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dissertation entitled

(^{3}H)Serotonin binding to cell fractions and dissociated cells of blastula, gastrula, prism and pluteus sea urchin (Arbacia punctulata) embryos

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[^{3}H]SEROTONIN BINDING TO CELL FRACTIONS AND DISSOCIATED CELLS OF BLASTULA, GASTRULA, PRISM AND PLUTEUS SEA URCHIN (ARBACIA PUNCTULATA) EMBRYOS

by

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ABSTRACT

[^{3}H]SEROTONIN BINDING TO CELL FRACTIONS AND DISSOCIATED CELLS OF BLASTULA, GASTRULA, PRISM AND PLUTEUS SEA URCHIN (ARBACIA PUNCTULATA) EMBRYOS

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The presence and location of serotonin binding sites in early sea urchin (Arbacia punctulata) embryos was investigated by the binding of radiolabelled serotonin to cell fractions of blastulae, gastrulae, prism larvae, early pluteus larvae and later pluteus larvae cell fractions and to dissociated cells of these stages. Specific [^{3}H]serotonin binding was demonstrated in some cell fractions from each developmental stage studied. Cell fractions were partially characterized by enzymatic and microscopical analyses. The localization of serotonin binding sites to particular subcellular components was suggested from comparisons between the binding activity and organelle composition of the various cell fractions. In the older plutei serotonin binding sites were tentatively determined to be located on cell surface membranes. In earlier embryos the

correlation between serotonin binding activity and the quantity of cell surface membranes in cell fractions was less distinct, and the possibility of serotonin binding sites on other cell organelles was examined. [^{3}H]Serotonin bound to nuclei of late blastula, gastrula and prism embryos while no binding to nuclear fractions of early and advanced plutei was detected. Specific [^{3}H]serotonin binding to dissociated cells of prism, early pluteus and older pluteus embryos was demonstrated, but no binding to late blastula cells was detected. Results of binding studies suggest that serotonin functions in early sea urchin embryos as it does in adult animal tissues by first binding to a receptor molecule. Possible serotonin functions and mechanisms of action are discussed.

DEDICATION

I would like to dedicate this work to my parents, Ray and Beverly Brown.

ACKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. John R. Shaver, for everything. Dr. Shaver is both an excellent scientist and a fine person. I was very fortunate to have been his graduate student.

For their help and advice I thank all of the members of my committee, Dr. Neal Band, Dr. Evelyn Rivera and Dr. Frank Welsch.

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INTRODUCTION

Several chemical compounds which function as neurotransmitters or hormones in tissues of adult vertebrate and invertebrate animals have also been detected in early embryos that have not yet developed nervous or endocrine systems. These substances include serotonin, acetylcholine, dopamine, epinephrine and norepinephrine. One or more of these compounds have been identified in embryos from the unfertilized egg through gastrula and postgastrula stages in animals of almost every animal phylum studied including echinoderms (Buznikov et al., 1964, 1968, 1972a), mollusks and nemerteans (Buznikov, 1973), polychetes (Emanuelsson, 1974), insects (Furneaux and McFarlane, 1965a, 1965b), amphibians (Baker, 1965), birds (Emanuelsson, 1976; Wallace, 1979) and mammals (Renson, 1971; Burden and Lawrence, 1973). The presence of neurotransmitters and hormones in embryos which have not developed neural or endocrine structures raises questions as to the function of these molecules in early embryos. The concentrations of these compounds fluctuate throughout development, but in general they are of the same order as in tissues of adult animals (Buznikov, 1971). This observation, along with studies which have demonstrated inhibition of early development in the presence of inhibitors of these compounds, suggest that they perform a regulatory role in early development.

The best case for a regulatory role of a neurohormone in early embryogenesis can be made for serotonin (5-hydroxytryptamine). In the nemertean, Tineus desori, and the polychete, Anaitides maculata, serotonin levels dropped twentyfold in the period between fertilization and the first cleavage divisions. This lower serotonin level was maintained through gastrulation except for a fivefold increase just prior to gastrulation (Buznikov, 1973). Serotonin has been detected in early embryos of several mollusc species. In the gastropod mollusc, Acanthodoris pilosa, serotonin levels increased up to the midblastula stage and then dropped to undetectable levels during gastrulation (Buznikov, 1973). Serotonin antagonists have been shown to inhibit cleavage in several nudibranch (Mollusca) species, and this inhibition could be reversed by the addition of serotonin into the embryo culture medium (Buznikov et al., 1970). In the loach, Misgurnus fossilis, drastic changes in serotonin content have been monitored during cleavage divisions. At later stages the serotonin level was barely detectable except for two short term increases at early and late gastrula stages (Buznikov et al., 1964). Baker (1965) has shown that serotonin levels in the frog, Xenopus laevis, were higher during blastulation and neurulation than during gastrulation. When neurula embryos of Xenopus laevis were exposed to the serotonin antagonist lysergic acid diethylamide (LSD), monoamine oxidase and 5-hydroxytryptophan decarboxylase activities (Baker, 1972) and serotonin levels (Baker, 1971) were altered compared with untreated controls. Exogenous serotonin stimulated morphogenesis in explanted neurula cells of the frog, Rana temporia; this effect was blocked by serotonin antagonists (Martynova and Balousov, 1978).

In the chick embryo serotonin was detected throughout gastrulation and neurulation (Emanuelsson, 1976). Injection of 5-hydroxytryptophan into chick gastrulae induced several malformations (Schowing et al., 1977). Serotonin antagonists induced disturbances in blastoderm expansion, primitive streak formation, neurulation, somitogenesis and brain formation in the chick embryo (Palen et al., 1979). Serotonin is present in rat ova, fertilized eggs, two-cell and four-cell embryos (Burden and Lawrence, 1973), and can reverse the depolarizing effect of the serotonin antagonist chlorpromazine on the membrane potential of mouse two-cell embryo blastomeres (Leonov et al., 1975).

Most of the investigations concerning the role of serotonin in early embryogenesis have been made with sea urchin embryos. Using a very sensitive biological assay based on the ability of serotonin to stimulate motility in larvae of different nudibranch molluscs, Buznikov et al. (1964) determined the serotonin levels in Strongylocentrotus drobachiensus sea urchin embryos from the fertilized egg through advanced pluteus larval stages. Serotonin levels peaked at each cleavage division until blastomeres started dividing asynchronously. Levels also peaked at hatching, when primary mesenchyme cells were separating from vegetal plate cells in the late blastula, during the initial invagination of the vegetal plate in the early gastrula, and at midgastrulation. Peaks were sharp, and embryos at stages inbetween peaks contained two to five times less serotonin. At the end of gastrulation, serotonin levels slowly rose and kept rising through advanced pluteus stages. In embryos of the sea

urchins, Strongylocentrotus drobachiensis and Strongylocentrotus intermedius, serotonin levels, measured by a fluorometric assay, remained unchanged from the unfertilized egg through the first three cleavages except for a four- to fivefold decrease at prometaphase of each division (Buznikov et al., 1972a). Using a histochemical fluorescence technique, Buznikov et al. (1972a) demonstrated the presence of serotonin in every cell of embryos of various stages up to the end of gastrulation, and the stage specific fluctuations in serotonin levels from this study agreed fairly well with levels determined by the biological assay discussed earlier. Using a different histochemical fluorescent technique, this study failed to detect serotonin until the early gastrula stage when it was shown to be specifically localized in the archenteron and ventral ectoderm region during gastrulation. Both histochemical fluorescence techniques demonstrated the presence of serotonin in pluteus stages only in the digestive tube, cells of the ventral ciliary band and possibly in pigment cells. Histochemical studies of serotonin localization in Psammechinus miliaris plutei confirm these observations (Ryberg, 1974). Toneby (1973, 1977a, 1977b), used fluorometric and thin layer chromatographic techniques and could not detect serotonin during cleavage, formation of primary mesenchyme cells and gastrulation in embryos of the sea urchins, Paracentrotus lividus and Strongylocentrotus fransiscanus. These results contradict those of Buznikov et al. (1964, 1972a). From the end of gastrulation until formation of a pluteus larva, serotonin was detected by Toneby (1973, 1977a, 1977b), and serotonin levels were shown to continually rise during this period. The inability of

Toneby to detect serotonin during cleavage and gastrulation may have been due to the lower sensitivity of the assays he used as compared with the biological assay of Buznikov, or it may reflect the greater specificity of the assays used by Toneby. Renaud et al. (1979) demonstrated the presence of 5-methoxytryptamine in cleavage stages of Paracentrotus and Sphaerechinus embryos when assayed by dansylation and thin layer chromatography but not of serotonin. The amount of 5-methoxytryptamine seemed to increase at the end of the cell cycle, and this compound was postulated to be the important molecule in the regulation of cell division. No spot corresponding to 5-methoxytryptamine was detected in the thin layer chromatograms of cleavage stage embryos as described by Toneby (1977a).

Several studies have examined the effect on early sea urchin development of serotonin and various antisertonin compounds by incubating embryos in sea water containing these substances. Molecules which have been shown to block serotonin function in adult animals have been shown to lower the membrane potential of Strongylocentrotus intermedius fertilized eggs (Korobtsov and Sorokin, 1974) and to inhibit both protein synthesis and cleavage in Strongylocentrotus drobachiensus, Strongylocentrotus nudus, Strongylocentrotus intermedius, Paracentrotus lividus, Scaphechinus mirabilis, Sphaerechinus granularis, Echinus melo and Arbacia lixula embryos (Buznikov et al., 1970, 1972b, 1974, 1976; Landau et al., 1981). Lower concentrations of these inhibitors blocked cytoplasmic divisions but allowed nuclear division to continue (Buznikov et al., 1970). Buznikov (1971) and Buznikov et al. (1971a) have demonstrated that various serotonin blocking agents in small concentrations

stimulate and in higher concentrations inhibit protein synthesis in Arbacia lixula and Strongylocentrotus nudus oocytes, fertilized eggs and early cleavage embryos. This action was not connected with the effect of antagonists on cell division, as indicated by a completely different shape of the dose-response curve. Early gastrulation events in Psammechinus miliaris embryos, including the migration of primary mesenchyme cells and the initial invagination of the vegetal plate, were blocked by some antiserotonins and serotonin synthesis inhibitors while other inhibitors delayed the onset of later gastrulation events by preventing the formation of secondary mesenchymal pseudopods (Gustafson and Toneby, 1970, 1971; Gustafson, 1973). In all inhibitor studies, the effects of inhibitors could be reversed by the addition of serotonin to the incubation medium. Serotonin stimulated ciliary movements in Psammechinus miliaris hatched blastulae (Gustafson, 1969). In low concentrations serotonin has been shown to inhibit hatching by blocking synthesis of hatching enzyme, and to increase the rate of protein synthesis in 2-16 cell stage Paracentrotus lividus embryos, and in higher concentrations serotonin completely inhibited cleavage (Deeb, 1972). Serotonin stimulated Psammechinus miliaris pseudopodal activity in primary mesenchyme cells, and these cells eventually formed a skeleton containing a spicule arrangement closer to that of another sea urchin genus Echinocardium (Gustafson, 1969).

Gustafson and Toneby (1970, 1971), Gustafson (1973), Buznikov (1971) and Buznikov et al. (1972a) have proposed the following functions for serotonin in sea urchin embryos from late blastula through pluteus larva stages:

1. Serotonin is involved in ciliary movement at hatching.

2. Serotonin produced by vegetal plate cells of late blastulae elicits their pulsatory and other movements that play a role in the release of primary mesenchyme cells and in the initial invagination of the vegetal plate during gastrulation. Serotonin may still be required for the formation and exploratory movements of secondary mesenchymal pseudopods, necessary for the continuation of gastrulation.

3. Serotonin, probably released from pigment cells (derived from secondary mesenchyme cells), induces the bubbling activity of the coelomic rudiment, an important activity in the morphogenesis of the coelom which begins in prism stage embryos.

