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Acid Mucopolysaccharide in Embryos of the Horseshoe Crab, *Tachypleus tridentatus* (Chelicerata, Arthropoda)[1]

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ABSTRACT — The distribution, type, quantity and biosynthesis of acid mucopolysaccharide (AMPS) in embryos of the horseshoe crab (Chelicerata, Arthropoda) were examined. In embryos at early developmental stages, most of the AMPS is sulfated type and fills in the perivitelline space between the chorion and the blastoderm. It is already found in unfertilized eggs and it is rarely synthesized after fertilization. When the germ disc appears (stage 7), it is separated from the blastoderm by the secretion of a membrane. It disperses into sea water after the rupture of the chorion (stage 18 or 19). The other type of AMPS is synthesized in the embryonic body and is non-sulfated. At the hatching stage (stage 21), the non-sulfated AMPS decreases and sulfated AMPS is found in the endoskeleton, the intestine and in the articulation of the appendages.

INTRODUCTION

In the cortical regions of the embryos of several marine animals belonging to the Annelida, Echinodermata and Vertebrata, there is a layer of acid mucopolysaccharide (AMPS) [1–4]. AMPS also exists in the intercellular matrix and it is related to many biological functions such as the construction of cells, the toughness and flexibility of tissues, calcification, the control of electrolytes and water, wound healing, lubrication, and the maintenance of stable transports [5].

In embryos of the horseshoe crab, a marine arthropod, the existence of AMPS in the cortical layer has been reported by Sekiguchi *et al.* [6] and Bennet [7], and the cortical reaction was examined in detail by Brown and Clapper [8], Bannon and Brown [9], and Brown and Barnum [10]. AMPS in the cartilage of adults has been described [11–13], as well as the effect of inhibitors of the synthesis of AMPS on the embryonic development [14, 15]. However, the exact distribution and type of AMPS present in the embryos at different developmental stages have not been elucidated thus far. This paper deals with the characterization of AMPS in embryos of Japanese horseshoe crab, *Tachypleus tridentatus.*

MATERIALS AND METHODS

Male and female horseshoe crabs, *Tachypleus tridentatus*, were collected in Saga and Fukuoka Prefectures in Japan. They were transferred to laboratories at the Shimoda Marine Research Center and Shizuoka University where the present studies were conducted. The mature eggs were obtained from the body cavity of females by dissection. The eggs were fertilized by artificial insemination of sperm obtained from male individuals. The fertilized eggs were reared in filtered sea water in a plastic tray. The stages of embryonic development were identified according to Sekiguchi [16].

For light microscopic studies, the embryos were fixed in Bouin's, Carnoy's, or FAA solution, embedded in paraffin, and sectioned from 5 to 20 μm in thickness. The sections were stained with Mayer's hematoxylin and eosin. The distribution

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of AMPS was determined by histochemical methods using 1% alcian blue (pH 1 or 2.5), 0.05–0.1% toluidine blue (pH 0.1 or 4.0) and Hale's reaction. The type of AMPS was determined by methylation and saponification. If a tissue contained non-sulfated AMPS, it was stained again after saponification. A digestion method using testicular hyaluronidase was also used for the determination of type of AMPS. Periodic-acid Schiff reaction (PAS) and digestion by amylase were used for the determination of distribution and type of neutral polysaccharide.

For electron microscopy, specimens were prefixed in 5% glutaraldehyde and 4% paraformaldehyde in cacodylate buffer (pH 7.4) and then post-fixed in 1% osmium tetroxide solution. These specimens were embedded in Epon 812 or Spurr low embedding resin (Polyscience Inc., USA), and sectioned with a Poter-Blum ultramicrotome. The sections were stained with saturated uranyl acetate and Reynold's solution.

The type, quantity and biosynthesis of AMPS were determined by the following biochemical methods. The AMPS was extracted from embryos at various developmental stages using the method of Aoki and Koshihara [17], and measured quantitatively by the method of Scott [18] using chondroitin sulfate as an internal standard.

