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An analysis of the role of sulfate in the embryonic differentiation of the sea urchin *(Paracentrotus lividus)*

by

John RUNNSTRÖM, Sven HÖRSTADIUS, Janis IMMERS and Molly FUDGE-MASTRANGELO [1]

The Wenner-Green Institute, University of Stockholm, and Zoological Institute, University of Uppsala

With 17 text figures.

Herbst (1904) first described the effect of lack of sulfate on the development of the sea urchin. He reared the larvae in artificial sea water devoid of sulfate ions. It turned out that sulfate belongs to the ions necessary for the sea urchin development, but the effect of its absence became evident only in late blastula and following stages. Herbst, who gave a careful description of the disturbances which occur, found that the larvae reared in sulfate-free sea water become radialized or animalized (according to Lindahl's (1933) terminology). In the former case, the proportions between ectoderm and endo-mesoderm are not or only slightly changed but the bilateral organization of the larva is suppressed. The normal predominance of the two centers of skeleton formation is lacking; a certain number of smaller triradiate pieces appear. No arms are formed and the acron region is hypertrophic to a varying extent. A pronounced animalization means that the endomesoderm is

[1] Present address: Dept. of Biology, Yale University, New Haven, Conn.

decreased or suppressed with a corresponding enlargement of the ectoderm in which the animal character is reinforced, particularly by an extension of the acron area. This area is carrier of the stereocilia and the degree of extension of the tuft of stereocilia has served as a semiquantitative measure of the degree of animalization (HÖRSTADIUS, 1935). Radialization represents a weaker degree of animalization. BÄCKSTRÖM (1953) found, for example, that eggs and larvae are sensitive to the animalizing action of 0-iodosobenzoate only during a certain limited period of the development. Radialization may result either because the agent was used in a concentration too low to produce the full effect or in periods of decreased susceptibility. Under the effect of sulfate free sea water similar transitions between different degrees of animalization and radialization were found.

LINDAHL (1936) made important contributions to the analysis of the role of sulfate in the sea urchin development, cf. further LINDAHL and STORDAHL (1937), SWEDMARK (1954) and LINDAHL's comprehensive survey of the developmental physiology of the sea urchin (1941). The main points established in the work referred to are the following: There is a decline in respiration when the effect of lack of sulfate becomes visible. This is due to the production of harmful substances in the vegetal region. These substances spread to and damage also the animal region. A separated animal fragment is more resistant to lack of sulfate than is the animal region of a non-fragmented egg, whereas vegetal halves differentiate less and are more strongly injured in sulfate free medium than are the animal halves. Eggs reared in sulfate free medium release more harmful substances than do eggs developing in normal sea water. Sulfate seems to serve as a detoxifying agent. SWEDMARK (1954) demonstrated that the incorporation of ^{35}S-labelled sulfate increases at the stage when the sea urchin embryo commences to be sensitive to lack of sulfate. These studies tended to demonstrate that specific differences in metabolism prevail between animal and vegetal regions of developing sea urchin eggs. The red pigment that appears in a number of mesenchyme cells is a typical product of vegetal metabolism; its dependence on the presence of sulfate was earlier demonstrated by HERBST. One of these writers, IMMERS (1961 a), has made a more elaborate study of the incorporation of ^{35}S-labelled sulfate and pointed out certain

correlations between rate of incorporation and morphogenetic events. Moreover a combined autoradiographic and histochemical study (IMMERS, 1961 b) has allowed certain inferences concerning localization and role of sulfated compounds. These have essentially the character of macromolecular sulfated polysaccharides which combine with proteins and probably also with lipids.

LINDAHL's work was an attempt at characterizing the different metabolic patterns prevailing in the animal and vegetal regions of developing sea urchin eggs and larvae. When continuing this work it seemed in first place urgent to make observations on a larger scale of the differences in morphogenesis in animal and vegetal halves reared in normal or sulfate free sea water. These studies should appropriately be combined with observations on incorporation of isotopically labelled precursors. First, ^{14}C-amino acids were tested. Their incorporation should give an indication of the rate of protein synthesis in developing eggs or fragments. The rate of incorporation would inform about the direct or indirect effect of sulfated compounds (mainly sulfated polysaccharides according to IMMERS (1962)) on the protein metabolism which is the basic event in differentiation. Although the morphogenetic studies were carried to a certain completion, the incorporation experiments are more incomplete. Work is in progress with the aim of extending the present study.

I. MATERIAL, METHODS and DESIGNATIONS

The gametes of *Paracentrotus lividus* served as material. The eggs were filtered through bolting silk and washed repeatedly in sea water. Dry sperm was collected from testes; it was diluted before use. Some minutes after insemination, the fertilization membrane was removed mechanically from the eggs that were to be separated into animal and vegetal halves. The separation was carried out with the glass needle technique (cf. HÖRSTADIUS, 1935, 1949, 1950) in the 16-32 cell stage after previous transfer to Ca-free sea water. After the operation the halves were transferred to artificial sea water, containing or lacking sulfate. The artificial sea water had the composition indicated by RUNNSTRÖM (1928) on basis of personal communication from BIALASCEWICZ. When sea

water devoid of sulfate was prepared, $MgSO_4$ was replaced by an equivalent amount $MgCl_2$. Hydrolyzed ^{14}C-labelled algal protein served as material for the study of the incorporation of amino acids. Each test contained 0.5 μC ^{14}C per ml. The exposure lasted for 30 minutes at 19°. The eggs were thereafter fixed in a modified Kahle solution (cf. IMMERS 1961 b); 5-10 minutes later the eggs were transferred to Carnoy's fixation liquid, thereafter in alcohol 95 per cent and finally in absolute alcohol. The embryos were thereafter put on round coverslips and dried (cf. MARKMAN, 1961). Furthermore they were extracted with water (for one hour) and subsequently with 5 per cent trichloracetic acid (70-80° C) for 45 minutes. The number of embryos on the coverslip (varying between 20-40) was determined and the number of counts assayed in a gas flow counter. The number of counts was referred to 100 larvae.

The operated embryos or larvae were outlined with the help of a camera lucida. A number of operated larvae were also studied in phase contrast. The treatment of an embryo with sulfate-containing or sulfate-free sea water will often be indicated below by putting ($+SO_4^=$) or ($-SO_4^=$) behind the designation of stage etc. The " animal halves " and " vegetal halves " (sometimes abbreviated An and Veg) were considered to represent half the volume of the original embryo. P is sometimes used as abbreviation of " polysaccharide ", PS of " polysaccharide sulfate " and Pr^1 of such proteins that combine with polysaccharide sulfate.

It is proposed to regard all stages, with inclusion of the prism stage, as embryonic, whereas the larval stages begin with the appearance of arms (1. larval or pluteus stage).

In the description of abnormal larvae, particularly vegetal halves in sulfate free sea water, certain difficulties arose. A region of the ectoderm that consists of continuous columnar epithelium will be called acron if it has an animal position and oral if it extends also in more vegetal direction. The remainder of ectoderm that consists of a pavement epithelium of characteristic form will be called " aboral ectoderm ". The designations " oral " and " aboral " may not seem appropriate in this context as the pertinent larva do not develop a mouth, but the regions are certainly homologous to the oral and aboral regions of normal larvae.

II. RESULTS

a) Development of whole eggs in SO_4-free sea water

This section presents some characteristic features in the effect of sulfate free sea water.

Fig. 1 represents a control larva in normal sea water, 26 hours after fertilization. It is in the prism stage with clearly developed bilateral symmetry. Figs. 2-4, on the other hand, represent larvae of about the same age that had been transferred from normal sea water to artificial sulfate-free sea water without washing in this latter. This means that the medium still contained low concentrations of sulfate ions. These larvae are rather radially symmetrical. This is manifested, for example, by the lack of the two symmetrical centers of skeleton formation. This agrees so well with Herbst's description that details are not necessary. However, attention was paid to the fact that a considerable extension of the ciliary tuft may occur, although the archenteron seems to be of normal or almost normal size. The following experiment was carried out in order to assure that the endomesoderm had its normal delimitation. In the 64-cell stage, the 8 veg_2 cells+micromeres (cf. Hörstadius, 1935) were separated from the rest of the egg, stained in Nile blue sulfate and then brought back to their normal place. Seven eggs so treated were reared to gastrulae and the boundary between ecto- and endoderm was found to be at the normal level as shown in Fig. 7a and b. Fig. 7b shows the extension of the ciliary tuft beyond the area it normally occupies.