4. Serotonin, probably released from pigment cells, stimulates cilia of the circumventral ciliary band which in turn stimulates swimming activity in the pluteus larva.

5. Serotonin, probably released from pigment cells of the pluteus larva, induces some ciliated ectodermal cells to enter the body cavity and send out pseudopods. These cells eventually become nerve cells, and the pseudopods become axons.

6. Serotonin functions as a neurotransmitter in serotonergic neurons of the advanced pluteus larva.

The functions proposed for serotonin in blastula and gastrula stages were determined from studies already discussed; those in postgastrular, preneural development (functions 3-5) were postulated from detailed time-lapse light microscopy and electron microscopical studies (Gustafson, 1969; Gustafson and Toneby, 1971) of Psammechinus miliaris embryos. From these studies it was determined that pigment cells were derived from secondary mesenchyme cells which are released from the archenteron tip at the completion of gastrulation. The cells accumulate pigment and show a chromaffin reaction, indicating the presence of a monoamine which is postulated to be serotonin. Pigment cells are initially located adjacent to the coelomic rudiment during its formation and are later localized adjacent to ciliated ectodermal cells. Agents which degranulate mast cells in adult mammals stimulate pigment cells to release their contents, and this results in a stimulation of ciliary activity in adjacent ectodermal cells. Addition of serotonin to the embryo culture mimicked the effect of degranulating agents. From these observations they postulated that serotonin, released from pigment cells, provided the stimulus for ciliary and coelomic activities in adjacent cells. In early plutei the ciliated ectodermal cells that are adjacent to pigment cells eventually break away from other ectodermal cells and form neurons. Cell processes of developing neurons accumulate a greenish substance which was presumably obtained from pigment cells. This transfer of serotonin was postulated to be the initiator for separation of future neurons from other ectodermal cells and for elaboration of neuronal processes. Ryberg (1977) used histochemical stains to identify neurons and confirmed the presence of nerve nets,

nerve strands and clustered cell bodies in Psammechinus miliaris pluteus larvae. Pharmacological studies with this species have suggested the presence of serotonergic and cholinergic neuronal components in advanced plutei (Gustafson et al., 1972a, 1972b).

In summary, the roles of serotonin in early sea urchin embryos cannot be totally determined from the available data. Consequently, the analysis of the mechanism by which serotonin functions is still at a theoretical level. Numerous pharmacological investigations, as well as biological and histochemical serotonin assays, suggest a role in cleavage and protein synthesis in cleavage stage embryos. Fewer studies of the same type, as well as microscopical analyses, have implicated a role of serotonin in ciliary activity in blastula through pluteus stages, in cell morphogenesis in the late blastula, during gastrulation and during postgastrular development, and as a neurotransmitter in the advanced pluteus larval nervous system. In postgastrula, preneural embryos the functions of serotonin released from pigment cells have been formulated principally from microscopical studies which need biochemical support. Although all investigations confirm a continual increase in serotonin levels between the end of gastrulation and formation of the pluteus larva, the chemical serotonin assays of Toneby, in contrast to the biological and histochemical assays, have not detected the compound in gastrula and pregastrula stages of development. It is possible that newly synthesized serotonin does not accumulate during or prior to gastrulation but is rapidly degraded after carrying out its function and that at these low levels serotonin could not be detected by Toneby's chemical methods. It is also possible that a different

monoamine, such as methoxytryptamine, may be the important regulatory agent in the earlier embryos.

The functional activity of neurotransmitter and hormone mediators in adult animals is realized by their reaction with corresponding receptor sites. If serotonin is directly involved in the regulation of early sea urchin embryogenesis, then embryos should contain reactive sites which could recognize and bind this substance. Determination of the subcellular location of serotonin binding sites could help answer the question as to how serotonin functions. For example, serotonin may function in cleavage and cell morphogenesis by interacting with the microfilaments and microtubules, cell organelles directly responsible for these events. Alternatively, serotonin may interact with other subcellular structures such as nuclei, yolk granules, mitochondria or endoplasmic reticulum. As a probable neurotransmitter in advanced pluteus larvae, serotonin would most likely function, as in adult animals, by interacting with cell surface binding sites. Cell surface binding sites, however, may also mediate serotonin activity in preneural embryos. The presence of serotonin binding sites in pregastrula and gastrula stages is supported by the experiments already discussed which have demonstrated developmental alterations in embryos exposed to serotonin binding site blockers accompanied by a reversal of the effects after addition of serotonin to the embryo culture. Results of several studies with early cleavage stage embryos suggest the presence of intracellular serotonin binding sites. All sea urchin species studied displayed the same sensitivity to certain antiserotonins, as determined by the ability of these compounds to block cleavage. Other antiserotonins elicited this

"normal" sensitivity with many species (Strongylocentrotus drobachiensus, Strongylocentrotus nudus, Strongylocentrotus intermedius, Paracentrotus lividus, Sphaerechinus granularis, Scaphechinus mirabilis) but were hyperactive against other "supersensitive" sea urchin species (Echinocardium cordatum, Arbacia lixula, Echinus melo, Echinus esculentus), inhibiting cleavage at concentrations 10-800 times less than those required to inhibit cleavage in the other species (Buznikov et al., 1974,1976,1979). In supersensitive species, neuroactive drugs of normal activity inhibited both protein synthesis and early cleavage in approximately the same concentrations, whereas hyperactive substances inhibited protein synthesis in concentrations much higher than those needed to block cleavage (Buznikov et al., 1972b). From these studies it was suggested that two types of intracellular serotonin receptor sites, supersensitive (S) and nonsupersensitive (N), were present in supersensitive embryos; hyperactive inhibitors could bind to either site, while normally active inhibitors bind only to N-receptors. It was also assumed that normally sensitive embryos contain superactive binding sites which are important for some cellular activities but which are uncoupled with the cleavage process. Buznikov et al. (1979) fragmented sea urchin fertilized eggs into halves and the halves into quarters and tested the sensitivity of various egg fragments to different antiserotonins which was measured by the ability of these compounds to block cleavage. All fragments from normally sensitive eggs exhibited normal sensitivity to all drugs. In supersensitive embryos, however, the centripetal pole egg halves displayed normal sensitivity to hyperactive drugs while centrifugal

pole halves displayed supersensitivity to the same drugs. If the centripetal pole half was split again by centrifugation, supersensitivity to hyperactive substances was restored in the centrifugal quarter while the other quarter displayed normal sensitivity to these substances. The investigators concluded that S-receptors were coupled to cell division by a sensitizer factor that moved centrifugally, and that normally sensitive embryos do not have the factor. This factor was postulated to be associated with yolk granules, the only organelle known to move during centrifugation in a manner similar to the postulated sensitizer factor. Landau et al. (1981) determined that hyperactive substances were in general more lipophilic than normally active antiserotonins and concluded that to reach supersensitive receptors the antiserotonins must first penetrate a lipid barrier and that the sensitizer factor may function by enhancing penetrability. Based on the effect of antiseritonins on early cleavage stage embryos treated with puromycin (Buznikov et al. 1971a; Buznikov, 1971) and antimycin A (Buznikov et al. 1972b; Buznikov and Markova, 1974b) N- and S-receptors were postulated to be localized on endoplasmic reticulum and mitochondria, respectively. Buznikov et al. (1975, 1977) and Manukhim et al. (1973, 1976) allowed serotonin and antiserotonins to attach to whole embryos and determined the amount bound by chemically assaying for the compounds in embryo pellet extracts, by spectrophotometrically measuring a decrease in the concentration of free ligands in the culture medium, or by lysing the embryo cells and determining the sensitivity of Arbacia lixula embryos to the lysate. In these experiments serotonin and antiserotonin binding occurred in two phases. They concluded

that the second phase apparently coincided with the uptake of serotonin and antiserotonins into embryo cells since dead or osmotically lysed embryos only showed the first phase of binding, and bound chemicals could not be released by washing embryos, but could be released by cell lysis.

From the experimental observations with cleavage stage embryos, serotonin receptive structures were postulated to be on mitochondria, endoplasmic reticulum and possibly on yolk granules. Localization of receptor sites with such methods is indirect. These studies have not determined that receptive structures are on these organelles, nor have they ruled out the possibility of receptive structures on other intracellular organelles or on the cell surface membrane. For this reason it is necessary to create cell free systems suitable for the direct study of structures that can specifically recognize and bind serotonin. The nature of serotonin receptive structures in embryos older than the early cleavage stages is almost totally unexplored. In the research described in this thesis, cell fractionation was performed on five developmental stages of the sea urchin Arbacia punctulata ranging from late blastulae to pluteus larvae. The presence and location of serotonin recognition sites was investigated by the binding of radiolabelled serotonin to various cell fractions and to whole dissociated embryo cells.

MATERIALS AND METHODS

Embryo Culture

Gametes of the sea urchin, Arbacia punctulata (Florida Marine Biological Specimen Co., Panama City, Fl.), were obtained by application of 15 volts AC to the oral surface of the animals. Eggs were collected in Herbst's (1904) artificial sea water as modified by Motomura (1954). Sperm were collected dry. Eggs were washed once in sea water by settling and fertilized with a dilute sperm suspension. Embryos were subdivided among two or three one-gallon jars to which sea water was added to a total volume of approximately 3500 ml in each jar such that the final embryo concentration was 2000 embryos/ml (7 ml washed, settled eggs per jar). Embryos were cultured at 15°C in a constant temperature chamber. In each jar embryos were continuously agitated by a teflon paddle rotated at 60 cycles per minute. In the case of 72 hour embryos, paddles were rotated at 30 cycles per minute after 60 hours of rotation at 60 cycles per minute. At the desired times - 24, 36, 48, 60 or 72 hours after fertilization, embryos were allowed to settle for one hour at 0°C. Excess sea water was poured off and embryos in the remaining sea water were collected by centrifugation in a Sorvall SS-34 rotor at 480xg for 3 min. Embryo samples were fixed in 3% formalin for later observation.

Cell Dissociation

Dissociation of embryo cells was performed according to the method of Guidice and Mutolo (1970) and is summarized in Figure 2. Embryos were washed three times in 20 vol calcium-free sea water (Okazaki, 1956), suspended in 5 vol 0.5M sucrose, 0.01M Tris-HCl buffer, pH 8.0, and homogenized in a 20 ml Dounce homogenizer with a loose-fitting glass pestle until dissociation was complete. The cells were collected by centrifugation at 100xg for 3 min in a Sorvall HB-4 rotor. Cell pellets were suspended in sea water for binding assays and cell counts. Cell counts were made in triplicate using a hemocytometer (Brightline, Americal Optical).

Cell Fractionation

The procedure for the separation of embryo cell fractions is summarized in Figure 1. Embryos were washed three times by suspension in 20 vol calcium, magnesium-free sea water (Marine Biological Laboratory, Woods Hole, Mass.) followed by centrifugation at 1000xg for 5 min in a Sorvall HB-4 rotor. Embryo pellets were then dispersed in 6 vol 0.25 M sucrose, 2mM $MgSO_4$, 5mM Tris-HCl, pH 8.0 (S-TM) (Chlapowski and Band, 1971), and homogenized in a 40 ml Potter-Elvehjem type teflon grinder with 15-20 strokes of the pestle rotated at 2500 rpm. The extent of homogenization was monitored by phase contrast microscopy and was continued until at least 95 percent of the cells had been disrupted. The homogenate was centrifuged at 2000xg for 15 min in a Sorvall HB-4 rotor. The supernatant was removed and saved, and the pellet was suspended in 20 vol 0.25M sucrose, 25mM KCl, 5mM Tris-HCl, pH 8.0 (S-TK), and centrifuged at 2000xg for 15 min in a Sorvall HB-4 rotor. The final pellet was

Figure 1. Procedure for the separation of sea urchin embryo cell fractions.

