap water in the laboratory, at a temperature of 14°-16°C., and used for this test at various stages of development as needed. In determining the osmotic pressure of the egg content, the freezing point depression was measured galvanometrically by the use of a Cu-constantan thermocouple. In order to reduce the possibility of error, a single egg was used in each case (cf. Krogh *et al*, 1938).

After the water had been removed from the egg surface with a peice of filter paper, the egg was placed in a small vial and gradually cooled by a freezing mixture of minute pieces of ice and NaCl. The temperature of the mixture was 8.0°±0.1°C. below zero. Galvanometric deflections, caused by change in temperature of the material under cooling, were recorded in scale dimension, arbitrarily taken. From the "freezing curve", obtained from such records, the freezing point of the material was determined in the scale dimension. The same method was applied to ovarial eggs and embryos of various stages, and also to adult serum, prepared by centrifugation technique. In tests made with the tadpole, pulp of ground material was employed.

In comparing the galvanometric data with cryoscopic freezing points, the measurements were conducted with egg or tissue *Brei*, each of about 120 specimens, adopting the usual micro-Beckmann method. For another purpose of study, permeability to water, and water content of egg and embryo were examined.

Experimental Results

Freezing point depression. The galvanometric data are given in Table 1, with Δ-values, obtained from cryoscopic determination, in ripe ovarial eggs, uncleaved fertilized eggs and larvae of fore-limb stage. In general, as shown in Table 1. the freezing point depression, which is, in turn, the osmotic pressure, is practically unchanged during the course of development in this animal, and coincides with that of the adult serum. The average freezing point depression of the eggs, and larvae, is estimated as −0.34°C., and is equivalent approximately to that of M/8 solution of NaCl, while Δ-value for tap water, in which the material was reared, is only −0.017°C., i. e., ca. M/220 NaCl eq. All of these data tends to indicate that the *Hynobius* egg develops homoiosmotically in fresh water.

Permeability to water and content of water. It is interesting to note the effect of the increase in osmolar consentration of surrounding media upon the osmotic pressure and the content of water in this developing animal. First, the freezing point of the morula stage eggs, immersed in tap water, and in M/10, M/20 and M/100 solutions of NaCl, was determined by use of the galvanometric method. The observations show that, independent of different osmolar concentrations of the media, the

Table 1

Developmental stages	Freezing point in galvanometric deflections	Δ in degree C.
Distilled water	260	
Tap water	261	—0.017
Ripe ovarial egg	278	—0.34
Uncleaved egg	275	—0.34
2-celled egg	273	
8-celled egg	275	
Morula	275	
Early gastrula	273	
Neurula	274	
Late neurula	273	
Early tail bud stage	279	
Tail bud stage	276	
Late tail bud stage	273	
Balancer stage	279	
Fore-limb stage	277	—0.34
21-days tadpole	273	
Adult serum	273	

freezing point depression is kept invariably constant, even in 24 hour immersion in any of these solutions. All of the data obtained indicates 274 or 275 in the same scale dimension as used in the preceding experiments. Second, the fertilized eggs were reared in the Ringer solutions[2] of M/15, M/60 and M/120 in concentration, and after they grew up into early balancer stage, the water content was closely examined every two days, for 14 days after fertilization. The data given in Table 2 indicate that the content of water increases with the age of the larvae, but decreases with concentration of the Ringer, in which the larvae had been reared. In spite of these variations in water content in the expeirmental condition, the freezing point depression in the larvae in the natural environment is kept constant. In consequence thereof, these facts suggest that the osmotic pressure in the larvae must be regulated in some way or another in the later development.

Table 2

		Water content in per cent			
Days after fertilization		8	10	12	14
Ringer solutions used	M/ 15	—	48.7	61.2	71.9
	M/ 60	51.6	55.2	70.2	76.9
	M/120	52.7	57.7	72.2	—
Control in tap water		53.1	—	71.4	—

2) The standard concentration is as follows : 1M NaCl, 100 parts ; 1M KCl, 2.8 parts and 2M/3 $CaCl_2$, 3.4 parts.

Discussion

As noted above, frog eggs develop poikilosmotically, while trout eggs develop homoiosmotically. In respect to osmotic behavior, as the preceding data show, the *Hynobius* specimens closely resemble the latter.