For qualitative analysis, the extracted AMPS was fractioned by Dowex 1-column chromatography. Hyaluronic acid, chondroitin sulfate and heparin were used as marker for the chromatography. Non-sulfated AMPS is mainly eluted by 0.25 M and 0.5 M NaCl, heparan sulfate by 1.25 M

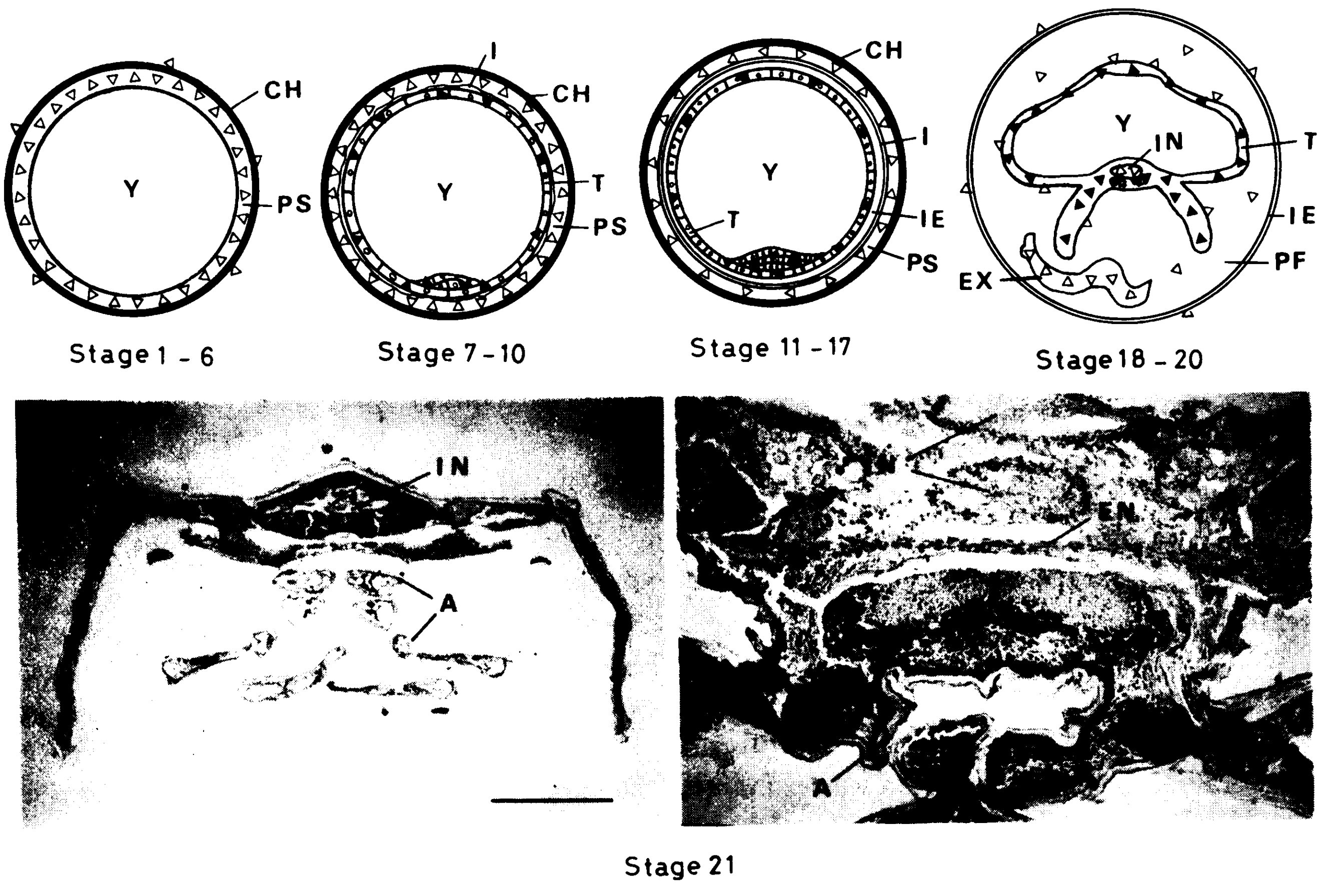
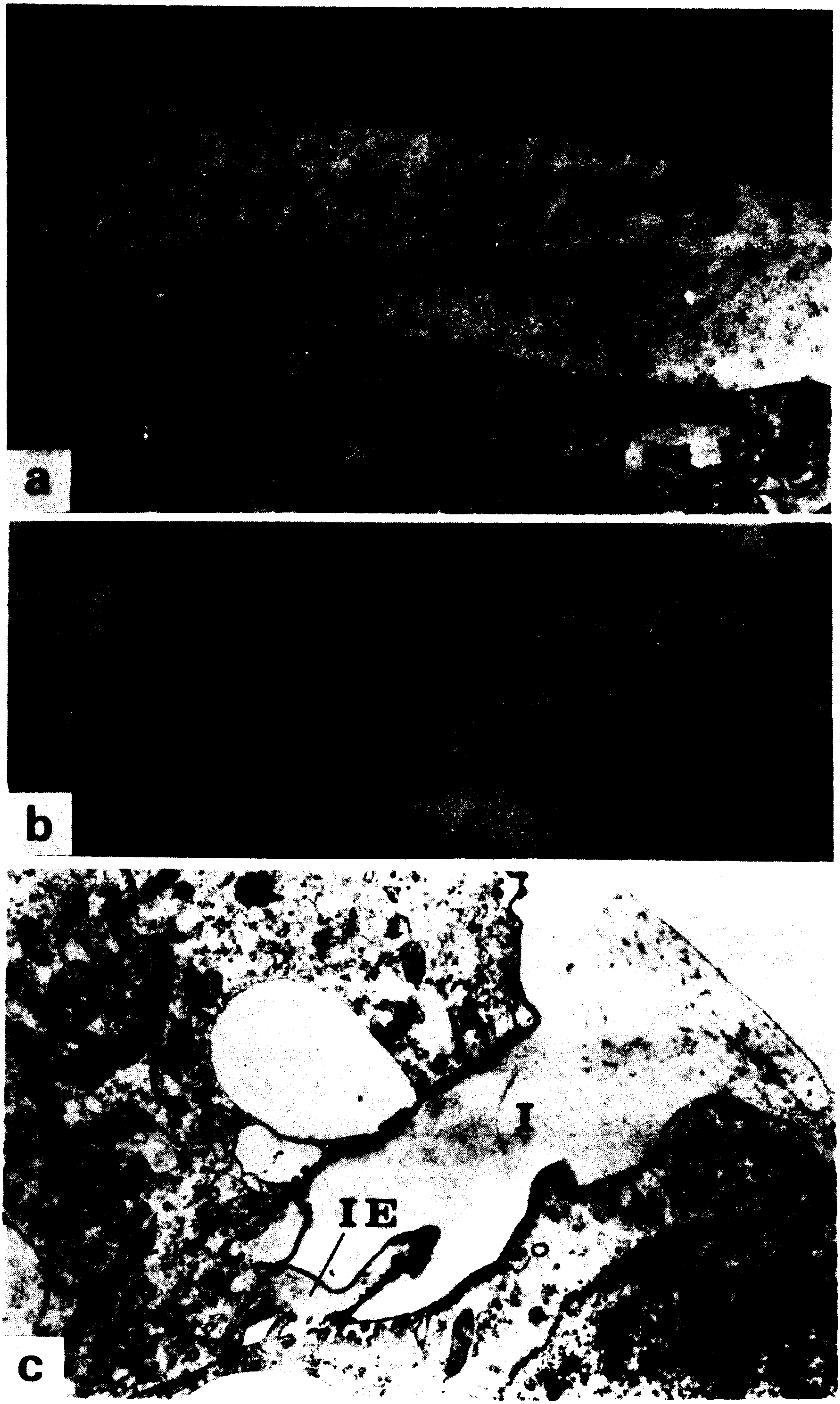


