Further Studies on Free Amino-Acids and Peptides in Eggs and Embryos of Different Sea-Urchin Species and Hybrids

In a previous study (CHEN and BALTZER[1]) it has been reported that eggs and embryos of three different sea-urchin species, that is to say *Paracentrotus lividus*, *Arbacia lixula* and *Sphaerechinus granularis*, differ distinctly in their patterns of free amino-acids and peptides. Such species-specific differences provide us with valuable criteria to judge the influences of both parental species on the developing hybrid-embryos. Recently in order to explore further possibilities of interspecific combinations we have investigated the free ninhydrin-positive substances in both eggs and embryos of three more sea-urchin species: *Genocidaris maculata*, *Psammechinus microtuberculatus* and *Echinocardium cordatum*. The first two species belong to the so-called regular form of *Echinoidea* while the third one is the irregular form. Our preliminary results showed that in all three species the patterns of free amino-acids and peptides are constant during early development and differ again from each other in a species-specific manner.

Procedures for performing two-dimensional chromatograms were the same as those described earlier (CHEN and BALTZER[1]). In addition the one-dimensional chromatographic technique has been used to study the total amount of free ninhydrin-reacting components (for a general description of the technique, see HADORN and STUMM-ZOLLINGER[2], CHEN and HADORN[3]). As a basis for comparison estimations of total nitrogen have been also carried out by using the ultramicro-Kjeldahl method of BOELL and SHEN[4]. It was found that for total free amino acids 1000–2000 eggs and for total nitrogen 250–500 eggs were enough for each determination.

As illustrated by the chromatograms in Fig. 1 and the list of ninhydrin-reacting substances summarized in Table I, there are both qualitative and quantitative differences in the three sea-urchin species. *E. cordatum* is especially rich in free amino-acids. For instance in all species so far analyzed by us, valine and leucine never occur in such large quantities as in the present one (compare spots No. 9 and No. 16 in Fig. 1 *A* and the corresponding spots of other species in the present study and in CHEN and BALTZER[1]). The *Echinocardium* eggs are further characterized by the occurrence of spot No. 17 which has been so far recorded only in *A. lixula*. However, they differ from the latter by their high contents of glycine (compare spot No. 8, Fig. 1 *B* in CHEN and BALTZER[1]). The pattern of free ninhydrin-positive substances showed no distinct changes at both gastrula and pluteus stages.

The chromatographic patterns of *G. maculata* and *Ps. micromaculatus* are very similar to *P. lividus* in so far that α-Alanine and Glycine represent the most concentrated spots in both species (Fig. 1 *B* and *C*). However, a closer examination of the chromatograms revealed certain differences. Spot No. 23 (peptide 4 in Tab. I) was found only in *Ps. microtuberculatus*. Its Rf-values (0,65 in water-saturated phenol and 0,47 in 70% n-propanol) are very close to those of the tripeptide spot specific for *S. granularis* reported in our earlier study (CHEN and BALTZER[1], spot No. 23 in Fig. 1 *C*), although the spot did not appear so regularly as in the latter species. In *G. maculata* a brown spot of low Rf-values (0,17 in 70% n-propanol and 0,21 in water-saturated phenol) was recorded (spot No. 21 in Fig. 1 *C*). Judging from its ninhydrin color and its position on the chromatograms this substance is probably identical to the peptide reported previously for *P. lividus* (see CHEN and BALTZER[1], spot No. 21 in Fig. 1 *A*).