In water, the frog egg swells up (Backmann and Runnström, 1912; Krogh *et al*, 1938) and decreases in electric conductivity (Picken and Rothschild, 1948) or the ions contained apparently diffuse and diminish (McClendon, 1915). It is, therefore, reasonable to cocnlude, that the surface of the frog egg is permeable to ions as well as to water. This property in protoplasmic surface is, undoubtedly, responsible for the osmotic change of the frog egg in fresh water (Backmann and Runnström, 1912; Krogh *et al*, 1938; Picken and Rothschild, 1948). However, in trout, the egg surface is hardly permeable to crystalloid, and even to water. This impermeability of the trout egg may play a fundamental part in the maintenance of its homoiosmoticity (Svetlov, 1929; Gray, 1932). Taking these facts into account, it is highly probable that the difference in osmoregulation, between these two animals, is intimately correlated to the difference in permeability systems involved in the egg surface. Therefore, as to the mechanism underlying the homoiosmoticity of the *Hynobius* egg, the permeability system must be taken into consideration.

The egg of *Hynobius* is enveloped with two membranes, namely capsule and chorion (or vitelline membrane). The capsules are permeable to water and crystalloids as well, but not to colloidal substance (Aoki, 1941, 1942a). Regarding permeability, in the writer's tentative experiments (Kusa, unpublished data), the chorion is found to be similar in nature to the capsules, and also to the chorions of frog and triton eggs (Harvey and Fankhauser, 1933; Briggs, 1939) as well as trout eggs (Sveltlov, 1929; Bogucki, 1930; Gray 1932) and salmon egg (Aoki, 1939, 1940, 1942b). Since these two membranes have thus nothing to do with osmotic behavior, the locus of the osmoregulation, in this material, appears to be confined to the egg surface.

According to Shinozaki (1949), the water content of *Hynobius* eggs does not alter, from the time of fertilization, and on up to early balancer stage, but begins to increase at the balancer stage, gradually increasing with further development. As shown in the preceding pages, even in 24-hour-immersion in M/10, M/20 and M/100 solutions of NaCl, the freezing point depression is almost unchanged in the earlier stage (morula) of development; and the water content (54.1%) of the larvae of early balancer stage, reared in M/15 Ringer solution, is almost equal to that (54.7%) of the corresponding stage of the control in tap water. These data favor the suggestion that, in the earlier stages of development, prior to the balancer, the surface of the specimens is impermeable, at least, to water. This impermeability may exercise a certain degree of control of the homoiosmoticity in the *Hynobius* specimens, similar to that of trout eggs. However, increase in water content of the larvae of advanced

stages of development is more or less retarded by an increase of osmolar concentration of the media experimentally used (Table 2). In consequence thereof it might be stated, that in the developmental stages, later than the early balancer, the impermeability to water can no longer be persisted in.

Nevertheless, this experiment tends to prove that the homoiosmoticity is still maintained in these later stages. It is evident, that this homoiosmoticity must be regulated by certain factors, other than the general protoplasmic surface of the organism. The possible factor or factors, which may or may not be responsible for the regulation, are : (a) adsorption of osmotically active substance by the embryo from the surrounding medium, (b) breakdown of inactive higher molecules into active lower molecules, (c) the functional development of special osmoregulatory mechanism or organ. At present, however, it is impossible to arrive at any definite conclusion on the problem of the homoiosmoticity in the later stages, until further analysis is obtained. In either event, it is unquestionably clear that the osmoregulatory mechanism is rather complicated in the advanced stages as compared with the earlier stages of development of this animal.

Summary

With use of the thermoelectric method and the micro-Beckmann method, the freezing point depression was determined with eggs and embryos of various stages of development in the salamander, *Hynobius retardatus*. *Hynobius* was determined to be homoiosmotic throughout the whole course of development, average freezing point depression, Δ, of the protoplasmic content being $-0.34°C$. The water content of the morulae is not affected by change in osmolar concentration of the surrounding media experimentally used, but it is more or less appreciably influenced in the larvae of more advanced stages than the balancer. Thus, in earlier stages, the mechanism underlying the homoiosmoticity is probably ascribed, at least in part, to impermeability of the protoplasmic surface to water. This impermeability to water can no longer be preserved in later stages, in which the homoiosmoticity may be supposed to be regulated by a certain factor or factors other than the general protoplasmic surface of this orgainism.

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