When the eggs were washed in sulfate free sea water before the final transfer to sulfate-free sea water the animalization of the larvae was much more pronounced than in the cases of Figs. 2-4 and 7. The acron region was enlarged so that the ciliary tuft often covered about half the surface of the larva. The primary mesenchyme cells remained attached to each other, and this group of cells detached themselves rather slowly from their site of immigration. A narrow invagination was confined to the central part of the vegetal region. It represented a rudimentary archenteron, on the top of which the group of primary mesenchyme cells was located. The general form of the larvae is shown in Figs. 5 and 6. In the

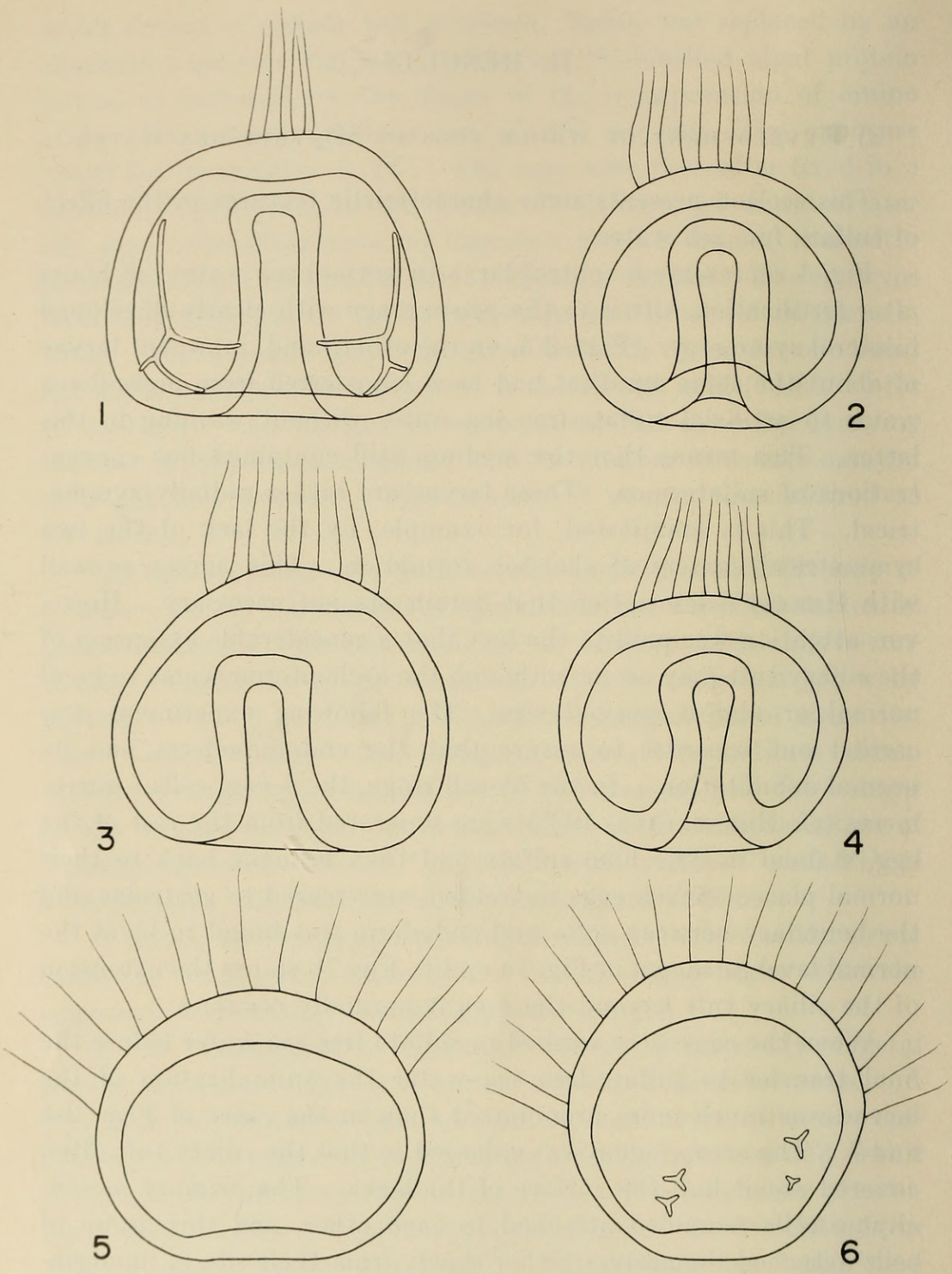

Fig. 1. — Bilateral late gastrula or prism stage from a culture reared in normal sea water. Fig. 2-4. — Gastrulae from a test culture transferred soon after fertilization into sulfate free sea water. The last traces of sulfate had not been removed by washing the eggs. Fig. 5-6. — Animalized embryos from a culture transferred soon after fertilization into sulfate free sea water followed by a washing in this medium. Fig. 1-6 320 ×.

latter some triradiate skeleton rudiments were distinguished. These arise when the primary mesenchyme cells eventually dissociate and spread from their original central position.

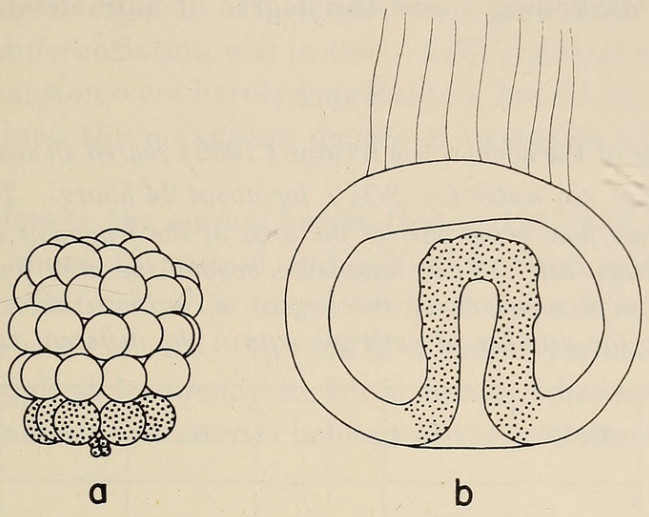

Fig. 7.

a, Staining of the veg 2 cell ring of an embryo treated with sulfate free sea water in the same way as the larve of Fig. 2-4; *b*, The whole endoderm stained in the late gastrula stage proving that no part of the presumptive endoderm has been converted into ectoderm. 320 ×.

b) Development of animal and vegetal halves in sulfate-free sea water

The first experiments were carried out in the spring of 1961, another series resulted from the work in the spring season of 1962. The two series were carried out by two different workers (M. Mastrangelo 1961 and S. Hörstadius 1962). Different judgement seems to have influenced the results only to a slight extent. It was obvious, however, that in the two seasons the material behaved in slightly different ways. In 1961, the animalization tested by the extension of the ciliary tuft was in general strong even in the animal fragments reared in normal sea water. This gave no good opportunity for studying an increase in animalization which was expected to occur as a consequence of rearing the fragments in sulfate-free sea water. Nevertheless it was evident that, even in the experiments of 1961, a certain increase in animalization occurred in the animal halves reared in sulfate-free sea water. These latter

appeared darker in phase contrast than the animal halves reared in normal sea water. As earlier demonstrated (cf. RUNNSTRÖM (1957) and (1961 a)), the ectodermic cells become darker when the larva becomes animalized and there is a certain correlation between this " darkening " and the degree of animalization.

TABLE 1.

Animal halves of Paracentrotus lividus *(1962) reared in normal ($+SO_4^=$) and sulfate free sea water ($-SO_4^=$) for about 24 hours. The figures 3/4, 4/4 etc. indicate how great part of the area of the fragment was covered by acron and ciliary tuft. Their extension beyond the area found in normal whole larves is a measure of the degree of animalization. The figures indicate the number of embryos within the different categories.*

	$^3/_4$-$^4/_4$	$^1/_2$	$^1/_3$	$^1/_4$	$^1/_5$-$^1/_6$	Total number of fragments
$+ SO_4^=$	10	46	38	54	33	181
$- SO_4^=$	109	45	23	1	0	178

TABLE 2.

Animal halves of Paracentrotus lividus *(1962) reared for about 45 hours in normal ($+ SO_4^=$) and sulfate free ($- SO_4^=$) sea water. The ciliary tuft (cf. Table 1) was replaced by motile cilia. A: the acron region occupied the whole area, Ba: more than half the area, Bb: less than half the area of the animal fragment. C: a ciliary band surrounded an oral field, D: as C but a stomodeum rudiment had appeared within the oral field. The figures indicate the number of embryos within the different categories.*

	A	Ba	Bb	C	D	Total number of fragments
$+ SO_4^=$	36	45	90	32	3	206
$- SO_4^=$	153	25	12	0	0	190

The tendency to animalization was less strong in the following spring season (1962). The degree of animalization was estimated

by the degree of extension of the ciliary tuft (cf. Hörstadius, 1935). It follows from Table 1, $+SO_4^{=}$, that about half the material has a comparatively weak extension of the ciliary tuft such as 1/6-1/4. In only 5 per cent of the material did the expansion attain the degree 3/4-4/4. It follows from the same table how different the differentiation was in the $-SO_4^{=}$-series where the lower degrees of extension were hardly represented, but 61 per cent of the embryos attained the maximum degree of extension of the ciliary tuft.