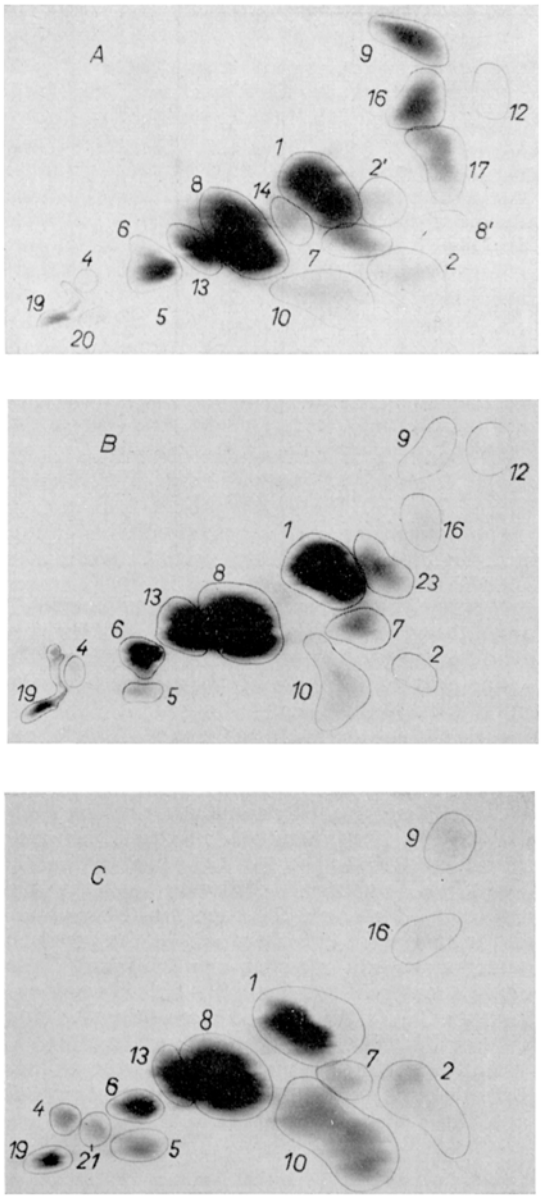

Fig. 1.—Free ninhydrin-positive substances in methanol extracts of different sea-urchin eggs. *A Echinocardium cordatum* (11250 unfertilized eggs); *B Psammechinus microtuberculatus* (12208 unfertilized eggs); *C Genocidaris maculata* (ca. 7500 unfertilized eggs). *1* α-Alanine; *2'* β-Alanine; *3* Arginine; *4* Aspartic acid; *5* Cystine; *6* Glutamic acid; *7* Glutamine; *8* Glycine; *8'* Histidine; *9* Leucine (and/or isoleucine); *10* Lysine; *12* Proline; *13* Serine; *14* Threonine; *15* Valine (and/or methionine); *17* 'Fast-aminobutyric acid'; *19–23* Peptides.

The protein residues after the methanol extraction were subjected to acid hydrolysis. In all three species the chromatograms of the hydrolysates showed the following amino-acids: α-alanine, arginine, aspartic acid, glycine, glutamic acid, histidine, leucine (isoleucine) serine, thre-

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[2] E. HADORN and E. STUMM-ZOLLINGER, Rev. suisse Zool. *60*, 506 (1953).
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Table I.—Free amino-acids and peptides in methanol extracts of different sea-urchin eggs and embryos. (Substances which occur in specifically high concentrations are indicated by a '!'.)

Free ninhydrin-reacting substances	A *Echinocardium cordatum*	B *Psammechinus microtuberculatus*	C *Genocidaris maculata*
1 α-Alanine	+	+	+
2′ β-Alanine	+	–	–
2 Arginine	+	+	+
4 Aspartic acid	+	+	+
5 Cystine	+	+	+
6 Glutamic acid	+	+	+
7 Glutamine	+	+	+
8 Glycine	+	+	+
8′ Histidine	+	–	–
9 Leucine (iso-Leucine)	+ !	+	+
10 Lysine	+	+	+
12 Proline	+	+	–
13 Serine	+	+	+
14 Threonine	+	–	–
16 Valine (Methionine)	+ !	+	+
17 'Fast-aminobutyric acid'	+ !	–	–
19 Peptide 1	+	+	+
20 Peptide 2	+	–	–
21 Peptide 3	–	–	+ !
23 Peptide 4	–	+ !	–

onine, tyrosine, proline, and valine (or methionine). This confirmed the earlier findings that, in spite of the species-specific differences in free ninhydrin-positive components, the amino-acid composition of the proteins remains at least qualitatively the same.

Since the chromatographic patterns of *E. cordatum* and *P. lividus* differ in spot No. 17 and the spots of valine and leucine, we made a brief study of the free ninhydrin-positive substances in the hybrid *Echinocardium* ♀ × *Paracentrotus* ♂ (EP). Our preliminary result of a few chromatograms showed that spot No. 17, which is specific for *Echinocardium*, appeared distinctly on the hybrid chromatogram. However, the concentrated spots of valine and leucine were not observed. At the present time it remains uncertain whether the EP-hybrid behaves entirely in a maternal manner as the hybrid combinations *Paracentrotus* ♀ × *Arbacia* ♂ and *Sphaerechinus* ♀ × *Paracentrotus* ♂ (see BALTZER, CHEN, and WHITELEY[5]).