Fig. 1. The distribution of AMPS at different developmental stages. △: sulfated AMPS, ▲: non-sulfated AMPS. The AMPS which was observed in the embryos at stage 21 was mainly sulfated AMPS. The bar represents 1 mm. A: articulation of appendages, CH: chorion, EN: endoskeleton, EX: exuvium of embryonic molting, I: intermediate membrane (I-membrane), IE: inner egg membrane, IN: intestine, PF: perivitelline fluid, PS: perivitelline space (layer of AMPS), T: general tissue of embryonic body, Y: yolk.

Fig. 2. The cortical region of horseshoe crab embryos. a. early blastula stage (stage 4), b. stage 9, c. stage 13. The bar represents 1 μm. CH: chorion, I: I-membrane, IE: inner egg membrane, N: nucleus, PS: perivitelline space (layer of AMPS).


a
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NaCl, chondroitin sulfate by 1.5 M NaCl, and heparin by 2.0 M NaCl. Electophoresis was also carried out using the methods of Seno *et al.* [19]. Digestion by hyaluronidase was also used.

For the examination of biosynthesis of sulfated AMPS, embryos at different stages were reared for 72 hr in sea water containing 5 μCi ^{35}S–Na_2SO_4. AMPS was extracted from the treated embryos, and the radioactivity was measured with a liquid scintillation counter. In addition, the extracted AMPS was fractioned on a Dowex column to examine the type of synthesized AMPS.

RESULTS AND DISCUSSION

Sulfated and non-sulfated AMPS were found in several portions of embryos at different developmental stages. The distribution of AMPS is summarized in Figure 1.