Table 2 refers to the animal halves that in 1962 had been reared for about 42 hours, in normal and sulfate-free sea water. Again a considerable difference in distribution of the material between the different classes occurs. In $+SO_4^{=}$ a more symmetrical distribution with highest frequency in Bb is found, whereas in $-SO_4^{=}$ the greatest part of the material belongs to the extreme category A.

Table 3.

Vegetal halves of Paracentrotus lividus *reared in normal (+ $SO_4^{=}$) and sulfate free sea water (— $SO_4^{=}$) for about 45 hours. The figures indicate per cent of the different categories of larvae.*

Material 1961	a Exogastrulae	b Ovoid larvae		c Tendency to pluteus organization	Total number of fragments
		I	II		
$+SO_4^{=}$	3	35	35 [1]	27%	66
		70			
$-SO_4^{=}$	36	60	4	—	70
		64			
Material 1962	I II [2]				
$+SO_4^{=}$	11 9		60	20	170
	20				
$-SO_4^{=}$	50 19		36	7	112
	69				

[1] I strictly ovoid, II transition forms of different degrees to category C.
[2] I full exogastrulation, II partial gastrulation.

Table 3 gives a survey of the distribution of ca. 45 hours old vegetal halves among three classes. Both the material of 1961 and that of 1962 were included in the table. The data demonstrate that the development of larvae with tendency to pluteus organization was kept back in $-SO_4^{=}$. Instead the number of exogastrulae is considerably greater in $-SO_4^{=}$ than in $+SO_4^{=}$. In the 1962 material the exogastrulae were divided into two subclasses: I, where the exogastrulation was complete, and II, where the exogastrulation was partial. The subclass I dominated in $-SO_4^{=}$, whereas in $+SO_4^{=}$ the material was rather equally distributed between the two subclasses. In 1961, on the other hand, the ovoid larvae were subdivided into two subclasses: I, with less differentiation, and II which is intermediary between ovoid larvae and those with tendency to pluteus organization. The subclasses I and II were equal in $+SO_4^{=}$ whereas the subclass I with less differentiation predominated in $-SO_4^{=}$. The higher organization in subclass II was manifested by the subdivision of the intestine, by a stronger development of the skeleton and by a tendency to formation of ciliary band.

The tendency to vegetalization was greater in the material of 1962 than in that of 1961. Even in $+SO_4^{=}$ there was a fair number of exogastrulae in the first mentioned material. This was correlated with the lower tendency for animalization that was mentioned above, cf. further HÖRSTADIUS (1935).

χ^2-tests showed that the differences between the distributions of the different classes of larvae was highly significant in Tables 1, 2 and 3. The data presented in the Tables of this section thus show definitely that rearing of the fragment embryos in SO_4-free sea water causes an additional animalization of the animal halves and a vegetalization of the vegetal halves.

c) SOME DETAILS CONCERNING MORPHOGENESIS IN ANIMAL AND VEGETAL HALVES

Tables 1 and 2 give sufficient information about the variation of the animal halves ($+SO_4^{=}$) and ($-SO_4^{=}$). The former were swimming more actively than the latter. This is in correlation to the greater extension of the tuft of non motile stereocilia in the animal halves ($-SO_4^{=}$). Often the elimination of the stereocilia

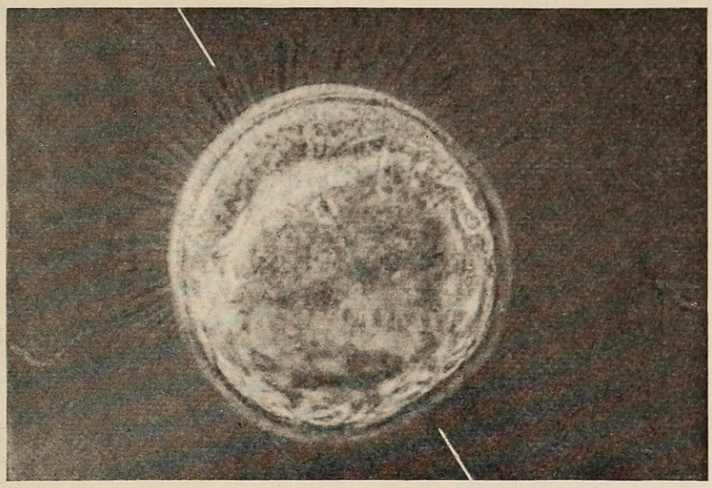

FIG. 8.

Animal half separated in the 16-cell stage and then reared for ca. 42 hours in normal sea water. Ciliary tuft replaced by motile cilia. 330 ×.

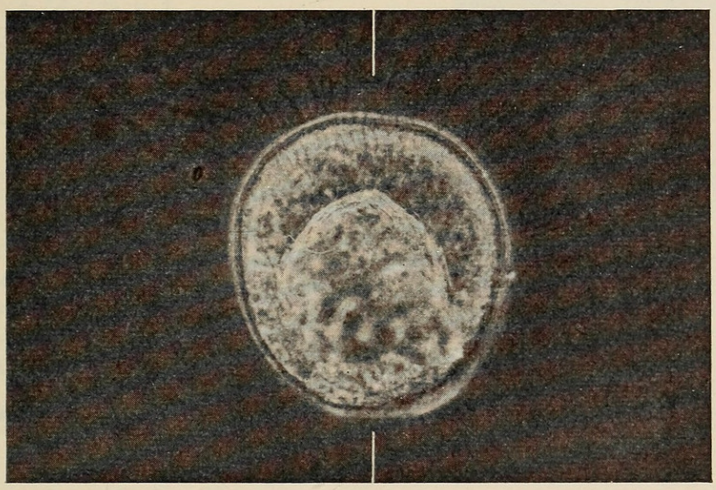

FIG. 9.

Animal half from the same egg material as that of Fig. 7. Separated in the 16-call stage and thereafter reared for ca. 42 hours in sulfate free sea water. Ciliary tuft still present. 330 ×.

was delayed in the animal halves ($-SO_4^{=}$). Fig. 8 represents an animal half ($+SO_4^{=}$) about 45 hours after insemination in which the cilia of the acron cells were short and motile, whereas in the animal half ($-SO_4^{=}$) of Fig. 9 the stereocilia were still present, and the acron covered a greater part of the area of the embryo than in the animal half ($+SO_4^{=}$). In the acron of the latter (Fig. 9) dark

patches were seen. These seemed to represent nuclei with surrounding dense material, as was born out by electron microscopic observations.

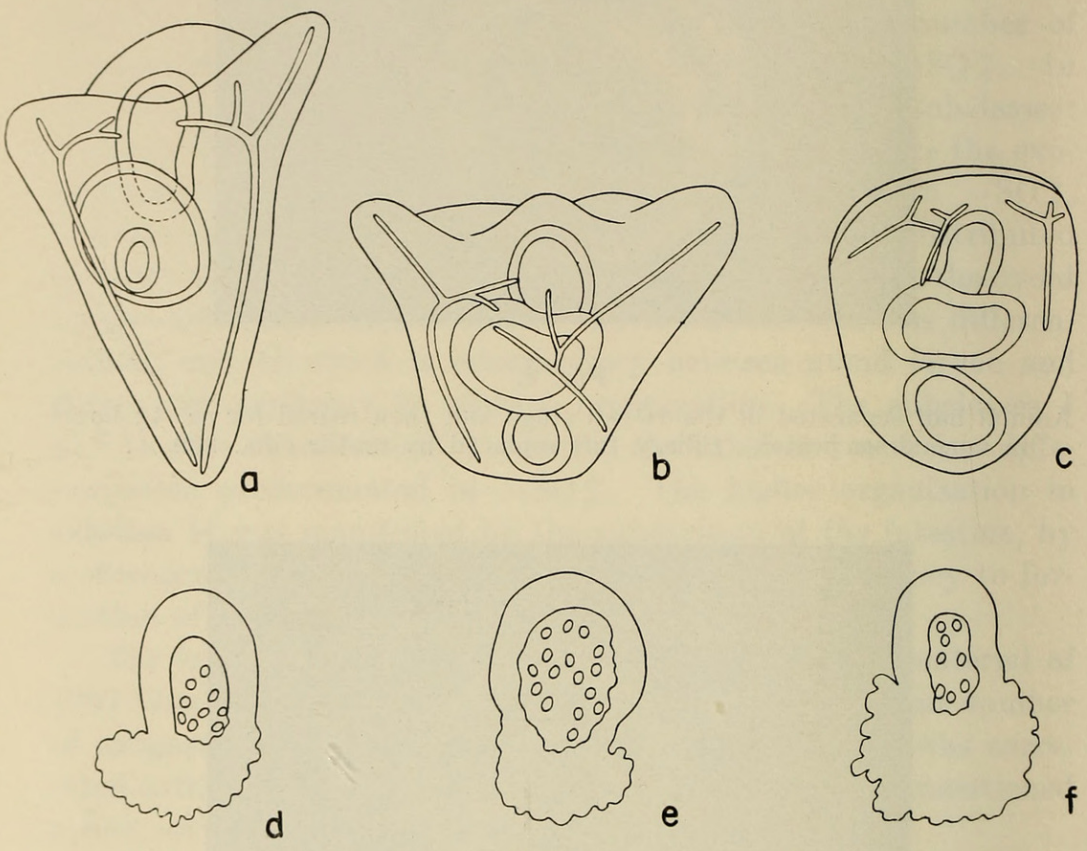

Fig. 10.

a, *b*, *c* represent animal halves reared in sulfate containing sea water; *d*, *e*, *f* represent some vegetal halves reared in sulfate free sea water. 320 ×.