In order to get more informations on the amino-acid metabolism during sea-urchin development we determined the total nitrogen and the total free amino-acids and peptides of four species thus far analyzed by us (see the more detailed report of BALTZER, CHEN and WHITELEY[5]). As shown by the data given for unfertilized eggs in Table II, there is a large variation in the egg sizes of the different sea-urchin species. *A. lixula* has the smallest and *E. cordatum* the largest eggs, while the egg sizes of *P. lividus* and *S. granularis* are equal. The same holds true for total nitrogen (compare HARVEY[6], for related data on *A. punctulata* and *P. lividus*). The egg volume of *A. lixula* is only 59% of that of *P. lividus*. With regard to total ninhydrin-positive components the values are high for *E. cordatum* and extremely low for *A. lixula*. The total content per egg for *Arbacia* is 20% of that for *Paracentrotus*. The difference remains pronounced even when the figures are compared on the basis of total nitrogen (Table II, E.U./γN). The *Echinocardium* egg has about 7 times more free amino-acids than the *Arbacia* egg (Table II, E.U./10^3 eggs). The values are still three times higher for the former species even when the data are expressed as extinction unit per μg total nitrogen. The morphogenetic role of the free amino-acids is largely unknown. Beside their function as building stones for the proteins they may serve as energy sources for the maintenance of the developing embryo. It has also been reported that glycine, which represents the most concentrated amino-acid in most sea-urchin species, is especially involved in the osmoregulative phenomenon (KAVANAU[7]). For a better understanding of the biochemical basis of morphogenetic development, such species-specific differences in the amino-acid contents deserve our special attention.

Another question which appears to be pertinent to the present study is whether these species-specific substances are derived from the yolk proteins deposited in the cell, or if they are directly related to the cytoplasmic part of the developing egg. During development there are no doubt such processes as the breakdown of egg proteins and the conversion of them into specific proteins of different tissues and organs. Efforts have been made by culturing the plutei for a longer period until their yolk reserve was exhausted and the feeding began. Our analysis of the larvae fed for 3–10 days showed that there is a pronounced accumulation of free glycine in *A. lixula*. Otherwise there is no essential modification of the amino-acid patterns. For instance spot No. 17, which is specific for *Arbacia* eggs, appeared regularly on the chromatogram even at these later stages. (No feeding experiment on *Echinocardium* was carried out.) In the other species glycine remains to be the dominating amino-acid. This indicates that the free ninhydrin-reacting compounds reported here

[5] F. BALTZER, P. S. CHEN, and A. H. WHITELEY, Symp. Soc. Cell Biol. (in press).

[6] E. B. HARVEY, *The American Arbacia and Other Sea Urchins* (Princeton University Press 1956), 193.

[7] J. L. KAVANAU, J. exp. Zool. *122*, 285 (1953).

Table II.—Size, total nitrogen, and total free ninhydrin-positive substances of different sea-urchin eggs

Species	Diameter mm/egg[1]	Volume relation (D^3) mm^3/egg	Total N $\gamma/10^3$ eggs	Total free ninhydrin-reacting substances	
				E. U./10^3 egg	E. U./γ N
Arbacia lixula	0·080	512×10^{-6}	8·50	0·080	0·0094
Paracentrotus lividus	0·095	857×10^{-6}	13·32	0·390	0·0293
Sphaerechinus granularis	0·095	857×10^{-6}	14·51	0·410	0·0282
Echinocardium cordatum	0·122	1816×10^{-6}	17·24	0·549	0·0318

[1] Measurements carried out by Prof. Dr. F. BALTZER. Compare data given by HARVEY 1956, pp. 63–67.

for the different sea-urchin species should not be considered merely as intermediate decomposition products of the yolk proteins, but as active components in the cytoplasm of the developing egg.

This study was carried out at the Zoological Station of Naples, Italy in cooperation with Prof. Dr. F. BALTZER, to whom I should like to express my sincere thanks for taking care of the materials and counting the samples, and for his valuable discussions in the course of the experimental work. Thanks are also due to Prof. Dr. L. VON UBISCH for supplying a part of the *Echinocardium* eggs investigated by us. The investigation was aided by a research grant from the *Schweizerischer Nationalfonds zur Förderung der wissenschaftlichen Forschung*. Finally I should like to thank the authorities at the Zoological Station of Naples for providing the working facilities.