There was a great deal of AMPS in the perivitelline space between the chorion (the first egg membrane, the vitelline envelope or the outer egg membrane) and the surface of the periplasm at the stages between the unfertilized egg and the blastula stage (Fig. 2–a). After stage 7, when the germ disc appeared, the AMPS was separated from the blastoderm by a membrane which was secreted from the blastodermal cells. Hereafter, the authors call this membrane the intermediate membrane (I-membrane). It corresponds to the thin membrane which was reported by Sekiguchi *et al.* [6] (Fig. 2–b). The I-membrane became thicker and was pushed up by an other membrane which began to be secreted at stage 11. The latter membrane is called the inner egg membrane (the secondary egg membrane or deutovum) [20] (Fig. 2–c).

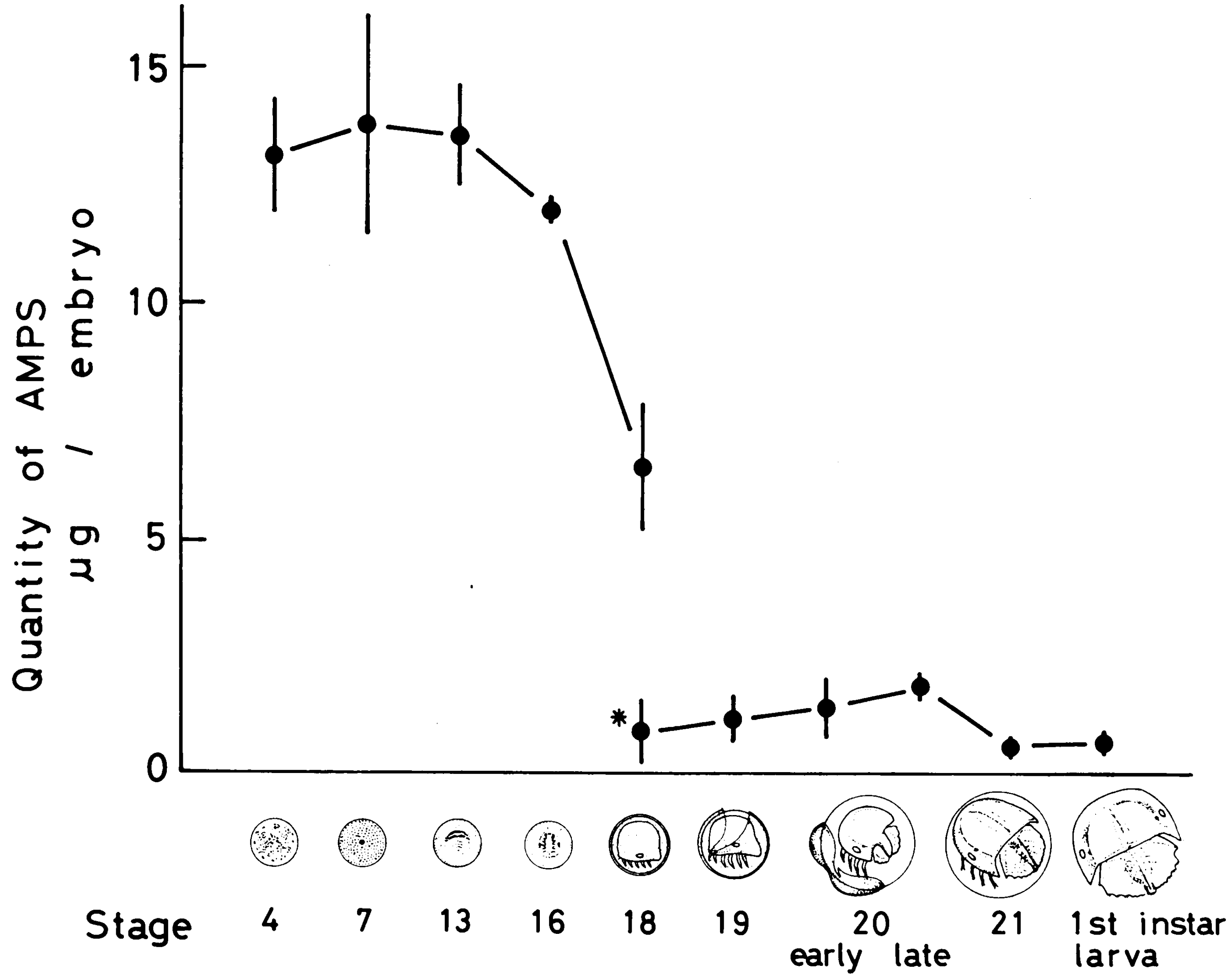

FIG. 3. The quantity of AMPS at different stages of horseshoe crab embryos. The abscissa shows the different developmental stages. In the horseshoe crab, the 1st instar larva has the same form as the embryos at stage 21. *: the quantity of AMPS extracted from the embryos after the rupture of the chorion. The AMPS which was attached to the ruptured chorion was only 0.3 μg/embryo. Sample number is more than 3 at each stage. Bars show standard deviation.