The vegetal halves ($+SO_4^=$) and ($-SO_4^=$), see Fig. 10 upper and lower row, had a greater range of variation than the corresponding animal halves. Larvae with tendency to pluteus organization and exogastrulae may be present within the same material. Fig. 10 upper row represents larvae ($+SO_4^=$) of the first mentioned kind. An oral field but no stomodeum had developed. The skeleton was almost typical (a) or typical on one side and very atypical on the other (b). A third larva (c) was ovoid with radially symmetrical arrangement of rudimentary skeleton pieces. The exogastrula ($+SO_4^=$) represented in Fig. 11 was from a batch of eggs also containing larvae of the type represented im the upper

row of Fig. 10. An aggregate of immobilized mesenchyme cells without recognizable boundaries was present within the everted intestine. Other cells had succeeded, however, in immigrating into the ectodermal region. These cells were separate from each other and several of them showed indications of pseudopodia.

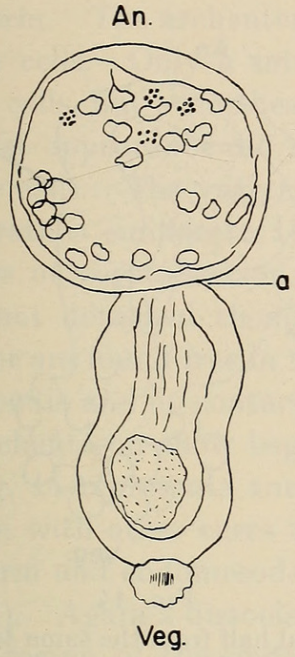

Fig. 11.

Vegetal half. Exogastrula with major part of the mesenchyme present beneath the ectodermic epithelium. An, Veg. animal and vegetal direction; *a*, ectoderm-endoderm boundary. 500 ×.

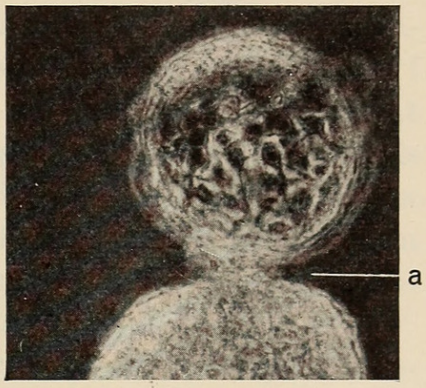

Fig. 12.

Larva from a test culture transferred 60 min. after fertilization to sea water containing 0.04 M Li^+ and 4×10^{-4} M glutathione, returned to sea water without washing after ca. 28 hours, fixed in 4 per cent formaldehyde 19 hours later; *a*, ectoderm-endoderm boundary. 500 ×.

The interaction between ectoderm and mesenchyme was studied in larvae which had undergone an exposure for 28 hours to sea water containing 0.04 M LiCl and 4×10^{-4}M glutathione. After this time the concentration of Li and glutathione was diluted to 1/5 by adding sea water to the culture. This treatment brought about a rather strong vegetalization of the larvae. These larvae offered good opportunities for studying the behavior of the mesenchyme cells. Fig. 12 shows the ectodermal and the adjacent endodermal region of one of these larvae in phase contrast. The whole endoderm is pale greyish. In the ectoderm there is a cap of more cylindrical cells which appear rather dark in phase contrast. Furthermore, mesenchyme cells with numerous pseudopodia show a strong phase contrast effect. These mesenchyme cells had migrated into the ectodermic region. Their site of origin was

within the endomesodermic region, where, as in the larvae of Fig. 10, aggregated pale mesenchyme cells without pseudopodia were visible. The immigration of the mesenchyme cells into the ectoderm was observed in a great number of larvae. Sometimes the neck between ectodermic and endomesodermic region was very narrow; the migrating mesenchyme cells were then elongated so as to assume a thread-like shape. This allowed the mesenchyme cells to pass through the neck.

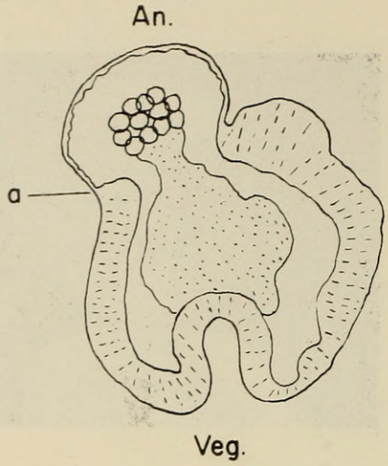

Fig. 13.

Vegetal half separated in the 16-cell stage and reared for ca. 42 hours in sulfate free sea water. An, Veg, animal vegetal direction; *a*, ectoderm endoderm boundary. 480 ×.

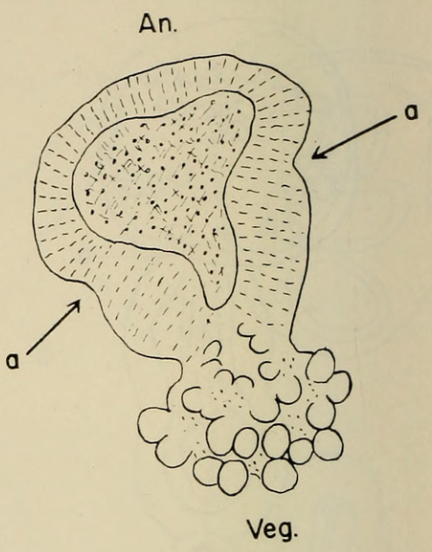

Fig. 14.

Vegetal half from the same test culture as the fragment represented in Fig. 13. An, Veg, animal and vegetal direction; a, ectoderm-endoderm boundary. 400 ×.

This digression on the behavior of the mesenchyme cells in Li treated larvae had the scope of demonstrating a process that plays a role also in the vegetal halves reared in normal sea water. When these have the character of exogastrulae, an immigration of mesenchyme cells into the ectodermal region occurs as described above (Fig. 11). The immigrated cells have a dark appearance in phase contrast. A certain opaqueness of the vegetal halves, however, makes its photographic demonstration more difficult than in the case of the Li larvae.

The behaviour of the mesenchyme cells constitutes a very important difference between the vegetal halves in sea water with or without sulfate. The careful examination of a number of vegetal halves showed that changes of form and migration of mesenchyme cells never or only rarely occurred in vegetal halves

($-SO_4^=$). The mesenchyme cells remained aggregated or, if some of them were dissociated, no formation of pseudopodia and no " darkening " in phase contrast occurred. In many cases, it was difficult to delimit ecto- and endomesoderm. This did not hold, however, for the vegetal half ($-SO_4^=$) represented in Fig. 13. In this case, the endomesoderm was extended at the expense of the ectoderm. The archenteron contained a large aggregate of mesenchyme cells. Only a small number were separated from the rest of the cells, but even these formed no pseudopodia and their phase contrast appearance did not differ from that of the rest of mesenchyme cells. The vegetal halves represented in Fig. 10 d, e and f were rather similar to that Fig. 13. They show some different degrees of vegetalization. A great part of the mesenchyme cells have not detached themselves from the archenteron. A certain number are found free in the blastocoel but they show no formation of filopodia and no contact with the ectoderm. The vegetal cells of the archenteron show beginning dissociation.

Fig. 14 represents another vegetal half ($-SO_4^=$) where a comparison with other cases makes it probable that the limit between ectoderm and endomesoderm corresponded to the shallow constriction (a). Again a dissociation of the cells had occurred in the more distal region of the endomesoderm. The mesenchyme formed a rather continuous pathological aggregate.

This type of vegetal halves ($-SO_4^=$) was rather common in certain egg batches. Sometimes the impression gained was that the development of the ectoderm had not lagged behind that of the endomesoderm but obviously there was no aboral pavement epithelium present in this type of larva.