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Zusammenfassung

Als Fortsetzung früherer Untersuchungen der freien Aminosäuren und Peptiden in Eiern und Embryonen verschiedener Seeigelarten, wurde das Muster der Ninhydrin-positiven Stoffe von *Echinocardium cordatum*, *Psammechinus microtuberculatus* und *Genocidaris maculata* papierchromatographisch untersucht. Die *Echinocardium*-Eier zeichnen sich durch ihre hohe Konzentration an Valin (Fig. 1*A*, Fleck 16) und Leucin (Fleck 9) aus, welche bei allen übrigen von uns untersuchten Arten nur in sehr geringer Menge auftreten. Ferner sind sie durch das Vorkommen eines spezifischen Stoffes charakterisiert, der nur bei *Arbacia lixula* nachgewiesen wurde (Fleck 17). *Psammechinus microtuberculatus* und *Genocidaris maculata* unterscheiden sich in den Peptiden. Die *Psammechinus*-Eier enthalten einen Stoff, der wahrscheinlich mit dem Tripeptid von *Sphaerechinus granularis* identisch ist (Fig. 1*B*, Fleck Nr. 23). In *Genocidaris*-Eiern wurde ein Peptid registriert, das auch bei *Paracentrotus lividus* vorkommt (Fig. 1*C*, Fleck Nr. 21).

Es wurden ferner Messungen von Eivolumen, Totalstickstoff und Totalmenge der freien Ninhydrin-positiven Substanzen des unbefruchteten Eies durchgeführt. Das Eivolumen und der Gesamtstickstoffgehalt sind bei *Echinocardium cordatum* am grössten, bei *Arbacia lixula* am geringsten. Die *Echinocardium*-Eier sind durch ihren hohen Gehalt an freien Aminosäuren gekennzeichnet. Sie enthalten rund 7mal mehr solche Stoffe pro Ei als die *Arbacia*-Eier; zwischen *Paracentrotus lividus* und *Sphaerechinus granularis* zeigen die Messwerte keinen deutlichen Unterschied.

Fat Formation and Glycolysis in Tissue Culture Action of Hydrocortisone[1]

The question of the role of adreno-cortical steroids in fat metabolism remains as yet unsettled. On the one hand it appears that hydrocortisone is the only physiological compound that can cause obesity when administered in excess of body needs and that this type of obesity is indistinguishable from that of Cushing's syndrom. A synergistic effect of insulin and cortisone in increasing fat synthesis from carbohydrate was reported[2]. But on the other hand, antagonistic effects of cortisone and insulin on fatty acid synthesis from labeled acetate was described, cortisone having an inhibitory effect[2]. It has therefore been thought that information gained from a study of the direct action of hydrocortisone on fat formation in tissue culture may shed some light on this question. Since, however, the tendency to accumulate fat is a characteristic feature of tissue culture cells in general, even of such cell types which in the organism do not show a tendency to fat accumulation, a preliminary study of fat formation in normal untreated tissue culture cells appeared necessary.

Now, for many years, 2 distinctive characteristics of cultured cells have been observed. First, the presence of more or less high glycolysis[3], even in the presence of oxygen[4], and, second, a striking tendency to fat accumulation in cytoplasmic vacuoles[5]. It is tempting to assume that these 2 special characteristics of cultured cells are not independent of each other. From investigation into the action of high glucose concentration on respiration and glycolysis on the one hand, and on formation of fat vacuoles on the other hand, a possible relationship between glycolysis and lipogenesis has emerged, and the assumption has been made that glycolysis as a reducing system favors fatty acid synthesis. Lipogenesis requires a reducing system for the regeneration of reduced pyridine nucleotide necessary for fatty acid synthesis.

Methods.– For observation of lipid granules, the hanging drop method and a medium consisting of plasma and chick amniotic fluid[6] was used. Control cultures in amniotic fluid show far less fat accumulation than in other culture media[6]. In the first 2–4 days of growth, primary cultures of various tissues in a medium of plasma and amniotic fluid showed less fat accumulation, as estimated by number and size of fat vacuoles observed with 120 × magnification, than cultures in a medium containing embryo extract as well. If the media were not changed for several days, fat vacuoles gradually increased in number and size; by renewal of medium, or aeration, fat vacuoles could partly be removed. Mitoses were observed in cells containing large fat vacuoles. The lipid appeared predominantly to be produced by the cell and not to come from the medium, because it could be removed or reduced by renewal of the medium. Furthermore, cells in pure saline with glucose and with explants removed also showed fat vacuoles. Lipid containing cells often continued to produce acid mucopolysaccharide, as revealed by the method of mucin clot formation[7]. Addition of 0·5 to 2% glucose to the medium resulted in considerable increase of fat accumulation in the cytoplasm, compared with cultures growing in a medium of plasma and amniotic fluid only.

For investigation of the action of higher glucose concentrations on respiration and glycolysis, strain L derived from a fibroblast of mouse subcutaneous tissue was used

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