TABLE 1. Histochemical reaction of embryos of the horseshoe crab

	Cortical region (the layer of AMPS)	Surface of chorion, exuvium, intestine, articulation	General tissues of embryos	Chorion	Inner egg membrane	Yolk
Alcian blue (pH 2.5)	⧻	⧻	⧺	—	—	—
Alcian blue (pH 1.0)	⧻	⧻	⧺	—	—	—
Toluidin blue (pH 4.0)	⧻	⧺	⧺	—	—	—
Toluidin blue (pH 0.1)	⧻	+	—	—	—	—
Hale's reaction	⧻	⧻	⧺	—	—	—
Alcian blue (pH 2.5) after Methylation	—	—	—	—	—	—
Alcian blue (pH 2.5) after Methylation+Saponification	—	—	⧺	—	—	—
Alcian blue (pH 2.5) after Hyaluronidase digest	⧻	⧻	+	—	—	—
Peridoic-acid Schiff (PAS)	—	—	⧺	—	—	⧻
PAS after Amylase digest	—	—	—	—	—	⧻

+ = Weak reaction; ⧺ = Moderate reaction; ⧻ = Strong reaction; — = No reaction.

The AMPS remained in the perivitelline space until stage 18. The first embryonic molting occurs at stage 18. At stage 18 or 19, the chorion is ruptured by the enlargement of the inner egg membrane. Then, the inner egg membrane wraps the embryo instead of the chorion. The AMPS in the perivitelline space was discharged following the rupture of the chorion. As the total quantity of AMPS in eggs decreased greatly after the rupture of the chorion (Fig. 3), we were able to determine that almost all AMPS in the eggs before the rupture of the chorion existed in the perivitelline space. This AMPS was sulfated AMPS and mainly eluted in the fraction of heparan sulfate (Table 1, Fig. 4). Nevertheless, sulfated AMPS was rarely synthesized during the period immediately after fertilization (stage 1) to the development of appendage rudiments (stage 16) (Fig. 5). The AMPS may be synthesized during oogenesis.

A small quantity of sulfated AMPS was found in an other place. It existed on the outer surface of the chorion at stage 1 and was gradually dis-