Fig. 15 represents another rather different example of a vegetal half ($-SO_4^=$). It was elongated in the animal-vegetal direction. The boundary between ecto- and endomesoderm may be at a; the subsequent region a-b should then represent a hind gut with cylindrical epithelial cells. Also the delimitation of the midgut is conjectural; it may extend between b and c. The terminal (most vegetal) region corresponds to oesophagus and coelom rudiment. The main part of the mesenchyme was present as a great rather undifferentiated clump within the midgut. Only vaguely were some cell boundaries distinguishable within the clump. Some twelve cells had, however, detached themselves and

immigrated into the anterior region of the larva. Their surface was somewhat irregular but no real lobo- or filopodia were distinguished (by contrast, see Fig. 11 and 12). Some of them had developed into pigment cells. No trace of skeleton was visible, not even in the polarization microscope. The more distal (vegetal) region of the everted endomesoderm presented a number of dissociated cells. The dissociation decreased along a vegetal-animal gradient, as was also the case in the vegetal half represented in Fig. 14.

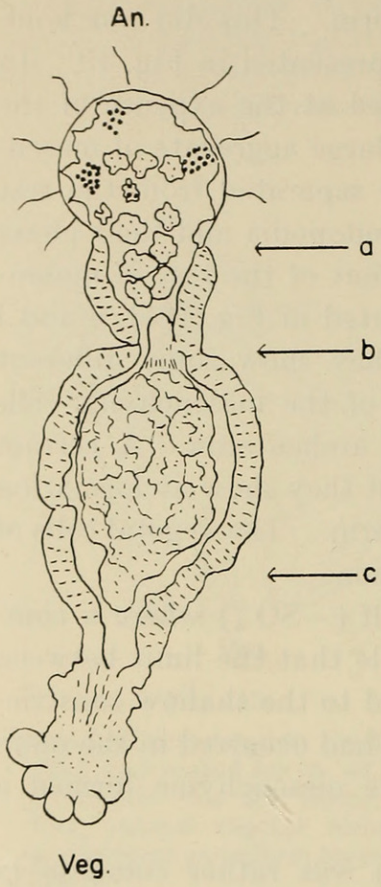

Fig. 15.

Vegetal half separated in the 16-cell stage and reared for ca. 42 hours in sulfate free sea water. An, Veg, animal and vegetal direction; *a*, *b* and *c* boundaries between ectoderm and hind gut, between hind- and midgut, and between midgut and oesophagus region. 680 ×.

Only rarely was a pronounced bilateral symmetry found in vegetal halves subjected to sulfate-free sea water. In these an oral (ventral) side was distinguishable by its cylindrical cells, whereas the aboral side was covered by a flat epithelium. On the oral side of the invaginated endomesoderm there was an aggregation of spherical mesenchyme cells, pale in phase contrast. In the opposite (aboral) direction the mesenchyme cells were fewer, darker in phase contrast and evidently capable of a certain movement. They had the characteristic feature of secondary mesenchyme. The same kind of bipolarity as that described was often observed in embryos reared from polyspermic eggs (unpublished observations). Observations of this kind indicate that the aboral pavement epithelium of the ectoderm has the main inductive effect on movements and differentiation of the secondary mesenchyme cells. This would also explain the degenerate state of the mesenchyme in embryos of the type represented in Fig. 13 and 14.

The type of vegetal half ($-SO_4^{=}$) represented in Fig. 16 was found in low frequency. In the animal direction a rather large

acron plate consisting of cylindrical cells was visible, whereas the aboral vegetal region was constituted of flat cells. In the interior a rather large endomesoderm was observed. It was subdivided into two compartments, oesophagus and midgut, which were thick-walled and bulging. They were very rich in cells. By striking contrast the hind gut was very small; it was built up of a rather small number of thin flat cells. This disproportion between the two anterior and the hind intestinal compartments may be conceived of as a vegetalization within the endomesodermal region with a tendency of suppressing the most animal compartment, the hindgut. The mesenchyme cells in the animal region were large and disordered. The fewer mesenchyme cells in the aboral region were at least one generation more advanced with respect to cell division. They showed also indications of form changes.

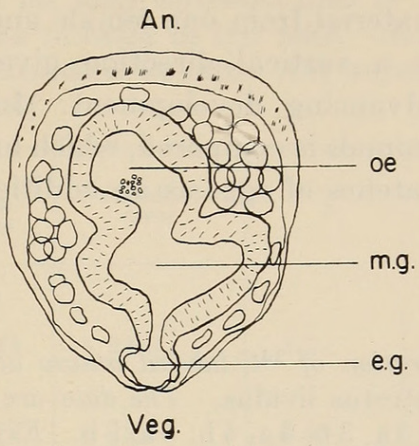

Fig. 16.
Vegetal half separated in the 16-cell stage and reared for ca. 42 hours in sulfate free sea water. An, Veg, animal and vegetal direction, oe oesophagus, m.g. midgut and eg. endgut. 450 ×.

In Table 3, the material of vegetal halves was roughly subdivided into three to four different groups. As follows from this section the subdivision could have been carried further. The purpose of this section was, however, to give some examples of the different types observed and to convey the impression of the great variability of the development of the vegetal halves, particularly when they were reared in sulfate-free sea water.

d) Incorporation of labelled amino acids into whole embryos, and into animal and vegetal fragments reared in normal and sulfate-free sea water

As described in section I embryos ($+SO_4^{=}$) and ($-SO_4^{=}$) of various stages were exposed for 30 minute periods to labelled amino acids (hydrolysate of algal proteins).

Table 4 brings together a number of single experiments with whole embryos and with animal and vegetal halves. Each one of these categories of embryos was reared in normal ($+SO_4^=$) and in sulfate-free ($-SO_4^=$) sea water. Each horizontal line refers to material from one female and one male. Reading of the columns in a vertical direction gives the change of incorporation with advancing development. In this way, material from different animals is compared, which brings in the variation between different batches of eggs as an additional source of error.

TABLE 4.

Incorporation of ^{14}C-labeled amino acids (algal protein hydrolysate) into embryos of Paracentrotus lividus. *The data are based on different experiments labeled* 1 a, 1 b, 2 a, 2 b, 3 a, 3 b, 4 a, 4 b, 5 a, 5 b. *Experiments with the same number were in the same stage:* 1, *early mesenchyme blastula*, 2, *middle mesenchyme blastula*, 3, *late mesenchyme blastula*, 4, *gastrula ~ 3/4 invagination*, 5, *late gastrulae. The experiments* a *were carried out in 1962, the experiments* b *in 1963. The figures represent counts per minute per 100 embryos.*

Exp.	Whole embryos		Animal halves		Vegetal halves	
	$+SO_4^=$	$-SO_4^=$	$+SO_4^=$	$-SO_4^=$	$+SO_4^=$	$-SO_4^=$
1 a	670	485	310	350	225	230
1 b	678	515	465	368	320	305
2 a	795	780	580	490	415	400
2 b	840	590	690	345	480	410
3 a	930	630	490	445	385	380
3 b	1140	1050	675	630	420	450
4 a	935	645	445	350	250	260
4 b	940	708	830	435	358	335
Mean	*866*	*682*	*522*	*427*	*357*	*346*
Standard error	*± 58.8*	*± 64.7*	*± 44.3*	*± 34.7*	*± 32.6*	*± 34.6*
Fiducial limits, 5 per cent level	*727-1005*	*529-835*	*418-627*	*344-510*	*280-433*	*264-428*
5 a	1175	680	570	520	480	390
5 b	1205	708	830	600	460	367
Mean	*1190*	*694*	*700*	*560*	*470*	*379*
Standard error	*± 15*	*± 14*	*± 130*	*± 40*	*± 10*	*± 12*
Fiducial limits, 5 per cent level	*1154-1226*	*661-727*	*392-1008*	*520-600*	*460-480*	*367-391*

TABLE 5.

Means of paired differences between values contained in Table 4. The different combinations are given in column 1. The pairing was always made between values from one and the same material i.e. values found on the same horisontal lines in Table 4, Exp. 1 a-4 b. The Exp. 5 a and b have not been included in this table. Soma data about differences within Exp. 5 a and b are discussed in text. An, animal half; Veg, vegetal half.