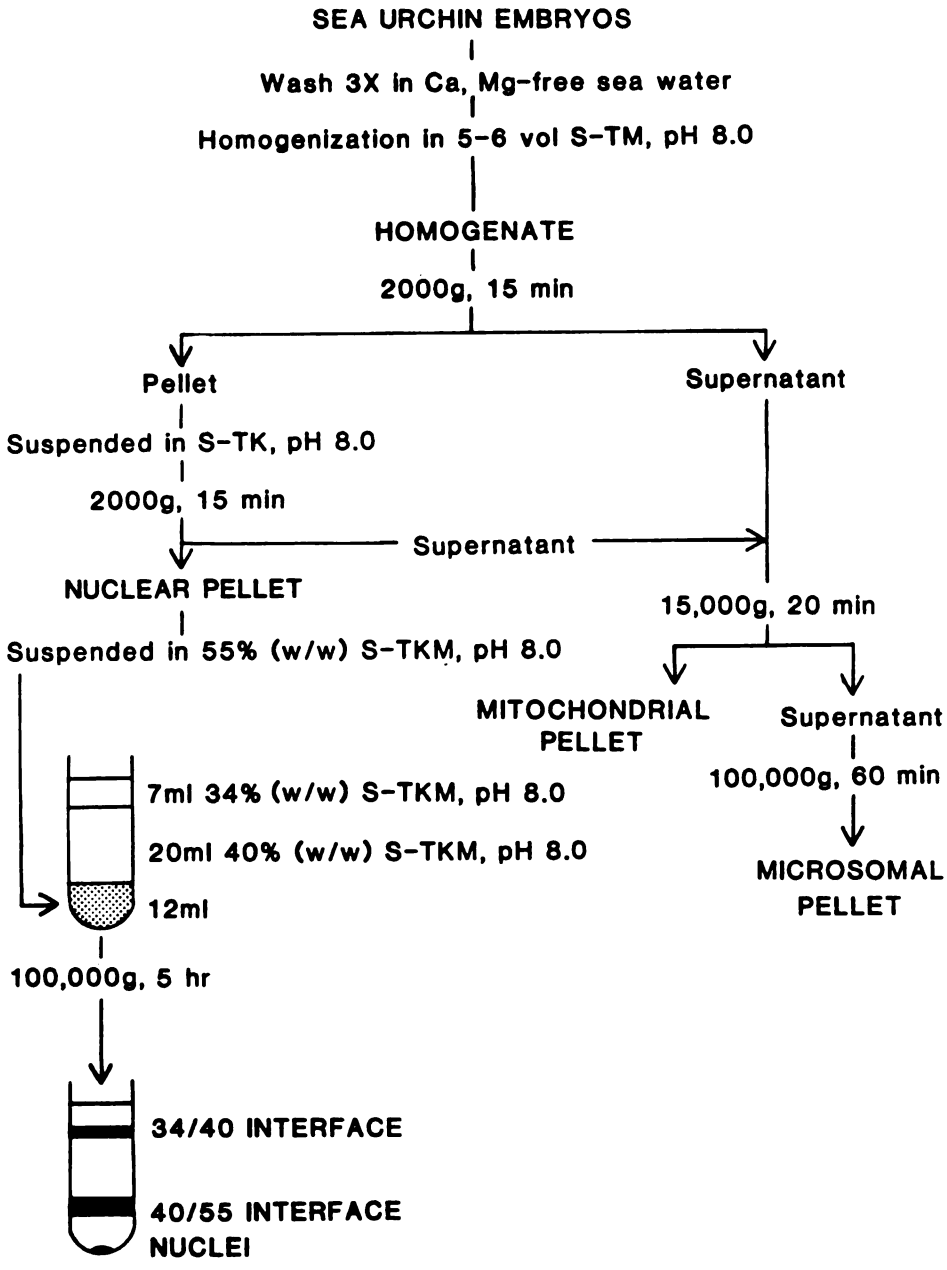
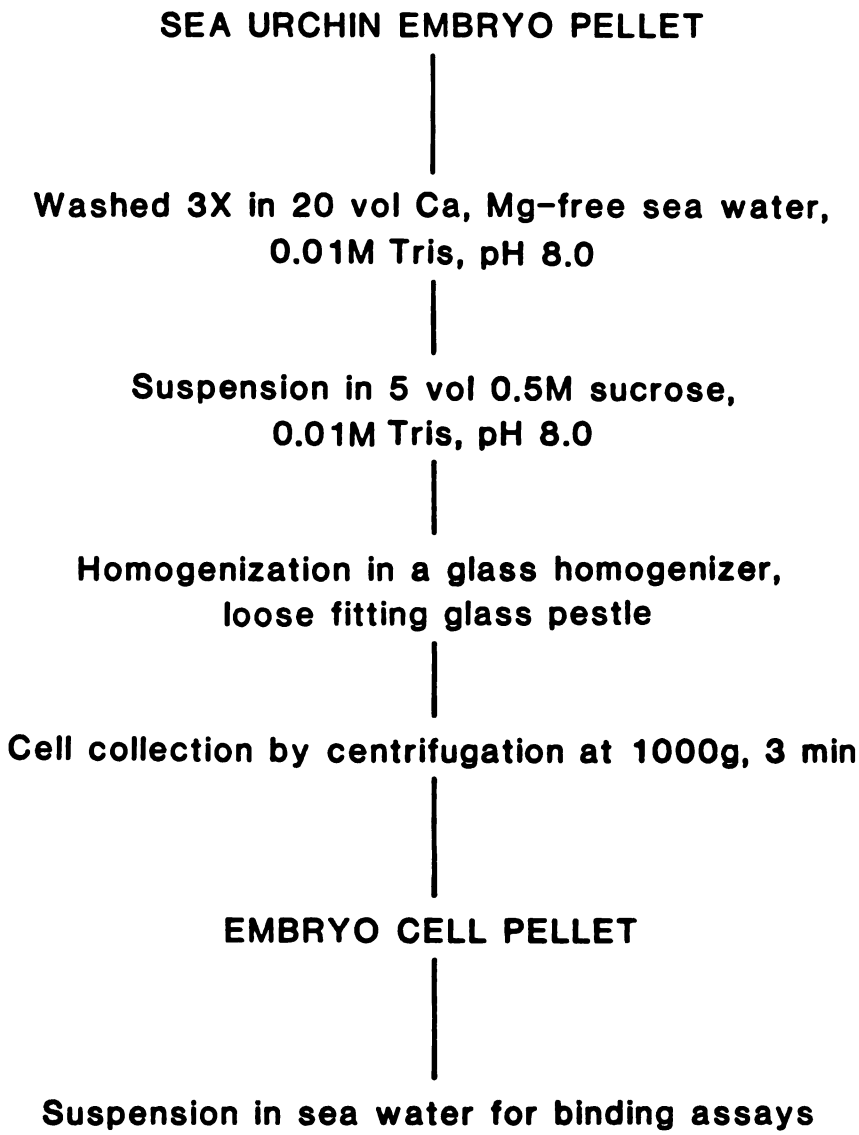

SEA URCHIN EMBRYOS
Wash 3X in Ca, Mg-free sea water
Homogenization in 5-6 vol S-TM, pH 8.0
HOMOGENATE
2000g, 15 min
Pellet
Supernatant
Suspended in S-TK, pH 8.0
2000g, 15 min
Supernatant
NUCLEAR PELLET
15,000g, 20 min
Suspended in 55% (w/w) S-TKM, pH 8.0
MITOCHONDRIAL PELLET
Supernatant
100,000g, 60 min
MICROSOMAL PELLET
7ml 34% (w/w) S-TKM, pH 8.0
20ml 40% (w/w) S-TKM, pH 8.0
12ml
100,000g, 5 hr
34/40 INTERFACE
40/55 INTERFACE
NUCLEI

Figure 2. Procedure for dissociating sea urchin embryo cells as in Giudice and Mutolo (1970).


SEA URCHIN EMBRYO PELLET
Washed 3X in 20 vol Ca, Mg-free sea water, 0.01M Tris, pH 8.0
Suspension in 5 vol 0.5M sucrose, 0.01M Tris, pH 8.0
Homogenization in a glass homogenizer, loose fitting glass pestle
Cell collection by centrifugation at 1000g, 3 min
EMBRYO CELL PELLET
Suspension in sea water for binding assays

the previous supernatant and centrifuged 15,000xg for 20 min in a Sorvall SS-34 rotor. The resulting pellet was referred to as the mitochondrial pellet. A microsomal pellet was obtained by centrifuging the post-mitochondrial supernatant at 100,000xg for 90 min in a Spinco Type 30 rotor. The post-microsomal supernatant was discarded.

The nuclear pellet was further fractionated by discontinuous sucrose gradient centrifugation. The pellet was dispersed in 20 ml 55% (w/w) sucrose, 25mM KCl, 2mM $MgSO_4$, 5mM Tris-HCl, pH 8.0, (55%(w/w)S-TKM) by homogenization in a 40 ml Potter-Elvehjem type teflon grinder with 4 strokes of a pestle rotated at 2500 rpm. Additional 55% (w/w) S-TKM, to a total volume of 74 ml, was slowly mixed in with a glass stir rod; 12.0 ml were delivered to each of six 38.5 ml cellulose nitrate centrifuge tubes, and 19.5 ml 40% (w/w) S-TKM and 7.0 ml 34% (w/w) S-TKM, respectively, were layered over the suspended nuclear pellet. The tubes were centrifuged in a Spinco SW-27 swinging bucket rotor at 100,000xg for 4 hours. The two interfaces were collected by pipetting from the top of the gradients with a straight-tipped Pasteur pipet. Each interface was mixed with an equal volume of 25mM KCl, 2mM $MgSO_4$, 5mM Tris-HCl, pH 8.0, (TKM) and sedimented by centrifugation at 100,000xg for 2 hours in a Spinco Type 30 rotor. The fractionation of the nuclear pellet resulted in three additional fractions - the two interfaces, designated as the 34/40 interface and the 40/55 interface and a pellet contianing nuclei at the bottom of the gradient tubes.

Cell fractions were washed one time in sea water prior to serotonin binding assays and transmission electron microscopy or one

in 0.05M Tris-HCl, pH 8.0, prior to enzyme assays. Nuclear and nuclear pellet fractions were subsequently collected by centrifugation at 5000xg for 20 min in a Sorvall HB-4 rotor while mitochondrial, microsomal, 34/40 interface and 40/55 interface fractions were sedimented by centrifugation at 100,000xg for 90 min in a Spinco Type 30 rotor. An aliquot of homogenate destined for use in enzyme assays was first dialysed against 1000 vol 0.05M Tris-HCl, pH 8.0, for 36 hours with three changes of dialysis solution.

All centrifugation operations required in embryo collection, cell dissociation, cell fractionation and transmission electron microscopical procedures were performed at 2°C-4°C. Sorvall SS-34 and HB-4 rotors were used in a Sorvall RC-5 centrifuge. Spinco Type 30, SW-27 and SW-50 rotors were used in Beckman L2-50 and L3-50 ultracentrifuges. Cell fractions were stored overnight at 2°C for use in serotonin binding assays and at -40°C for no longer than 14 days for microscopic and enzymatic studies.

[^{3}H]Serotonin Binding Assays

Binding to Cell Fractions. Cell fraction pellets were dispersed in sea water by homogenization in a 10 ml Potter-Elvehjem type teflon grinder. Cell fraction protein (0.1 mg-0.5 mg) was incubated with 5nM (30,000 dpm) radiolabelled serotonin (5-hydroxy [G-^{3}H]tryptamine creatine sulfate, New England Nuclear, 16.6 Ci/mmole) in a total volume of 0.2 ml containing 10 mM pargyline, a monoamine oxidase inhibitor. Control samples also contained 10 mM unlabelled serotonin. All chemicals were made up in sea water before addition to assay tubes. Pargyline was preincubated for 15 min with the cell

fractions before addition of other chemicals. Samples were incubated in triplicate in 15mm x 75mm disposable glass tubes on a shaker table for one hour at 23°C.