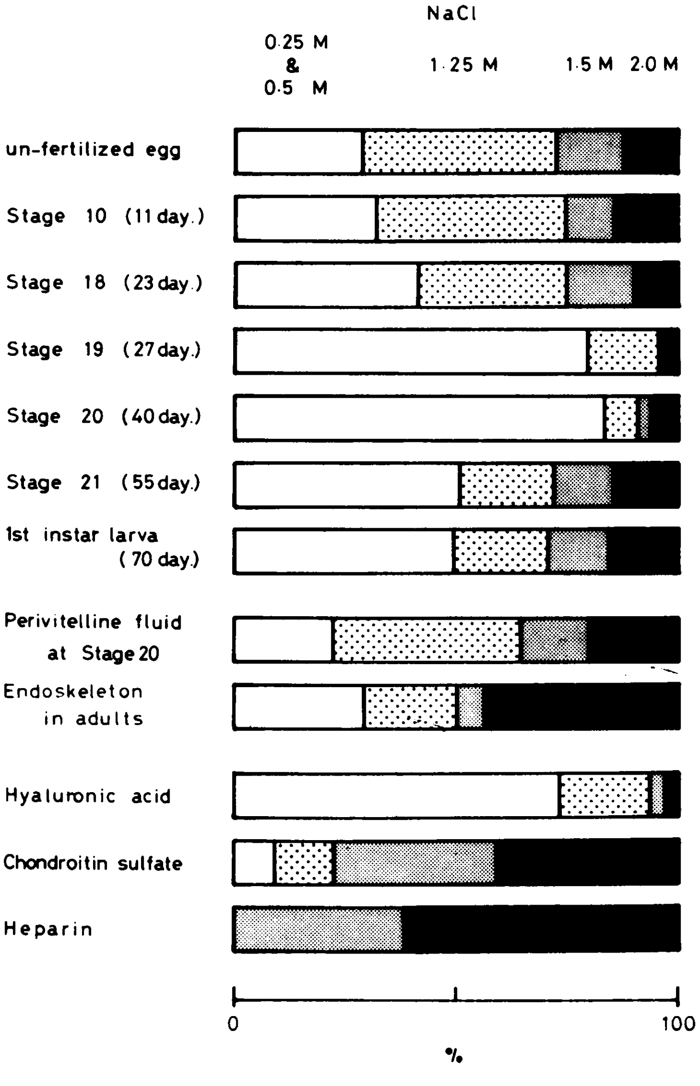

FIG. 4. The rate of each fraction of AMPS by column-chromatography. AMPS was extracted from horseshoe crab embryos at each stage shown in the ordinate. The AMPS was fractioned by NaCl. In the case of the extraction from the embryos at stage 18, their chorions were not ruptured. ( ): the days after the insemination.

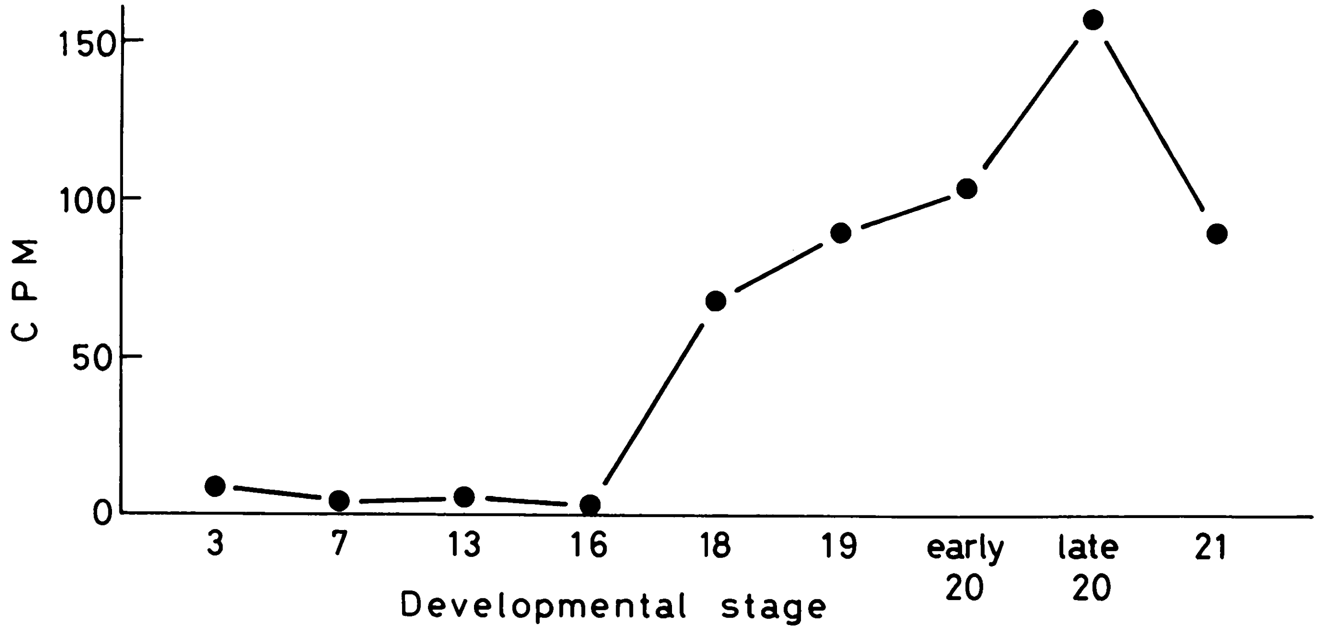

FIG. 5. The biosynthesis of sulfated AMPS in horseshoe crab embryos. Incorporation of ^{35}S–Na_2SO_4 into AMPS which was extracted from the embryos at the different stages shown in the abscissa. The ordinate shows the counts per minute (CPM)/total AMPS in an embryo.

persed into the sea water (Fig. 1, Table 1).

The synthesis of sulfated AMPS was recognized after the first embryonic molting (stage 18) (Figs. 4 and 5). In the period from stage 18 to 20, the sulfated AMPS was found on the outer surface of the embryonic body, the exuvium of the embryonic molting, and the internal surface of the intestine. In the perivitelline fluid which exists between the inner egg membrane and the embryonic body, sulfated AMPS was also found at these stages. The sulfated AMPS which was synthesized at these stages was mainly eluted into the heparan sulfate fraction (Figs. 4 and 6). However, the synthesized sulfated AMPS seemed to be small in quantity. As mentioned later, biochemical and histochemical observations showed that a major part of AMPS at stage 19 and 20 was non-sulfated AMPS.