Difference in C.P.M./100 embryos	Mean of difference	Standard error	t	Significance
1) between whole embryos (+ $SO_4^=$) and (— $SO_4^=$)	184	± 34.5	5.3	× ×
2) " 2 × An (+ $SO_4^=$) and whole embryos (+ $SO_4^=$)	177	± 73.0	2.42	×
3) " 2 × An (+ $SO_4^=$) and whole embryos (— $SO_4^=$)	361	± 68.5	5.3	× ×
4) " 2 × An (— $SO_4^=$) and whole embryos (+ $SO_4^=$)	— 102	± 59.7	1.7	0
5) " 2 × An (— $SO_4^=$) and whole embryos (— $SO_4^=$)	176	± 28.4	6.2	× ×
6) " 2 × Veg (+ $SO_4^=$) and whole embryos (+ $SO_4^=$)	— 153	± 20.5	7.5	× ×
7) " 2 × Veg (— $SO_4^=$) and whole embryos (— $SO_4^=$)		± 41.1	0.31	0
8) " 2 × An (+ $SO_4^=$) and 2 × An (— $SO_4^=$)	190	± 78.1 (35)	2.43 (3) [1]	×
9) " Veg (+ $SO_4^=$) and Veg (— $SO_4^=$		10.4	1	0

[1] The figures within parenthesis refer to the non doubled values of An (+ $SO_4^=$) and An (— $SO_4^=$).

In order to facilitate the statistical treatment of the material Exp. 1 a-4 b (Table 4) have been regarded as being one population. This is not strictly correct. MARKMAN's (1961) curve shows that from the youngest stage included in the table (Exp. 1 a and b), the early mesenchyme blastula stage, to the gastrula stage with about $^2/_3$ invagination (Exp. 4 a and b) there is an increase in incorporation of labelled amino acid into eggs in sea water. This

follows also from an inspection of the second column of Table 4. Differences in mean values may thus partly be due to differences in rate of increase of the incorporation instead of differences between values belonging to the same level. The outcome of the statistical treatment justifies the procedure adopted. The values referring to embryos with achieved gastrulation (Exp. 5 a and b) have, however, not been included in the pooled material. It is included separately in the lower part of Table 4. The results presented in Table 4 has to be regarded as preliminary; the writers have, however, some supporting material at their disposal and, at the first opportunity, continued measurements will be carried out.

In Table 5, the mean of differences paired in nine combinations have been recorded. The first mean refers, for example, to the differences between whole embryos reared in normal and in sulfate-free sea water (column 2 and 3 of Table 4). The members of one pair belong throughout to the same material. This means that only values recorded on the same horizontal line in Table 4 have been paired.

The *t* value is a test of null hypothesis (see SNEDECOR, 1946, p. 66). The deviation from the value zero of the mean of differences was highly significant (++), significant (+) or not significant (0). The incorporation values for the half embryos have been doubled in order to make them directly comparable to the whole larvae. This was not done, however, in the difference between vegetal halves ($+SO_4^=$) and ($-SO_4^=$) where even the difference between the non doubled values proved to be non-significant.

For the embryos that had accomplished gastrulation only two pairs of values were available for each category of embryos (Exp. 5 a and 5 b). Thus they do not lend themselves to statistical treatment. However, it is obvious that the fiducial limits do not overlap in whole embryos $+SO_4^=$ and whole embryos $-SO_4^=$ (Table 4, column 2, and 3). This indicates a high significance of the paired differences. The mean of this is considerably higher than in the pooled material of Exp. 1 a-4 b. This does not mean a sudden increase of mean difference in the last stage of the incorporation experiment (Exp. 5 a and b). It depends partly on the non-uniform character of the pooled material of the earlier stages.

A significant difference seemed also to prevail between vegetal halves in Exp. 5 a and b. This could mean that, in isolated

vegetal halves, the amino acid incorporation gradually becomes dependent on $SO_4^=$. This does not seem incompatible with the morphogenetic events. This point has to be more carefully tested in future work.

As above the combination 2 x An ($-SO_4^=$) and whole embryo ($+SO_4^=$) presented no significant difference. The range of fiducial limits for An ($+SO_4^=$) is very great in Exp. 5 a and b; the values are therefore useless for testing differences.

III. DISCUSSION

The uptake of sulfate ions begins to be pronounced approximately 6 hours after fertilization. It increases then but not continuously. There is one peak soon after hatching, another in the second period of gastrulation (Immers, 1961 a). Histochemical work indicated that sulfate combines with polysaccharides. As a consequence, sulfated polysaccharides arise within the embryo and larva (Immers, 1961 b, 1962). These are strongly linked to proteins. Work in progress has shown that proteins must be removed by enzymic digestion in order to release the sulfated polysaccharides in homogenates of eggs.

The main amount of sulfate taken up by the embryo probably becomes linked to polysaccharides. Most convincing in this regard is the experience that the acid polysaccharides of the hyaline layer do not appear in embryos reared in sulfate free sea water (Immers, 1956). Other sulfated substances may be present in low concentrations, among those intermediate donors of sulfate to the polysaccharides.

It is unknown whether the polysaccharide molecule is built up prior to the formation of linkages with sulfate or whether this latter process is taking place by steps during the synthesis of the polysaccharide molecule. Even in the first case the non-sulfated polysaccharide will have physical-chemical properties widely different from those of polysaccharide sulfate. A macromolecule of the former type will not fit into the cytoplasmic organization; consequently, disturbances may arise. More work is needed to substantiate this view. In the following the conception will nevertheless be maintained that, in last instance, lack of sulfate

prevents the formation of certain polysaccharide sulfate protein compounds. A role of sulfate as a precursor to sulfur-containing amino acids can be excluded. Like the chick embryo (Lash, 1963), the sea urchin embryo seems to lack the enzymes necessary for sulfate reduction.

The most conspicuous effect of lack of sulfate on whole larvae is an animalization to variable extent. Evidently both immigration of mesenchyme cells and invagination of the archenteron are inhibited in the vegetal region. This is in keeping with the conclusions drawn by Immers (1961 b, 1962) concerning the role of polysaccharide sulfate (PS) in the formation of pseudo- or filopodia in mesenchyme cells and the particular importance of sulfate in the second period of gastrulation (Immers, 1961 a). According to the results on whole larvae the vegetal region is more sensitive to lack of sulfate than is the animal one. The animal region exerts a continuous induction pressure to which the vegetal region yields in absence of PS. The PS seem thus to exert a stabilizing action on the vegetal differentiation.

In the animal region itself a displacement of boundaries occurs; the most obvious one is the displacement of the boundary between acron region and the region with motile cilia. Also within this latter, different regions may be distinguished; the most characteristic is the region to which the primary mesenchyme cells attach themselves. They are here induced to form the two bilaterally arranged groups united by an oral and an apical chain of cells. The extension of the acron brings about a change also in the region of ectoderm with motile cilia. This is no longer able to induce the bilateral arrangement of the primary mesenchyme cells. These and the resulting skeleton pieces become radially arranged (see Fig. 6). It is remarkable that an extension of the acron is possible even in embryos with such well developed archenterons as those represented in Figs. 3-5. Hörstadius (1935) has shown that even the cell layer veg_1 (separated at the transition to 64 cell stages) is able to arrest the extension of the acron and ciliary tuft (l.c. p. 323-4). The whole embryos of Figs. 3-5 contain more vegetal material (also veg_2+micromeres). The disturbance of balance manifested in the acron extension may be due to an impairment of the vegetal " moderator " that normally limits the extension of the acron region.

The treatment of animal and vegetal halves with sulfate-free sea water had the effect of animalizing the former while vegetalizing the latter (Tables 1-3). In view of the results obtained with whole eggs an animalization of the animal halves should be expected. In the animal halves ($+SO_4^{=}$) there is sufficient synthetic activity along vegetal pathways that the extension of the ciliary tuft is blocked to some extent. If lack of sulfate causes a further inhibition of the vegetal pathways in the animal halves the animalization caused by the separation from the vegetal region will be enhanced (see RUNNSTRÖM, 1961 b).

The enhancement of vegetalization of the vegetal halves ($-SO_4^{=}$) is probably due to several convergent factors. The number of exogastrulae was considerably higher in these vegetal halves than in those reared in normal sea water. This may indicate a decrease of the volume of the ectoderm but also an impaired activity of mesenchyme cells. After an initial autonomous phase of gastrulation there is a second phase in which pseudopodia are emitted from the tip of the archenteron. These pseudopodia fixing themselves at the ectoderm contribute to the invagination during the second phase of gastrulation (GUSTAFSON and KINANDER, 1956). As shown above, lack of sulfate practically abolished the capacity of the mesenchyme cells for forming pseudopodia in vegetal halves (as also to a large extent in whole embryos and larvae). The contact with the ectoderm is thus not established or at least not to the same extent as in the vegetal halves ($+SO_4^{=}$). The migration of mesenchyme cells into the ectodermal region of these latter was described above; it was evident that the mesenchyme cells are not only attracted by the ectoderm but also differentiate under the inductive influence of the ectoderm, an effect probably mediated by a secretion from the ectoderm (perhaps mainly from the aboral ectoderm). The similar conditions in Li treated larvae were referred to above (section II c). RUNNSTRÖM (1933, 1957) described further how ectodermic patches may arise within the evaginated endoderm of Li larvae. These patches attract primary and secondary mesenchyme cells and induce skeleton formation by the former.