After incubation, fractions were quickly filtered over 2.4 cm Whatman GF/B glass fiber filters. Sample tubes and filter wells were rinsed once with 1.0 ml of sea water followed by two additional 1.0 ml rinses of the filter wells. Samples were prepared for scintillation counting according to Kobayaski (1980). Filters were dried overnight, placed in scintillation vials and incubated with 0.5 ml Protosol tissue solubilizer (New England Nuclear) at 60°C for 1.5 hours. Vials were cooled and 20 1 glacial acetic acid followed by 10 ml Econofluor (New England Nuclear) were added to each vial. Radioactivity content in [^{3}H]serotonin stock solutions was determined by counting aliquots of these solutions in 10 ml Biofluor (New England Nuclear).

Binding to Whole Cells. In the whole cell binding experiment the assay volume and chemical composition and the incubation method were the same as used in cell fraction binding studies. After incubation, 1.0 ml sea water was added to each of the assay tubes which were then centrifuged 1000xg for 2.5 min. Supernatants were removed and cell pellets were partially dispersed in 1.0 ml sea water and collected by centrifugation at 1000xg for 2.5 min. Supernatants were again removed and the sides of the tubes wiped clean. Centrifugations were performed at 2°C in a table-top centrifuge (Damon/IEC). Samples were digested and prepared for scintillation counting according to Kobayaski and Mandsley (1974). Cell pellets were each suspended in 0.5 ml Protosol tissue solubilizer (New England Nuclear)

and transferred to glass scintillation vials. An additional 1.0 ml Protosol was used to rinse each assay tube before being transferred to the vials. After incubation at 55°C for 48 hours, 0.1 ml 30% H_2O_2 was added to each vial and vials were incubated at 55°C for an additional 30 min. After cooling, 10 ml Econofluor (New England Nuclear) was added to each vial before being counted.

Scintillation vials from serotonin binding experiments were counted for 100 min each in a Beckman LS-100C liquid scintillation counter. Vials from cell fraction and whole cell binding experiments were counted with average efficiencies of 44% and 40%, respectively.

Transmission Electron Microscopy

Cell fraction samples were suspended in fixative for 1.5 hours at room temperature. The fixative solution was composed of 2.5% glutaraldehyde, sea water (pH 8.0) diluted in half and 0.09M NaCl. The osmolality of the fixative was 935 milliosmoles which closely approximates the osmolality of sea water (Maser et al., 1967). Fixed samples were collected by centrifugation at 85,000xg for 1.0 hour in a Spinco SW-50L rotor. The fixative was poured off and cell fraction pellets were partially dispersed in a few drops of 2% (w/v)agar (Noble). After cooling, the agar was sliced into small pieces which were washed in sea water and post-fixed in a solution containing 1% osmium tetroxide, seawater (pH 8.0) diluted in half, and 0.225M NaCl for 2.0 hours at room temperature. The osmolality of this fixative was 940 milliosmoles (Maser et al., 1967).

Post-fixed samples were rinsed briefly in 50% ethanol and dehydrated in an ethanol series - 50%, 70%, 95% and 3X in 100%, 15

min each. Samples were cleared by two changes of propylene oxide, 15 min each, placed in 2:1 followed by 1:2 mixtures of propylene oxide:plastic, 30 min each, and then into plastic for 30 min. The samples, in a final change of plastic, were placed in a desiccator jar for 17 hr followed by 20 hr in a 40° oven and 24 hr in a 60° oven. Sections were cut on a MT-2B Sorvall microtome using glass knives made with a LKB knife breaker, picked up on uncoated grids, and stained with 2% aqueous uranyl acetate followed by 5mM lead citrate, 0.05M NaOH. Sections were examined and micrographed in a Philips 300 electron microscope operated at 60 kv.

Enzyme Analysis and Protein Determination

5'-nucleotidase. The assay procedure was according to Cestelli et al. (1975). 5'-nucleotidase was assayed at 37°C in a total volume of 0.5 ml containing 5mM AMP (Sigma), mM EDTA and 0.05M Tris-HCl, pH 8.0. The reaction was stopped after 30 min by the addition of 1.0 ml 12% (w/w) cold trichloroacetic acid. Proteins were removed by centrifugation, and inorganic phosphate was determined by the method of Bonting et al. (1961). A 0.5 ml aliquot of phosphate solution was mixed with 0.5 ml color reagent made by adding 730 mg $FeSO_4 \cdot 7H_2O$, just prior to use, to 10 ml of 1% (w/v) ammonium molybdate, 1.15N H_2SO_4. After 10 minutes at room temperature, the optical density at 700 nm was measured and compared with standard phosphate to determine the amount of substrate hydrolysed. Enzyme activity was expressed as nanomoles of substrate hydrolysed per minute per milligram protein.

NADH Oxidase. This enzyme was assayed according to Avruch and Wallach (1971) as modified by Cestelli et al. (1975). NADH oxidase was assayed in a 1.0 ml volume containing 0.35 moles NADH (Sigma), 16 moles Tris-HCl, pH 7.4, 0.66 moles potassium ferricyanide, and enzyme suspension. The oxidation of NADH was followed by monitoring the decrease in optical density at 340 nm. Enzyme activity was expressed as nanomoles of substrate oxidized per minute per milligram protein.

Cytochrome c Oxidase. The assay procedure was according to Wharton and Tzagaloff (1967). One percent (w/v) ferrocytochrome c was produced by dissolving cytochrome c (Sigma) in 0.01M potassium phosphate buffer, pH 7.0, and reducing with a few milligrams of potassium ascorbate. Excess ascorbate was removed by dialysis against 1000 vol 0.01M potassium phosphate buffer, pH 7.0, for 24 hours with three changes of buffer. To each of two 1.0 ml cuvettes was added 0.1 ml of 0.1M potassium phosphate buffer, pH 7.0, 0.07 ml 1% ferrocytochrome c and 0.83 ml water. The blank cuvette was oxidized with 0.01 ml 0.1M potassium ferricyanide and the cuvettes were incubated at 37°C for 3 min. The reaction was initiated by the addition of 25 ul enzyme, and the decrease in optical density at 55 nm was measured. Enzyme activity was expressed as nanomoles of substrate oxidized per minute per milligram protein.

Protein Determination. Protein determination was performed by the methods of Lowry et al. (1951) and Bradford (1976) using bovine serum albumin (Sigma) as a standard.

All enzyme and protein assays were done in duplicate. In 5'-nucleotidase and NADH oxidase assays 0.05 mg - 0.8 mg protein were added per assay tube. In the cytochrome c oxidase assay 0.02 mg - 0.2 mg protein were added to each assay tube. All measurements were done with a Gilford 2400-2 automatic recording spectrophotometer. NADH oxidase and cytochrome c oxidase activities were monitored using a Gilford Electronik 196 chart recorder.

RESULTS

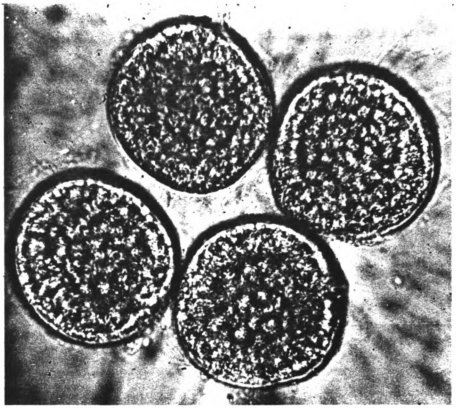
Embryo Culture

Embryos were cultured at a concentration of 2000 embryos/ml sea water, or approximately $7x10^6$ embryos per culture jar containing 3500 ml sea water. Denser cultures resulted in some embryo death and greater variation in extent of developement between embryos in the same culture vessel. Developmental variation due to temperature fluctuation was minimized by culturing embryos in a constant temperature chamber. In cell fraction serotonin binding experiments it was necessary to culture at least 2×10^7 embryos to the desired developmental stage. This made it possible to perform serotonin binding, microscopic, and enzymatic studies on the same batch of embryos and allowed for a better comparison between binding capacity and organelle composition of a particular fraction than would have been possible if these studies were performed on different embryo batches. In whole cell serotonin binding studies, approximately 4×10^6 embryos of each developmental stage were used.

Embryos were cultured for 24 (Figure 3), 36 (Figure 4), 48 (Figure 5), 60 (Figure 6) or 72 (Figures 7 and 8) hours after fertilization, corresponding to swimming blastula (SB), gastrula (G)(gut complete), late gastrula or prism (LGP)(change of larval axis from ovoid to polygonal), early pluteus larva (P2) and pluteus larva (P3), respectively. Staging and stage abbreviations are designated according to Harvey, 1956. Morphogenetic processes characteristic of

Figure 3. Unstained light micrograph of Arbacia punctulata swimming blastula embryos (24h). x 1580.

Figure 4. Phase contrast micrograph of an Arbacia punctulata gastrula embryo (36 h). x 630.



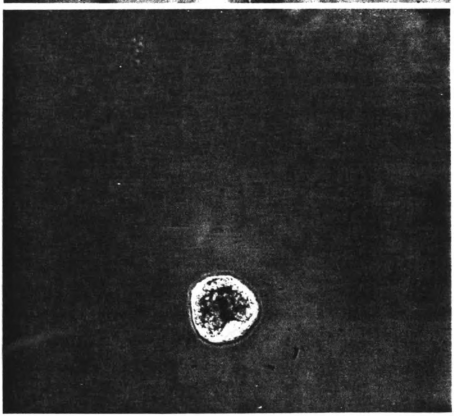
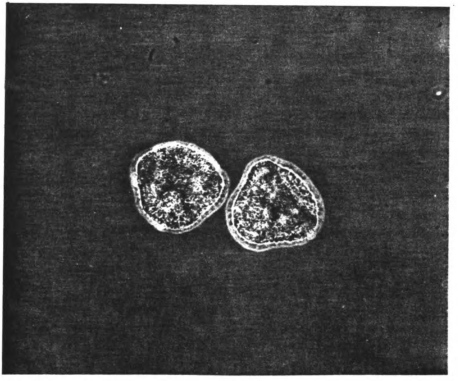


Figure 5. Phase contrast micrograph of Arbacia punctulata prism embryos (48h). x 810.

Figure 6. Phase contrast micrograph of an Arbacia punctulata early pluteus larva (60 h). x 1010.



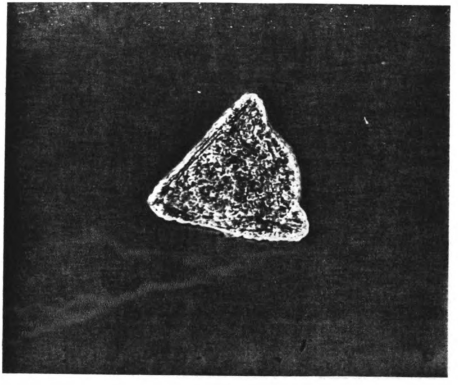
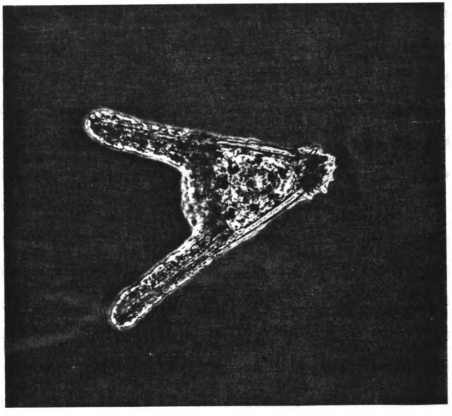


Figure 7. Unstained light micrograph of Arbacia punctulata pluteus larvae (72h). x 390.

Figure 8. Phase contrast micrograph of an Arbacia punctulata pluteus larva (72 h). x 1010.



these embryonic stages and significant in this study, as discussed in the introduction, are: formation of primary mesenchyme cells in 24 hour embryos, completion of gastrulation and release of secondary mesenchyme cells in 36 hour embryos, bending of the gut tube toward the oral surface and the initiation of coelom formation in 48 hour embryos, continuation of coelom formation and initiation of nerve cell formation in 60 hour embryos, and elaboration of neuronal cell processes in 72 hour embryos.