In the embryonic body at stage 21 (the stage after the 4th embryonic molting, or the stage of hatching), sulfated AMPS was found in the endoskeleton, the epithelium of the intestine and in the articulation of appendages (Fig. 1, Table 1).

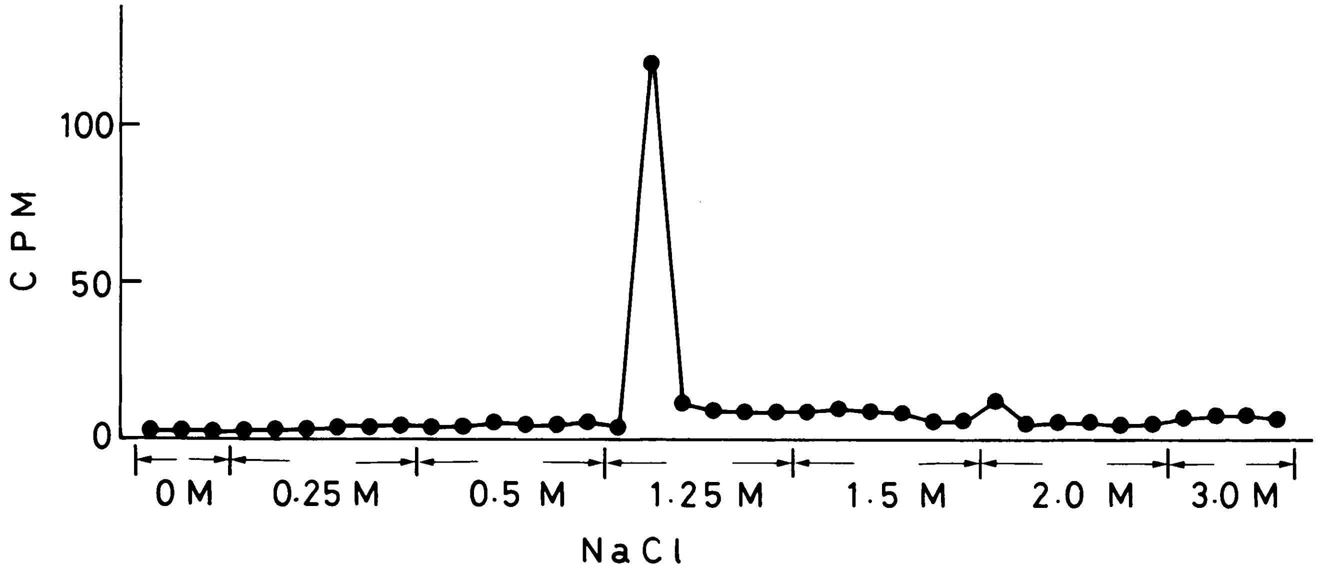

FIG. 6. The column-chromatography of AMPS incorporating ^{35}S–Na_2SO_4. The AMPS was extracted from the embryos at late stage 20, and was fractioned by NaCl. The radioactivity of each fraction was counted. The ordinate shows CPM/embryo. The abscissa shows each fraction of NaCl. The radioactivity was mainly found in the AMPS eluted by 1.25 M NaCl, that is, the fraction of heparan sulfate.

In addition, it was found in the perivitelline fluid at stage 21 as well as the period from stage 18 to 20.

As sulfated AMPS has an active polyanionic nature in controlling electrolytes and water in extracellular fluid and has a role in lubrication [21], the sulfated AMPS in the perivitelline space may protect the horseshoe crab embryos from the high salinity of sea water and the mechanical force. Embryos of many other marine animals have a great deal of sulfated AMPS in the perivitelline space, too. The cartilage of marine animals contains a great deal of sulfated AMPS as compared with animals living on land or in fresh water [22–26]. These facts support the above-mentioned supposition.

Non-sulfated AMPS was found in the tissues of the embryonic body at the stages before 20. It was uniformly distributed in the embryonic body and was thought to be mainly hyaluronic acid, judging from histochemical and biochemical examinations (Table 1 and Figs. 4 and 7). When AMPS was extracted from embryos at stage 19 or 20 and examined by electrophoresis, only one band was observed. The band was identified with that of hyaluronic acid and was digested by hyaluronidase (Fig. 7). Histochemical observations also showed that the AMPS of embryonic bodies at stages before 20 was mostly non-sulfated AMPS (Table 1). The non-sulfated AMPS decreased greatly at stage 21 (Figs. 3 and 4).