The intimate relations between ectoderm and mesenchyme are abolished by lack of sulfate; in particular this is pronounced in vegetal halves.

As described above, lack of sulfate may cause a dissociation of the endomesoderm cells, a change progressing from the most vegetal region in animal direction. MARKMAN (1963) brought vegetal halves (—$SO_4^=$) into 4 x 10^{-4}M uridine. The exposure to this began 4 hours after insemination. Addition of uridine prevented fully the cell dissociation and conferred a more healthy appearance on the cells. On the other hand, the uridine treatment did not change the proportion of exogastrulae among the vegetal halves (—$SO_4^=$). It was superior to the number of exogastrulae in vegetal halves (+$SO_4^=$) (MARKMAN, personal communication).

The number of exogastrulae was not only augmented in vegetal halves (—$SO_4^=$) but these tended also to be more vegetalized than in vegetal halves (+$SO_4^=$). HÖRSTADIUS (1935), LINDAHL (1936) and v. UBISCH (1950) emphasized that a tendency to pluteus organization could become prevalent in vegetal halves only after readjustments of the opposite gradient systems. In the language adopted by RUNNSTRÖM (1961 b), the animal synthetic pathways have to be reinforced, whereas the vegetal synthetic pathways have to be moderated. As evident from Table 3 the tendency to pluteus organization was almost completely suppressed in vegetal halves (—$SO_4^=$). This means, in general terms, that the readjustments of the gradient systems is strongly inhibited in the vegetal halves (—$SO_4^=$). This may depend on several factors; one very important is transportation that, in vegetal halves (—$SO_4^=$), may be inhibited by the described behavior of the mesenchyme which, normally, is an important mediator of exchange processes within the embryo or larva. The cell movements are another factor to be considered. An accumulation and increased adhesion of the cells of the most animal region and a differentiation of the pavement epithelium of aboral region seem to be prerequisite for attainment of a fuller regulation in vegetal halves. When the action of these and probably other factors is reduced, as in the vegetal half (—$SO_4^=$), the original conditions are maintained. Consequently, the prevalence of the vegetal trend of differentiation will remain.

The data concerning the incorporation of amino acids (Table 4, Exp. 1a - 4b and 5) have been condensed in a simplified manner in Fig. 17 where the different kinds of embryos are arranged on three different levels. The values for the halves have, as in Table 5, been multiplied by two in order to make them directly comparable

with the whole embryos. The highest level of incorporation, ~ 1000 c.p.m./100 embryos, was found in the animal halves ($+SO_4^=$); the next level, ~ 850 c.p.m./100 embryos, was found in whole embryos ($+SO_4^=$) and 2 x An ($-SO_4^=$); the lowest level, ~ 700 c.p.m., is represented by Veg ($+SO_4^=$), whole embryos ($-SO_4^=$) and Veg ($-SO_4^=$). It may be allowed to consider the

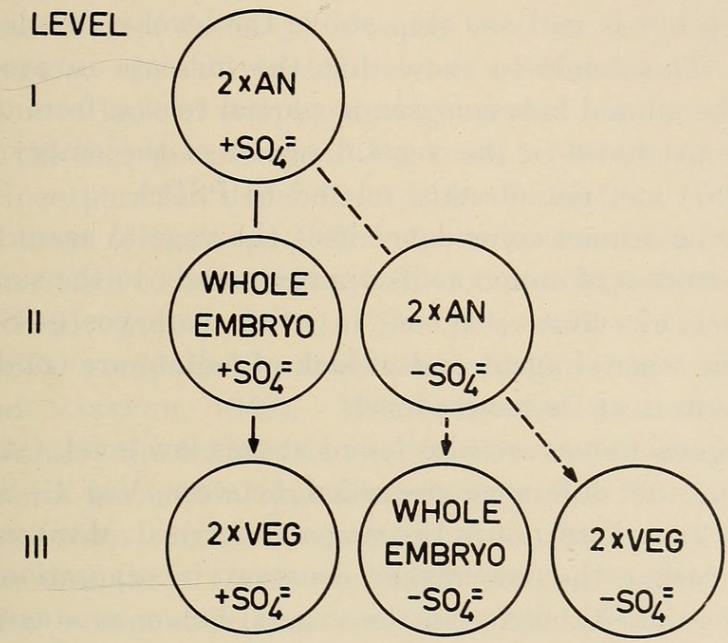

Fig. 17.

A diagram representing the different levels of incorporation of amino acids in different categories of embryos. Level I corresponds to ca. 1000, level II to ca. 850 and level III to ca. 700 c.p.m./100 embryos. Based on the data of Table 4 and 5.

incorporation data as a measure of the protein synthesis. It is then obvious that in animal halves and in whole embryos lack of sulfate brings about a decrease in protein synthesis, whereas there is no difference between the vegetal halves ($+SO_4^=$) and ($-SO_4^=$). As indicated previously in this section the sulfated polysaccharides are bound to proteins. An acceptable explanation of the difference, e.g. between whole embryo ($+SO_4^=$) and whole embryo ($-SO_4^=$), would be that the proteins (called here Pr^1) normally bound to the polysaccharide sulfate (PS) are not formed in the latter embryos. The situation would be the same as in chondrogenic cells where

according to CAMPO and DZIEWIATKOWSKY (1962) the PS moities of the polysaccharide sulfate protein compounds (PSPr), and the protein component, Pr^1, are synthesized simultaneously (cf. also DORFMAN and SCHILLER, 1962).

The increase in incorporation of amino acids in animal halves ($+SO_4^=$) above the level prevalent in whole embryos ($+SO_4^=$) was earlier found by MARKMAN (1961). The diagram, Fig. 17, shows that the level of incorporation in animal halves ($-SO_4^=$) is lowered by one step but is still one step above the level in whole embryos ($--SO_4^=$). This tends to show that the increase in protein synthesis in the animal halves is due to partial release from a moderating agent produced in the vegetal region of the embryo (RUNNSTRÖM, 1957) and not directly related to $PSPr^1$.

It may be a mere coincidence that the vegetal agent decreases the incorporation of amino acids approximately to the same extent as does lack of sulfate. When, in whole embryos ($--SO_4^=$), the effect of the vegetal agent and of lack of sulfate are combined the incorporation is at its lowest level.

The vegetal halves are also found at this low level. As already pointed out, no difference prevailed between Veg ($+SO_4^=$) and Veg ($-SO_4^=$), at least not in the stages concerned. MARKMAN (1961) described earlier the low rather constant incorporation of both ^{14}C-adenine and ^{14}C-leucine in the vegetal halves in stages ranging from early cleavage to gastrula. RUNNSTRÖM (1961 a) presented the view that from about the stage of late mesenchyme blastula or early gastrula stage the vegetal region is supplemented from the animal region with some agent that the endomesoderm and mesenchyme are unable to build up. The scheme, Fig. 17, suggests that the supplementing agent directly or indirectly has to do with the protein synthesis. One attractive possibility is that the supplementation may primarily concern agents necessary for the binding of sulfate to polysaccharide. In the absence of these agents no sulfated polysaccharides would be synthesized and consequently the proteins Pr^1 would fail to appear.

In vegetal halves ($+SO_4^=$) a certain amount of $PSPr^1$ may gradually be built up by a regulation leading to the differentiation of a functioning ectoderm. This regulation does not occur in the vegetal halves ($-SO_4^=$). The possibility of a regulation of the amino acid incorporation is to some extent supported by data of

Table 4, Exp. 5 a and b (cf. comments in section II d, last paragraph but one).

According to the suggestion made the lack of sulfate should specifically eliminate certain proteins Pr^1. The relative high incorporation of amino acids prevailing even at level III favours the view that the decrease in incorporation is a specific and not a general one.

The vegetal halves ($+SO_4^{=}$) cannot incorporate more than do the vegetal halves ($—SO_4^{=}$) because of their isolation from the animal region. In the vegetal halves ($+SO_4^{=}$) the low incorporation is due to lack of supplementing agents. In the whole larvae ($—SO_4^{=}$) the incorporation is at the same level but in this case the reduced incorporation depends on lack of sulfate.

Substances of the type $PSPr^1$, heparin, chondroitin sulfate etc. have been shown to be inhibitors of blood clotting (cf. for references Jorpes, 1946; Gibian, 1954). The jelly coat of the sea urchin egg (a $PSPr^1$ compound) was shown to inhibit blood clotting (Immers and Vasseur, 1949). This substance was also shown to delay or inhibit the fertilization or the elevation of the fertilization membrane in sea urchin eggs (Runnström and Wicklund, 1950; Harding, 1951). In blood clotting, these substances inhibit the activation and the activity of the proteolytic enzyme thrombin. The $PSPr^1$ compounds also act, however, on other proteolytic enzymes like cathepsin, trypsin and pepsin. Lundblad (1954) showed that heparin inactivates the proteolytic enzyme E II in sea urchin eggs. Lundblad and Runnström (1962) considered that E II is the enzyme causing cytoplasmic gelation. A number of non proteolytic enzymes are also inhibited, among those ribonuclease (cf. Gibian, 1959, Table p. 83).