Cell Fractionation

Explanation of Methods. A cell fractionation technique was developed that resulted in the separation of six cell fractions from an embryo homogenate (Figure 1). The entire cell fractionation procedure was monitored by phase contrast microscopy. Most critical in obtaining the best separation and integrity of cell organelles was the homogenization procedure. The magnesium ion concentration in the homogenization medium was varied between zero and 5mM. Without magnesium or any other divalent cation in the homogenization medium cells were easily lysed, but most cell surface membranes disintegrated to microsomal size and nuclei clumped. With 5mM magnesium ions only 70% homogenization could be obtained. A compromise concentration of 2mM magnesium sulfate allowed for 95% homogenization, little nuclear clumping, and cell surface membrane fragments large enough to pellet with the nuclei and unbroken cells at low centrifugal force in the nuclear pellet fraction. Homogenization was performed immediately after suspension of embryos in the homogenization medium. Delayed

homogenization resulted in less complete cell lysis such that embryos only 75% of the cells of dispersed in homogenization medium for 5 min prior to homogenization could be broken.

The low speed pellet of the homogenate was quickly washed in a divalent cation free medium, which removed much of the mitochondrial and microsomal size particles adhering to cell surface membrane pieces and nuclei. While standard mitochondrial and microsomal fractions were being prepared, the nuclear pellet fraction was quickly dispersed in 55% (w/w) S-TKM. The time delay between homogenization and dispersal of the nuclear pellet fraction in 55% sucrose had to be made as short as possible since longer time periods resulted in some nuclear swelling and lysis, and loss of cell-surface membrane to the post nuclear supernatant. Sucrose concentrations used in the discontinuous gradient centrifugation were chosen to obtain the best separation from other organelles of nuclei and cell surface membranes in the nuclear and 34/40 interface fractions, respectively. Gradients were centrifuged for 4 hours at 100,000xg. Longer centrifugation times did not alter the distribution of particles in the gradient. Dispersal of the nuclear pellet fraction in sucrose concentrations greater than 55%, or centrifugation of the gradients for longer than 4 hours resulted in distorted nuclei due to nuclear shrinkage.

After sucrose gradient centrifugation, extensive variation in the patterns of gradients prepared from different stage embryos was observed. The 34/40 interface of 24, 48 and 72 hour embryos contained a grainy component as compared with the homogeneous appearance of this interface prepared from 37 hour and 60 hour

embryos. Approximately 40 times as much material was collected at the 34/40 interface of 37 hour and 48 hour embryos than at the same interface of 24, 60 and 72 hour embryos. This interface was bilayered only in 24 hour embryos. The 40/55 interface in 24, 37, 48 and 72 hour embryos contained three layers which could not be separated from each other during collection of this interface. The same interface in 60 hour embryos was composed of four layers. The top layer could be separated from the bottom three, and these two fractions were designated as 40/55 interface-top and 40/55 interface-bottom. Gradient profiles were reproducible; the same cell fractionation procedure, used in experiments not covered in this text, produced the same stage specific gradient profiles as those discussed above.

Enzymatic Analysis

The enzymes 5'nucleotidase, NADH oxidase and cytrochrome c oxidase were assayed as markers for cell surface membranes (Touster, 1970; Cestelli et al., 1975; McClay et al., 1977), endoplasmic reticulum and outer mitochondrial membranes (Sottocasa et al., 1967; Huber and Morrison, 1973) and inner mitochondrial membranes (Sottocasa et al., 1967), respectively. Enzyme activities, expressed as nanomoles of substrate hydrolyzed or oxidized per minute per milligram protein, were calculated, and the results of enzymatic analyses are summarized in Tables 1-5. Standard errors for 5'nucleotidase, cytochrome c oxidase and NADH oxidase assays averaged 3.8%, 5.9% and 5.4%, respectively. Differences in appearance of the same cell fractions isolated from different embryonic stages, as judged by

gross inspection and microscopy, were reflected in differences in organelle distribution within the cell fractions of various embryonic stages as determined by enzymatic analysis. Enrichment in the concentration of various subcellular particles in cell fractions over their concentration in the homogenate is given in parentheses in Tables 1-5.

5'-nucleotidase. Densities of cell surface membranes in the 34/40 interface fraction, as calculated from 5'-nucleotidase activities, were 1.6, 2.6, 2.6 and 5.6 times that of the homogenate in 36, 48, 72 and 24 hour embryos, respectively. At these developmental stages this fraction contained the greatest enrichment in cell surface membranes over the homogenate except for the microsomal fraction of 72 hour embryos with a 5'-nucleotidase specific activity of 3.4 times that of the homogenate (Table 5). In 60 hour embryos (Table 4) the highest 5'-nucleotidase activity was demontrated in the top layer of the 40/55 interface; the 34/40 interface fraction activity was only 0.8 times that of the homogenate. Cell surface membranes were distributed fairly evenly between microsomal, mitochondrial and crude nuclear pellet fractions of 24 (Table 1), 36 (Table 2) and 48 (Table 3) hour embryos. Of these fractions, the highest 5'-nucleotidase specific activity was located in the mitochondrial fraction of 24 hour embryos (4 times the homogenate), the microsomal fraction of 36 hour embryos (1.3 times the homogenate) as well as the microsomal and nuclear pellet fractions of 48 hour embryos (1.3 times the homogenate). In 60 and 72 hour embryos cell surface membrane distribution between these three fractions was not as uniform, and highest 5'-nucleotidase

Table 1

[1]Binding and enzymatic data were obtained from the same batch of embryos and are averages of triplicate and duplicate measurements, respectively, from one experiment.

[2]Each number in parentheses is a ratio of the specific activity of a fraction over that of the homogenate.

Distribution of [^{3}H] Serotonin-Binding Activity in Subcellular Fractions Isolated from the Homogenate of 24-Hour Sea Urchin Embryos

Cell fraction	Specific binding of [^{3}H]serotonin (DPM) per mg protein	Enzyme Specific Activity (nmoles/min/mg)		
		5'-Nucleotidase	Cytochrome c oxidase	NADH oxidase
Homogenate	136 ± 82	2.0	8.0	34.2 *
Mitochondrial	567 ± 159	7.9 (4.0)	69.6 (8.7)	204.7 (6.0)
Microsomal	693 ± 287	7.1 (3.6)	7.7 (1.0)	79.8 (2.3)
Nuclear pellet	343 ± 273	5.9 (3.0)	14.4 (1.8)	192.1 (5.6)
34/40 Interface	1739 ± 207	11.2 (5.6)	6.6 (0.8)	31.3 (0.9)
40/55 Interface	0 ± 450	10.7 (5.4)	15.6 (2.0)	108.6 (3.2)
Nuclei	788 ± 400	1.3 (0.6)	3.3 (0.4)	192.4 (5.6)

Table 2

[1]Binding and enzymatic data were obtained from the same batch of embryos and are averages of triplicate and duplicate measurements, respectively, from one experiment.

[2]Each number in parentheses is a ratio of the specific activity of a fraction over that of the homogenate.

Distribution of [^{3}H] Serotonin-Binding Activity in Subcellular Fractions Isolated from the Homogenate of 36-Hour Sea Urchin Embryos

Cell fraction	Specific binding of [^{3}H] serotonin (DPM) per mg protein	Enzyme Specific Activity (nmoles/min/mg)		
		5'-Nucleotidase	Cytochrome c oxidase	NADH oxidase
Homogenate	48 ± 84	5.1	33.6	95.6
Mitochondrial	1169 ± 266	5.9 (1.2)	127.3 (3.8)	542.1 (5.7)
Microsomal	0 ± 144	6.8 (1.3)	15.6 (0.5)	252.6 (2.6)
Nuclear pellet	542 ± 387	4.9 (1.0)	25.9 (0.8)	613.3 (6.4)
34/40 Interface	210 ± 135	8.2 (1.6)	25.0 (0.7)	121.2 (1.3)
40/55 Interface	0 ± 319	4.4 (0.9)	67.9 (2.0)	618.2 (6.5)
Nuclei	1023 ± 78	not detected	11.2 (0.3)	600.0 (6.3)

Table 3

[1]Binding and enzymatic data were obtained from the same batch of embryos and are averages of triplicate and duplicate measurements, respectively, from one experiment.

[2]Each number in parentheses is a ratio of the specific activity of a fraction over that of the homogenate.

Distribution of [^{3}H] Serotonin-Binding Activity in Subcellular Fractions Isolated from the Homogenate of 48-Hour Sea Urchin Embryos

Cell fraction	Specific binding of [^{3}H] serotonin (DPM) per mg protein	Enzyme Specific Activity (nmoles/min/mg)		
		5'-Nucleotidase	Cytochrome c oxidase	NADH oxidase
Homogenate	0 ± 223	8.2	11.2	28.1
Mitochondrial	0 ± 117	5.4 (0.6)	34.0 (3.0)	55.4 (2.0)
Microsomal	0 ± 300	10.3 (1.3)	4.4 (0.4)	436.0 (15.6)
Nuclear pellet	0 ± 320	11.0 (1.3)	24.0 (2.1)	145.3 (5.2)
34/40 Interface	958 ± 416	21.2 (2.6)	19.0 (1.7)	23.7 (0.8)
40/55 Interface	685 ± 369	13.1 (1.6)	44.6 (4.0)	76.7 (2.7)
Nuclei	1371 ± 599	5.1 (0.6)	30.2 (2.7)	267.2 (9.5)

Table 4

[1]Binding and enzymatic data were obtained from the same batch of embryos and are averages of triplicate and duplicate measurements, respectively, from one experiment.

[2]Each number in parentheses is a ratio of the specific activity of a fraction over that of the homogenate.

Distribution of [^{3}H]Serotonin-Binding Activity in Subcellular Fractions Isolated from the Homogenate of 60-Hour Sea Urchin Embryos

Cell fraction	Specific binding of [^{3}H]serotonin (DPM) per mg protein	Enzyme Specific Activity (nmoles/min/mg)		
		5'-Nucleotidase	Cytochrome c oxidase	NADH oxidase
Homogenate	295 ± 44	4.8	5.6	118
Mitochondrial	1524 ± 251	9.1 (1.9)	12.1 (2.2)	317 (2.7)
Microsomal	1223 ± 226	6.8 (1.4)	6.1 (1.1)	264 (2.2)
Nuclear pellet	158 ± 152	3.5 (0.7)	12.8 (2.3)	454 (3.8)
34/40 Interface	1423 ± 654	3.8 (0.8)	not detected	275 (2.3)
40/55 Interface				
Top layer	2183 ± 250	12.5 (2.6)	7.4 (1.3)	467 (4.0)
Bottom layer	0 ± 264	3.6 (0.8)	9.0 (1.6)	597 (5.0)
Nuclei	0 ± 96	1.1 (0.2)	9.6 (1.7)	371 (3.1)

Table 5

[1]Binding and enzymatic data were obtained from the same batch of embryos and are averages of triplicate and duplicate measurements, respectively, from one experiment.

[2]Each number in parentheses is a ratio of the specific activity of a fraction over that of the homogenate.

Distribution of [^{3}H]Serotonin-Binding Activity in Subcellular Fractions Isolated from the Homogenate of 72-Hour Sea Urchin Embryos

Cell fraction	Specific binding of [^{3}H]serotonin (DPM) per mg protein	Enzyme Specific Activity (nmoles/min/mg)		
		5'-Nucleotidase	Cytochrome c oxidase	NADH oxidase
Homogenate	273 ± 142	3.6	29.0	34.6
Mitochondrial	1068 ± 107	6.3 (1.8)	46.5 (1.6)	55.5 (1.6)
Microsomal	593 ± 112	12.3 (3.4)	24.8 (0.9)	123.0 (3.6)
Nuclear pellet	236 ± 150	3.3 (0.9)	9.9 (0.3)	62.2 (1.8)
34/40 interface	650 ± 139	9.3 (2.6)	15.0 (0.5)	8.6 (1.4)
40/55 interface	568 ± 109	5.7 (1.6)	35.3 (1.2)	110.5 (3.2)
Nuclei	0 ± 41	1.0 (0.3)	25.3 (0.4)	67.4 (2.0)

specific activities were located in the mitochondrial and microsomal fractions, respectively. 5'-Nucleotidase specific activities in the nuclear fraction ranged from undetectable in 36 hour embryos to 0.6 times that of the homogenate in 24 hour and 48 hour embryos.