According to Meyer [27], non-sulfated AMPS controls water and protects animals against bacterial attack. The non-sulfated AMPS in horseshoe crab embryos may have these roles and may also play a role in cell construction during morphogenesis and in Vertebrata, as well [28].

The tissues of the embryonic body also contained neutral polysaccharide. As it was digested by amylase, it was probably glycogen. In the yolk, there was no AMPS, just neutral mucopolysaccharide. In the chorion and inner egg membrane, AMPS and neutral polysaccharide

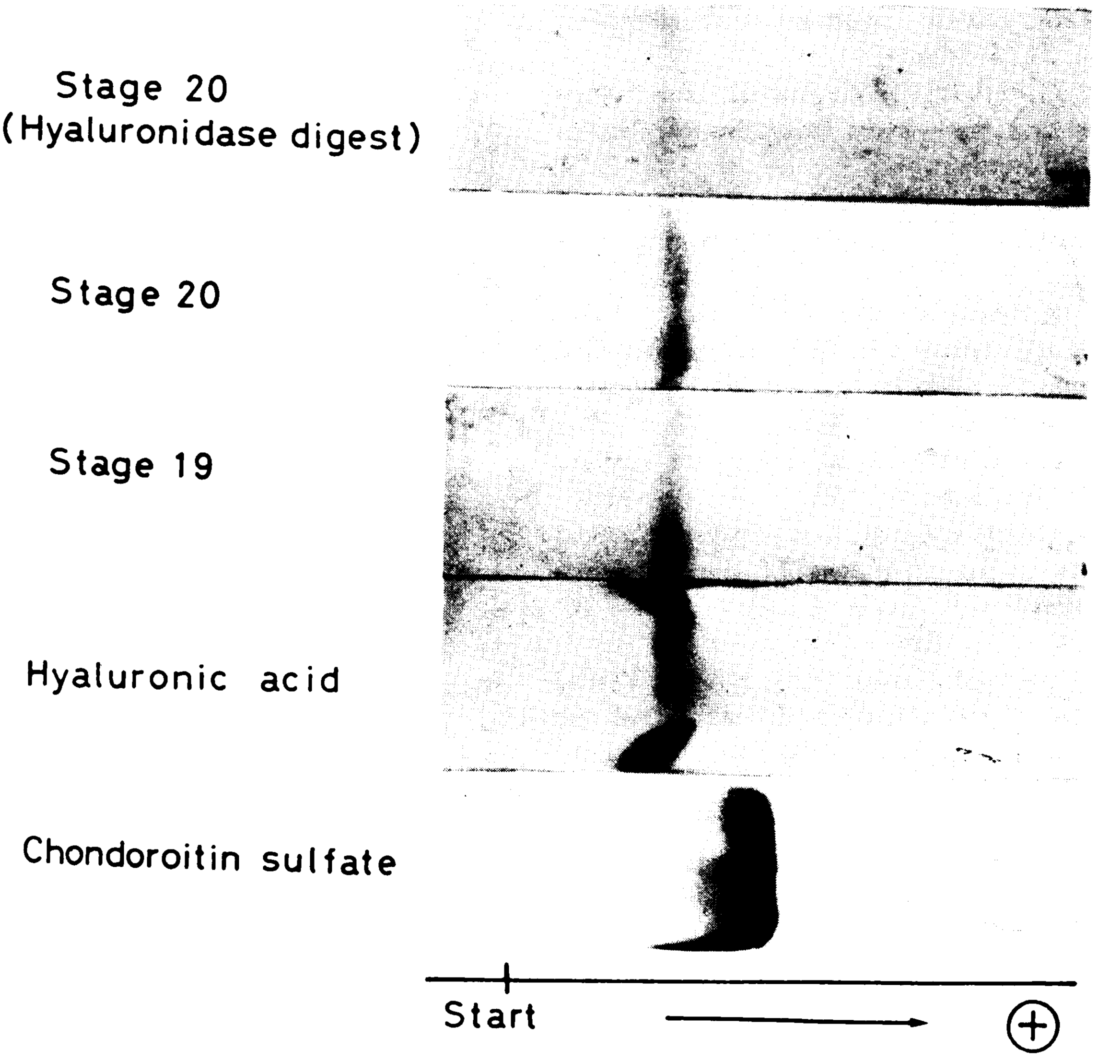

FIG. 7. The electrophoresis (cellulose acetate strips) of the extracted AMPS.