An important consequence of absence of $PSPr^1$ may thus be an increased release or activation of hydrolytic, particularly proteolytic enzymes. The morphogenetic effect of lack of sulfate resembles in several respects that obtained by pretreatment of the eggs with low trypsin doses (Runnström, 1961 c and 1962). This pretreatment brings about the activation of a gelating enzyme. Lack of sulfate may likewise cause activation or release of enzymes that cause slight changes in the structure of proteins that are products of vegetal synthetic pathways (Runnström, 1961 b). Owing to these changes they lose some property necessary for their inter-

action in the embryo. In this way the vegetal region becomes less protected, certain of its molecular groups losing their capacity of producing the moderator of animal pathways. Results reported in section IIa prove definitely that this must be the first change that occurs as a consequence of lack of sulfate. In the larvae represented in Figs. 2, 3, 4 and 7, the endomesoderm has its normal extent but, nevertheless, an extension of the acron beyond its normal area (cf. Fig. 1) has occurred. Moreover this extension has disturbed the balance within the ectoderm as evidenced by the lacking capacity of the posterior region of the ectoderm to induce a bilaterally symmetric arrangement of the primary mesenchyme cells. Also groups of " vegetal " components that are present in the animal half are attacked by the activated and released enzymes. The effect of this is visualized, for example, by the increased degree of animalization in the animal halves (—SO $\frac{=}{4}$).

This gives evidence of a basic assumption involved in the double gradient concept. Products of vegetal pathways—primarily proteins or products of their activity or transformation—are present also in the animal half of the embryo in concentrations decreasing in animal direction. The conditions of stability of these vegetal products seem to be the same as when they are present in the vegetal region of the embryo. This points to the conclusion that at a certain level a mixture of products of animal (an) and vegetal pathways (veg) are present side by side. In early development the an/veg quotient may be decisive for the differentiation at the different levels in the embryo, as was postulated by RUNNSTRÖM (1957 and 1961 a). From a certain stage—probably corresponding to the mesenchyme blastula stage—the situation becomes more complicated by secondary interactions. One of these is visualized by the necessity for a factor from the animal region to facilitate the growth and differentiation of the vegetal region.

The protein molecules which are products of the animal synthetic pathways must be more resistent to hydrolytic enzymes than are the protein molecules built up through vegetal pathways. This striking difference in resistance is found also after pretreatment of the eggs with low doses of trypsin (RUNNSTRÖM, 1961 c, 1962).

The increased tendency to hydrolytic processes in the embryos reared without sulfate may not essentially influence the amino

acid incorporation. On the other hand they may, e.g. by causing gelation, inhibit the morphogenetic movements, diffusion processes, etc., as discussed above.

An unpublished experiment (carried out with kind assistance of Dr. T. Hultin) showed that the gelation caused by pretreatment of the eggs with low trypsin doses did not inhibit the strong rise in incorporation of amino acids that occurs on fertilization (cf. Hultin, 1961). It is admitted that a prolonged lack of sulfate will cause damage that may have a more unspecific effect on protein synthesis. A release of nucleases occurs also as a consequence of treatment with sulfate-free sea water but this phenomenon will be described and discussed elsewhere.

According to Lindahl (1936) whole embryos reared in sulfate free sea water showed no change in respiration until the late blastula stage (about 12 hours after fertilization). In the next four hours these embryos showed a respiration that was at the most 10-15 per cent lower than in the control embryos. It is essentially a slowing down of the increase in respiration that occurs during this period (1.c. Fig. 106, p. 324). This decrease may correspond to a decreased drain on ATP (cf. Immers and Runnström, 1960) as a consequence of the decrease in protein synthesis occurring in sulfate-free sea water. Thus the figures of Lindahl do not prove that a delay in the *formation* of the respiratory enzymes occurs. The slight decrease may rather be due to an increased control of respiration. The results obtained by Lindahl thus rather support the view that lack of sulfate has not primarily a general inhibitory effect of protein synthesis but merely inhibits the synthesis of a special fraction of embryonic proteins.

SUMMARY

An attempt has been made to analyze the role of sulfate ions in the developing sea urchin embryo. Sulfate is stored mainly in bound form within the egg but an uptake of sulfate ions from the sea water begins to be pronounced approximately 6 hours after fertilization. With respect to whole larvae the study confirmed that a radialization or animalization of the larvae takes place when they

are reared in sulfate-free sea water. The first effect of lack of sulfate is a weakening of the vegetal factor (moderator) that prevents the extension of the acron region beyond its normal area. Animal halves separated in the 16-32 cell stage became more strongly animalized in sulfate-free than in sulfate-containing sea water (cf. Tables 1 and 2); the vegetal halves, on the other hand, became more vegetalized in sulfate-free than in sulfate-containing sea water (cf. Table 3).

The incorporation of ^{14}C-labelled amino acids (hydrolysate of algal proteins) into the different categories of embryos was tested, beginning with the early mesenchyme blastula and ending with a late gastrula stage. The original values are given in Table 4. The first four of the five stages tested were treated as a uniform material and the differences caused by different treatments were examined statistically (cf. Table 5). The results are represented in a simplified way in Fig. 17. Three different levels of incorporation of amino acids were distinguished. Two main factors seem to be at work: 1) the moderator built up in the vegetal region of the embryo and spreading from there, and 2) sulfated polysaccharides (PS) that increase the incorporation because they influence the protein synthesis in the direction of certain proteins Pr^1, which combine with polysaccharide sulfate to form $PSPr^1$. It is contended that some link necessary for building up PS originates in the animal embryonic region and spreads from there. Thus early isolation of a vegetal half should leave this with a low content of PS. The amino acid incorporation is in fact—at least until an advanced gastrula stage—equal in vegetal halves reared in sulfate-free or sulfate-containing sea water. Later a regulation may possibly occur in the former. The stronger vegetalization in vegetal halves in sulfate-free medium, Veg ($-SO_4^{=}$), is accounted for by the decrease in motility of the cells necessary in regulation processes. The mesenchyme cells that play a role in gastrulation and in connecting the different germ layers are mostly not motile and tend to aggregate with consequences for transportation and interactions. The morphogenetic difference between Veg ($-SO_4^{=}$) and Veg ($+SO_4^{=}$) is thus due to the lower capacity of regulation in the former. This leaves the vegetal halves ($-SO_4^{=}$) mostly in a state of animal-vegetal unbalance similar to that prevailing immediately after the operation. Conversely, the vegetal halves ($+SO_4^{=}$)

are capable of a regulation which may bring about a tendency to normal animal-vegetal balance (cf. Table 3).

The basic role of PSPr[1] is a protection of structure particularly in the vegetal region of the embryo. The protection may be based on the well known property of compounds of this type (e.g. heparin, chondroitin sulfate) to inhibit activation or activity of proteolytic and other hydrolytic enzymes.

When sulfate is lacking these enzymes may be relased and cause structural changes e.g. gelation of the cytoplasm. Moderate changes of this kind may not influence amino acid incorporation but cause pronounced changes in morphogenesis as evidenced by a comparison of vegetal halves ($+SO_4^{=}$) and ($—SO_4^{=}$).

The animalization of whole larvae and of animal halves in sulfate-free medium is explained as being due to a high sensitivity of certain vegetal proteins to the activated or released enzymes whereas the " animal " proteins are resistent to the attack of these enzymes.

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ZUSAMMENFASSUNG

Aus Seewasser aufgenommenes Sulfat wird von Seeigelembryonen hauptsächlich an Polysaccharide gebunden. Ein Mangel an sulfathaltigen Polysacchariden äussert sich morphogenetisch — im Vergleich zu in normalem Seewasser gezüchteten Hälften — in der weiteren Animalisierung von isolierten animalen bzw. in der Vegetativisierung isolierter vegetativer Hälften.

Die verminderte Aufnahme von ^{14}C-markierten Aminosäuren beruht wahrscheinlich auf dem Verlust von Proteinen, die normalerweise mit sulfathaltigen Polysacchariden konjugiert sind.

RÉSUMÉ

Les embryons d'oursin fixent à des polysaccharides les sulfates qu'ils absorbent de l'eau de mer. Comparées à des moitiés d'embryon isolées dans de l'eau de mer normale, les moitiés animales sont animalisées et les moitiés végétatives végétativisées par un manque de polysaccharides sulfatés.

L'incorporation réduite d'acides aminés marqués au C^{14} est probablement due à un déficit en protéines normalement conjugées aux polysaccharides sulfatés.

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