Cytochrome c oxidase. Mitochondrial densities, as determined from cytochrome c oxidase activities, was greatest in the mitochondrial and 40/55 interface fractions. Specific activity values ranged from 1.6 to 8.7 times the homogenate in 24 to 72 hour embryo mitochondrial fractions. Mitochondrial densities in microsomal, 34/40 and nuclei fractions were, in general, less than the homogenate. Exceptions were the 34/40 interface fraction of 48 hour embryos (1.7-fold over the homogenate), the microsomal fraction of 60 hour embryos (1.1-fold over the homogenate) and the nuclei fractions of 48 hour (2.7-fold over the homogenate) and 60 hour (1.7-fold over the homogenate) embryos.

NADH oxidase. A quantitative determination of the endoplasmic reticulum purity in a particular cell fraction cannot be made from NADH oxidase specific activity values when it is assumed that this enzyme is localized in both endoplasmic reticulum and mitochondria. Relative purity over the homogenate of endoplasmic reticulum in various cell fractions can be estimated by subtracting the cytochrome c oxidase enrichment ratio value from the NADH oxidase enrichment ratio value. Using this calculation it can be demonstrated that the endoplasmic reticulum density in mitochondrial and 34/40 interface fractions was less than that of the homogenate except in the 36 hour

mitochondrial and the 60 hour 34/40 interface fractions. Endoplasmic reticulum densities were, on the average, highest in nuclear pellet, 40/55 interface and nuclear fractions, and of these three fractions highest endoplasmic reticulum densities were demonstrated in the nuclei fraction of 24, 36 and 48 hour embryos.

Microscopy

Phase contrast and electron micrographs were taken of some representative sucrose gradient fractions. Judging from phase contrast observations, the gradient did achieve an effective separation of nuclei and cell surface membranes in nuclear and 34/40 interface (40/55 interface-top in 60 hour embryos) fractions, respectively. Nuclear fractions of 24, 36, 48, 60 and 72 hour embryos are given in Figures 9-13, respectively. Nuclei appear intact and relatively free of cell surface membrane pieces or whole cells. The shiny rod shaped structures in 48 and 60 hour embryo nuclear fractions are skeletal spicules. The 72 hour embryo nuclear fraction was centrifuged 480xg for 2 min in an HB-4 rotor, and the nuclei were pipetted off from the heavier spicule layer before being micrographed. The 34/40 interface fractions (Figure 14) contained cell surface membrane fragments and few nuclei, while the 40/55 interface fractions (Fig. 15) contained a considerable amount of cell surface membrane as well as whole cells and nuclei. Electron micrographs of the 34/40 interface of 24 hour and 72 hour embryos are given in Figures 16 and 17, respectively, and confirm 5'-nucleotidase assays which indicated a greater purity of cell surface membranes in this fraction in 24 hour embryos than in 72 hour embryos.

Figure 9. Phase contrast micrograph of the 24 hour embryo nuclear fraction. x 2520.

Figure 10. Phase contrast micrograph of the 36 hour embryo nuclear fraction. x 1580.

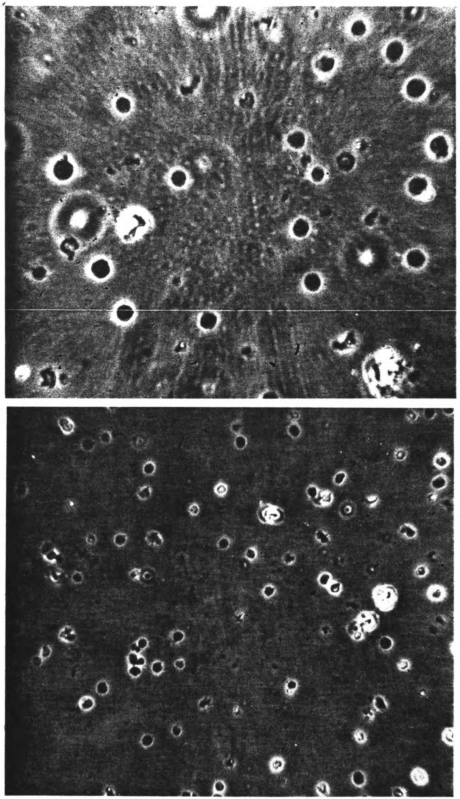


Figure 11. Phase contrast micrograph of the 48 hour embryo nuclear fraction. x 2020.

Figure 12. Phase contrast micrograph of the 60 hour embryo nuclear fraction. x 1010.

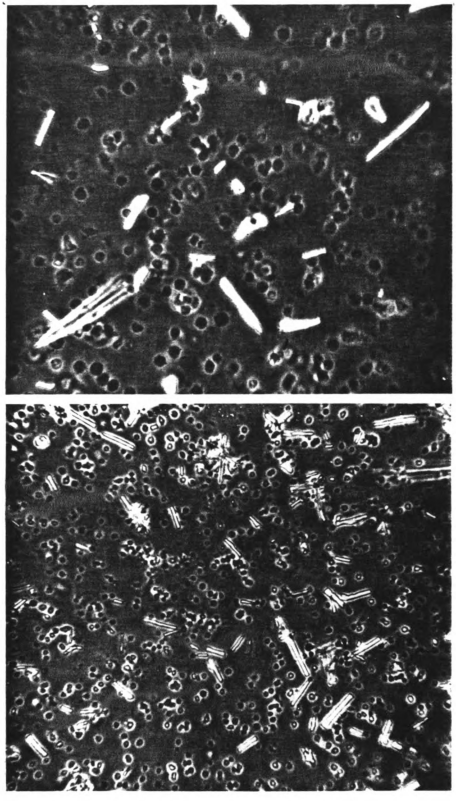


Figure 13. Phase contrast micrograph of the 72 hour embryo nuclear fraction. x 2520.

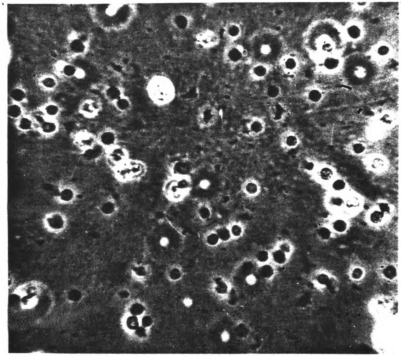


Figure 14. Phase contrast micrograph of the 48 hour embryo 34/40 interface fraction. x 2520.

Figure 15. Phase contrast micrograph of the 48 hour embryo 40/55 interface fraction. x 2520.

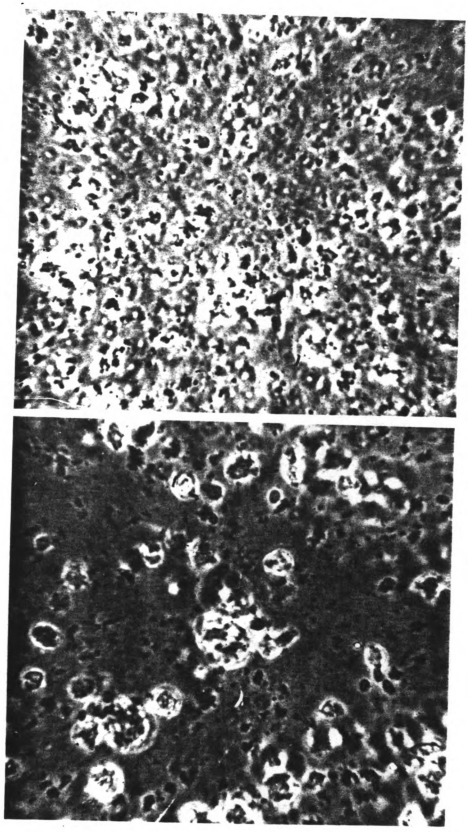
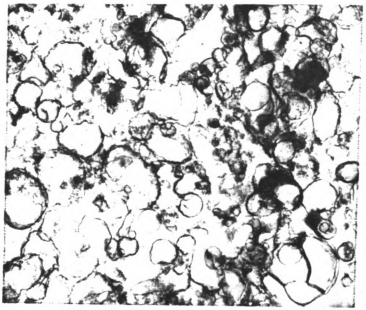
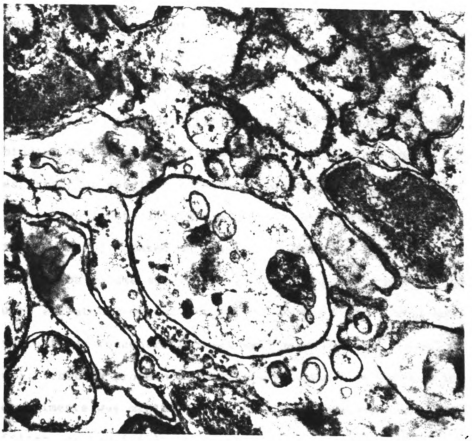


Figure 16. Electron micrograph of the 24 hour embryo 34/40 interface fraction. x 38,200.

Figure 17. Electron micrograph of the 72 hour embryo 34/40 interface fraction. x 53,800.





[^{3}H] Serotonin Binding Assays

Specific or saturable serotonin binding, defined as total binding (the amount of radioligand bound in the absence of excess unlabelled ligand)minus nonspecific binding (control) (the amount of radioligand bound in the presence of excess unlabelled ligand), (Bennett, 1978). In a pilot cell fractionation serotonin binding experiments 3.4 ug, 8.5 ug and 34 ug gastrula cell surface membrane fraction protein were each incubated with 10^{-9}M, $7x10^{-9}$M and 10^{-7}M radiolabelled serotonin in a total incubation volume of 1.0 ml. In this experiment, specific serotonin binding could not be detected at 10^{-9}M or 10^{-7}M radioligand. At 10^{-7}M [^{3}H] serotonin nonspecific binding was too high to obtain a significant difference from binding in controls. Specific binding could only be detected using 34 ug protein and 7 x 10^{-9}M radioligand, the dissociation constant for serotonin and rat brain serotonin receptors as estimated by Bennett and Snyder (1976); however, at this concentration of radioligand a considerable amount of specific binding to filter material was also detected. Several examples of saturable or specific binding to nonbiological materials are documented in the literature (Cuatrecasas and Hollenberg, 1975; Snyder, 1975). Based on results of the pilot experiment, the total incubation volume and radioligand concentration used in this study were set at 0.2 ml and 5 x 10^{-9}M, respectively. With this radioligand concentration and smaller incubation volume the amount of cell fraction protein or whole cells needed to perform binding experiments was minimized and specific serotonin binding to the filter was negligible.

Binding to Cell Fractions. Tables 1-5 list the specific binding of [^{3}H] serotonin, in disintegrations per minute (dpm) per milligram protein, to different cell fractions from 24, 36, 48, 60 and 72 hour embryos. Of the 30,000 dpm added to the incubation medium containing 0.1 mg-0.5 mg protein, a total of 500 dpm-1700 DPM were bound (100 dpm = 1 pg = 2.7 fmol) and specific binding approximated 20% of the total. Specific binding was detectable in various cell fractions from all embryonic stages studied.

In 72 hour embryos (Table 5)[^{3}H]serotonin bound specifically to all cell fractions except the nuclear fraction. The amount of radioligand bound to cell fractions from this stage correlated positively with 5'-nucleotidase activities of the fractions. Table 6 shows the specific [^{3}H]serotonin binding to 72 hour embryo cell fractions per milliunit of 5'-nucleotidase activity. Except for the mitochondrial fraction, these values were similar, suggesting that binding was to cell surface membranes. Binding to the mitochondrial fraction per milliunit of 5'-nucleotidase activity was approximately double that of the other cell fractions. This suggests that in addition to cell surface membrane sites other subcellular binding sites may be present in the mitochondrial fraction of 72 hour embryos. The total 72 hour embryo [^{3}H]serotonin binding content in cell fractions was calculated as the product of the specific binding per milligram protein and the total milligrams of protein in each fraction. Binding content data are given in Table 7. Approximately 60% of the total homogenate binding content was recovered in the mitochondrial (33%), microsomal (10%), and nuclear pellet (17%) fractions. Of the total nuclear pellet binding, 89%, 7% and 0% were

Table 6

Comparison between [^{3}H]Serotonin Binding and 5′-Nucleotidase Activity in Subcellular Fractions Isolated from 72-Hour Sea Urchin Embryos

Subcellular fraction	Specific binding of [^{3}H]serotonin per munit of 5′-nucleotidase activity [1]
Homogenate	75.8
Mitochondrial	169.5
Microsomal	48.0
Nuclear pellet	71.5
34/40 Interface	69.9
40/55 Interface	99.6
Nuclei	-

[1] One unit of enzyme activity is defined as the activity that liberates 1 μmole of P_i from AMP per minute.

Table 7

[^{3}H]Serotonin Specific Binding Recovery from 72-Hour Sea Urchin Embryo Cell Fractions

Cell fraction	Total protein (mg)	Specific binding (fmoles/mg protein)	Binding capacity (fmoles)	Recovery (% of homogenate)
Homogenate	614	7.4	4528	100.0
Mitochondrial	52	28.8	1500	33.2
Microsomal	27	16.0	432	9.5
Nuclear pellet	119	6.4	761	16.8
34/40 Interface	3	18.3	55	1.2
40/55 Interface	40	15.6	624	13.8
Nuclei	64	0	0	0

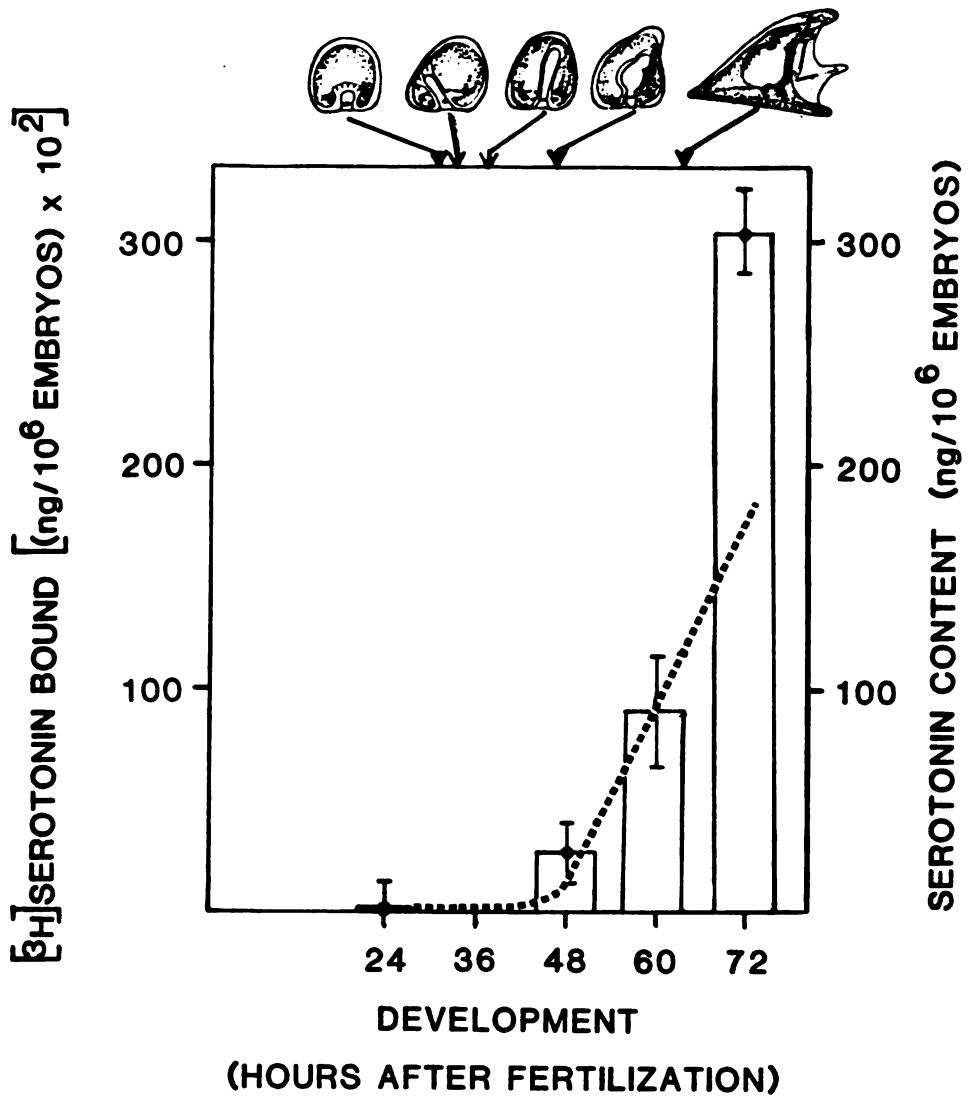
recovered in the 40/55 interface, 34/40 interface and nuclear fractions, respectively.

In 60 hour embryos (Table 4) specific [^{3}H]serotonin binding was demonstrated in all fractions except 40/55 interface-bottom and nuclei. Embryo cell fraction serotonin binding and 5'-nucleotidase activities showed less correlation than in 72 hour embryos. For example, binding to the 34/40 interface fraction was 4.8 fold over the homogenate, while the 5'-nucleotidase activity of this fraction was only 0.8 times that of the homogenate. In 48 hour embryos (Table 3) specific serotonin binding was demonstrated only in the 34/40 interface, 40/55 interface and nuclear fractions. Specific binding to the 48 hour embryo nuclear fraction per milliunit 5'-nucleotidase activity was approximately 5 times the values for 34/40 interface and 40/55 interface fractions. [^{3}H]serotonin bound specifically to 36 hour embryo mitochondrial, nuclear pellet, 34/40 interface and nuclear fractions, and the binding did not correlate with 5'-nucleotidase activities (Table 2). Binding was highest in mitochondrial and nuclear fractions. In 24 hour embryos (Table 1) specific serotonin binding was demonstrated in all fractions except the 40/55 interface. The amount of serotonin bound per milliunit 5'-nucleotidase activity was very similar for homogenate, mitochondrial, microsomal and nuclear pellet fractions. The 34/40 interface and nuclear fractions bound two and eight times as much serotonin, respectively, per milliunit 5'-nucleotidase activity than did the other fractions. [^{3}H]Serotonin binding did not correlate with mitochondrial densities in cell fractions from any of the embryonic stages studied.

Binding to Whole Cells. In this study 24, 48, 60 and 72 hour embryos were used. Total [^{3}H]serotonin binding to 2-9 x 10^6 whole, dissociated embryo cells was 1500 dpm - 4000 dpm, and specific serotonin binding, amounting to 14%-38% of the total, was only demonstrated for stages older than 24 hours. Figure 18 shows the binding capacity of Arbacia punctulata embryos, as determined from whole cell binding data, and serotonin content of Paracentrotus lividus embryos of similar developmental stages. Serotonin content data, determined by fluorometric and thin layer chromatographic techniques, were taken from Toneby (1977a). Both serotonin binding and serotonin content increased between 48 and 72 hours. Toneby could not detect serotonin in Paracentrotus lividus blastula and, in contrast to cell fraction binding studies, specific serotonin binding to Arbacia punctulata blastula cells could not be demonstrated even though more cell protein of this stage than other stages was added to assay tubes.

Figure 18. Comparison between serotonin content (---) in Paracentrotus lividus embryos and [^{3}H]serotonin binding to dissociated cells of Arbacia punctulata embryos. Serotonin content data and embryo figures were taken from Toneby, M. (1977a).

Numbers of cells from 24, 48, 60 and 72 hour embryos used in incubation media were 9.3×10^6, 2.1×10^6, 5.5×10^6 and 6.0×10^6 corresponding to 0.70, 0.07, 0.16 7 and 0.13 milligrams of protein, respectively.


[3H]SEROTONIN BOUND [(ng/10^6 EMBRYOS) x 10^2]
SEROTONIN CONTENT (ng/10^6 EMBRYOS)
300
200
100
24
36
48
60
72
DEVELOPMENT
(HOURS AFTER FERTILIZATION)

Discussion

Cell Fractionation

The relative ease with which sea urchin eggs can be obtained in large quantities, fertilized, and cultured has resulted in the widespread use of the sea urchin embryonic system in developmental research, especially in biochemical studies. Isolation of subcellular organelles, however, has proven difficult for embryonic systems in general, and despite the large volume of research on the sea urchin embryonic system, few cell fractionation techniques have been reported. Cell surface membranes have been isolated from fertilized and unfertilized Lytechinus pictus and Strongylocentrotus purpuratus eggs (Barber and Foy, 1973) as well as Paracentrotus lividus (Cestelli et al., 1975) and Lytechinus variegatus and Tripneustes eschulentes (McClay et al., 1977) gastrula. Cell cortices have been obtained from unfertilized eggs of Hemicentrotus pulcherrimus, Anthocidaris crassispina (Ohnishi, 1963), Pseudocentrotus depressus (Sakai, 1960), Strongylocentrotus purpuratus and Strongylocentrotus franscisianus (Vacquier, 1975; Begg and Rubhun, 1979); Detering et al. (1977) have obtained a cortical granule fraction from Strongylocentrotus purpuratus and Arbacia punctulata unfertilized eggs. Yolk granules from Strongylocentrotus purpuratus, Strongylocentrotus drobachiensus and Lytechinus pictus oocytes (Harrington and Easton, 1980) and nuclei from

from Strongylocentrotus purpuratus unfertilized egg through gastrula stages (Hinegardner, 1962) have been isolated. Several of these techniques can be applied only to specific species and/or embryonic stages. For example, the nuclear isolation technique of Hinegardner cannot be successfully applied to postgastrula stages (Hinegardner, 1962), and it has been difficult to apply the cell surface membrane isolation techniques of McClay and Cestelli to Arbacia punctulata gastrulae (author's results and personal communication with William Eckberg, Howard University, Washington, D. C.). In most studies, just as in the present one, it would be advantageous to obtain several cell fractions from the same embryo homogenate. Of the few organelle isolation procedures reported, many exclude the possibility of obtaining other cell fractions. The cell fractionation of unfertilized Strongylocentrotus interme [illegible] nvvh [illegible]helomin and Svetashev, 1978) is one of a very few studies which have succeeded in the separation and partial characterization of several crude subcellular fractions from a common sea urchin egg or embryo homogenate.

During the course of experiments described in this thesis, a subcellular fractionation procedure was developed, and cell fractions from Arbacia punctulata embryos were partially characterized by enzymatic and phase contrast as well as electron microscopical analyses. The same cell fractionation procedures were applied to five stages of embryonic development. This method resulted in the enrichment of various organelles in particular cell fractions, although the distribution of organelles in some cell fractions was stage specific. In general, the mitochondrial and microsomal

fractions were enriched in these organelles by the procedures used, and the discontinuous gradient centrifugation of the nuclear pellet fraction successfully separated nuclei from cell surface membranes in the nuclear and 34/40 interface fractions, respectively. In 60 hour embryos cell surface membranes were enriched in the top layer of the 40/55 interface. Although the cell fractions produced by this method are relatively crude, complete purification of cellular organelles is often not an absolute prerequisite for a variety of studies if the integrity of various organelles is maintained by the fractionation procedure and if their distribution and concentration are properly characterized. In this study, homogenization, washing and gradient media were carefully chosen to best preserve the integrity of various cell particles as judged by phase contrast and electron microscopy. Furthermore, marker enzymes were present in sufficient quantities to establish the usefulness of the cell fractionation methods used.

The merit of this fractionation technique as an initial step to the final purification of cell organelles must be considered for particular organelles of specific embryonic stages. In all stages studied a fairly clean nuclear fraction was obtained which could be further purified. The advantage over the Hinegardner (1962) nuclei isolation technique discussed previously is the ability to fractionate nuclei from postgastrula as well as gastrula and pregastrula stages. Only in 24 hour embryos are cell surface membranes sufficiently concentrated in a cell fraction (34/40 interface) and relatively free of other organelles to consider the use of this fractionation procedure as an initial step in the purification of cell surface membranes. The fractionation results provide a possible explanation

for the problems that have been encountered by several workers in obtaining a pure cell surface membrane fraction from sea urchin embryos, and from embryos in general, by differential and sucrose density gradient centrifugation methods. With the exception of 24 hour embryos, the concentrations of cell surface membranes in nuclear pellet fractions and corresponding homogenates were similar. In all stages cell surface membranes were fairly evenly distributed between the nuclear pellet, mitochondrial and microsomal fraction. This indicated that despite efforts to produce predominantly large membrane fragments which could subsequently be concentrated in the nuclear pellet fraction, approximately equal quantities of mitochondrial and microsomal size cell surface membrane fragments were also produced by the homogenization procedure.

[^{3}H]Serotonin Binding to Embryo Cell Fractions

[^{3}H]Serotonin bound specifically to some cell fractions from each of the five embryonic stages studied which ranged from late blastula to pluteus larva. The identification of serotonin binding sites in pregastrula and gastrula embryonic stages (24 and 36 hour embryos) and the detection of serotonin reported previously at these stages by bioassay (Buznikov et al., 1964) and histochemical fluorescence (Buznikov et al., 1972a) methods correlate well with studies which have demonstrated an inhibition of primary mesenchyme cell movement and gastrulation by mammalian serotonin receptor blocking agents (Gustafson and Toneby, 1970, 1971; Gustafson, 1973). Taken together, these studies strongly suggest that serotonin has morphogenetic function(s) in these embryos and that the inability of Toneby (1973,1977a,1977b) to

detect serotonin in these early embryos by chemical methods was probably due to assay sensitivity limitations. Alternatively, the present observations and all of the other studies discussed, including the serotonin assays of Toneby, would support the concept of the existence of a functional serotonin analog in 24 and 36 hour embryos if the analog and serotonin were similar enough chemically to compete for the same binding sites. In postgastrula stages all serotonin content studies have demonstrated a continuous increase in serotonin levels from the end of gastrulation through formation of the pluteus larva, and in the present investigation [^{3}H]serotonin binding sites were demonstraed during this developmental period in 48, 60 and 72 hour embryo cell fractions. Results of this cell fraction binding study, along with serotonin content and inhibitor studies, suggest that serotonin, or an extremely closely related serotonin analog, functions as in adult tissues by first binding to a distinct biological recognition site (receptor molecule).

Comparisons between [^{3}H]serotonin binding capacity and organelle distribution in cell fractions were made in an attempt to determine the subcellular location(s) of binding sites, knowledge of which would contribute to a better understanding of the role of serotonin in early sea urchin development. In 72 hour embryos a positive correlation between serotonin binding and 5'-nucleotidase activities in various cell fractions (except the mitochondrial fraction) was demonstrated. This suggests that [^{3}H]serotonin bound to cell surface membranes, although the possibility that binding sites were localized on some other cell component that distributed similarly to cell surface membranes among the various cell fractions

cannot be unequivocally ruled out. Along with studies which have demonstrated, in embryos of this stage, the beginnings of a nervous system (Ryberg, 1977; Ryberg and Lundgren, 1979) which probably eventually contains a serotonergic neuronal component as suggested from pharmacological studies on more advanced plutei (Gustafson et al., 1972a), the results of this study suggest that the binding sites detected in 72 hour embryos are specific cell surface serotonin receptors which may mediate or eventually mediate serotonergic neurotransmission. The correlation between [^{3}H]serotonin binding and its associations with the presence of cell surface membranes is less distinct in embryos younger than 72 hours.

At several developmental stages at which serotonin binding and 5'-nucleotidase specific activities seemed to parallel one another in some cell fractions, binding to 34/40 interface, mitochondrial or nuclei fractions per milliunit 5'-nucleotidase specific activity was greater than in other fractions. This suggests that binding sites other than or in addition to cell surface sites were present in these fractions. Several serotonin inhibitor studies discussed in the Introduction led the investigators to propose that mitochondrial serotonin receptors were present in early cleavage state embryos. In the present experimental observations no correlation between serotonin binding and the presence of mitochondria, as determined from cytochrome c oxidase content, was observed in any cell fraction from any of the developmental stages examined. While the possibility that serotonin recognition sites are located on mitochondria and also on other organelles which are distributed among cell fractions differently than mitochondria cannot be ruled out, the results

suggest that mitochondrial binding sites are not present in these embryos.

Ten-Cate (1952) pointed to an incongruity between the proteins synthesized in the early sea urchin embryo and the composition of the yolk granules expended, leaving a surplus of aromatic amino acids not accounted for by protein synthesis. Gustafson and Toneby (1971) postulated that these aromatic amino acids were precursors for serotonin production in yolk granules of sea urchin embryos. Although the presence of yolk granules in mitochondrial fractions was not examined in the present investigation, the same fraction, prepared from both unfertilized and fertilized sea urchin eggs by experimental procedures similar to those used in this study (Chemomin and Svetashev, 1978), was shown to contain a greater yolk granule density than nuclear pellet or microsomal fractions. Yolk granule serotonin storage sites may account for a significant amount of serotonin binding to mitochondrial fractions and possibly to other fractions as well.

Most interesting is the relatively large amount of [^{3}H]serotonin binding to 24, 36 and 48 hour embryo nuclear fractions when contrasted with the total absence of binding to this fraction in 60 and 72 hour embryos. In younger embryo nuclear fractions, the density of endoplasmic reticulum was greater than in 60 and 72 hour nuclei. The possibility that binding sites on endoplasmic reticulum accounted for the binding to nuclear fractions of the younger embryos is not supported since no binding to 48 hour and 36 hour embryo microsomal fractions was detected. Furthermore, the microsomal fraction from 36 hour embryos was more enriched in

endoplasmic reticulum than any other fraction from any developmental stage. The likelihood of the existence of nuclear serotonin binding sites in 24, 36 and 48 hour embryos which disappear at later stages is supported by these data. This may indicate the involvement of serotonin in nuclear processes in these earlier stages. Buznikov (1971) postulated that in early cleavage stage embryos serotonin may affect protein synthesis by reducing nuclear membrane permeability to mRNA and tRNA. Emanuelsson (1974,1976) demonstrated the presence of serotonin in nuclei of early cleavage stage Ophryotrocha polychaete embryos as well as gastrula and postgastrula chick embryos and suggested a nuclear function for serotonin in these embryos. These reports also localized serotonin in lipid droplets, plasma membranes and microfilaments and demonstrated the greatest serotonin concentration in yolk granules.

[^{3}H]Serotonin Binding to Dissociated Embryo Cells

The increase in the amount of serotonin bound to dissociated cells of embryos between postgastrula and pluteus stages correlated with an increase in sertonin content between these stages in other sea urchin species as measured by the chemical assays of Toneby (1973,1977a, 1977b) and the biological assays of Buznikov et al. (1976). This agreement strengthens the conclusions drawn from the cell fractionation binding studies which demonstrated specific serotonin binding to several cell fractions from 48, 60 and 72 hour embryos. Surprisingly, no binding to late blastula (24 hour) embryo cells was detected even though significant binding was demonstrated

in most cell fractions from this stage. Judged on the basis of cell fractionation binding studies, the [^{3}H]serotonin binding capacity of 24 hour embryos was roughly one-half that of 72 hour embryos as estimated from homogenate binding activities. In the 24 hour embryo intact cell serotonin binding assay, 50% more cells, corresponding to approximately 3 times the number of embryos and 5.5 times the amount of protein, were used than in the 72 hour whole cell binding assay. If the binding sites labelled in the 24 hour cell fraction binding study were equally accessible to [^{3}H]serotonin in the whole cell binding assay, they should have been detected in this study.

Among the possible explanations for the discrepancy between 24 hour embryo cell fraction and whole cell [^{3}H]serotonin binding data, the most obvious one would be to assume that 24 hour embryos contain only intracellular binding sites which, due to poor cell surface permeability to [^{3}H]serotonin, remained inaccessible to the label after a 60 minute incubation period. This explanation cannot be supported or ruled out since information concerning the permeability of blastula sea urchin embryos to the radiolabelled creatine sulfate-serotonin complex used in this thesis work is not available; however, studies have shown that serotonin is readily permeable to cleavage stage embryos. In early cleavage stage embryos exogenously added serotonin at 50 g/ml has been shown to rapidly accumulate into Arbacia lixula, Strongylocentrotus intermedius and Strongylocentrotus drobachiensus embryo cells, reaching a maximum concentration of 500 ng/10^6 embryos in 10 min (Manukhin et al., 1973). Some antiserotonins could arrest cleavage after a 50 min incubation with embryos, and this effect was diminished or eliminated

if serotonin was also added to the embryo culture (Buznikov et al, 1970). This observation, if it is assumed that the antidotal effect of serotonin is due to its interaction with intracellular receptors as discussed in the Introduction, also suggests that serotonin can readily penetrate into cells of early cleavage stage embryos. It is also possible that the cell dissociation procedure either removed or altered the accessible serotonin binding sites in 24 hour embryo cells.

One other explanation for the lack of [^{3}H]serotonin binding to dissociated 24 hour embryo cells deserves attention. Buznikov et al., (1972b,1975,1976) described a sensitivity phenomenon related to embryo density in which an increase in the number of cleavage stage embryos in the culture reduced their sensitivity to antiserotonins. The inhibitory effects of antiserotonins on cell division and protein synthesis could be completely reversed by raising the embryo concentration, and further increases in embryo density often resulted in a stimulatory effect. Density sensitivity was demonstrated in embryos up to the late blastula stage and was a result of nonspecies specific protective factor(s) (AN-factor(s)) which were secreted into the culture medium by embryo cells (Buznikov et al., 1971b). Embryos that had been previously lysed by osmotic shock could not release AN-factor(s) as judged by their inability to protect intact embryos from the cytotoxic actions of various antiserotonins (Buznikov et al., 1977). It was demonstrated that AN-factor(s) were normally released into the medium by untreated embryos, but this release was accelerated if antiserotonins were also present in the culture. Although serotonin was released by antiserotonin stimulated embryos,

its concentration was too low to account for the protective effects observed. An endogenous ganglioside fraction was isolated from cleavage stage Arbacia punctulata, Strongylocentrotus intermedius, Stronglylocentrotus nudus and Paracentrotus lividus embryos that mimicked the effects of the protective factor(s) (Buznikov et al., 1973a, 1973b). Buznikov et al. (1973c) have postulated that the AN-factor(s) participate in the regulation of the functional activity of intracellular serotonin as its endogenous antagonist which decreases or stops the effect(s) of serotonin on corresponding receptive structures. In the light of these studies, the lack of serotonin binding to late blastula dissociated cells could be due to the formation of endogenous serotonin binding site competitors (AN factor(s)) which would be heavily concentrated in the dense cell suspensions used in this cell binding study.

Serotonin in Embryonic and Nonembryonic Systems

The function(s) of serotonin in embryonic systems may be better understood by first examining the information available concerning its roles in nonembryonic systems. The proposed roles for serotonin in sea urchin embryos between the blastula and pluteus stages as reviewed in the Introduction involved the stimulation of ciliary activity, cell movements and cell shape changes, and neurotransmission. All of these functions are also mediated by serotonin in many nonembryonic systems. In adult vertebrate and invertebrate animals serotonin is a neurotransmitter in leech giant Retzius cells (Erwin and Thies, 1975), snail central neurons (Gerschenfeld and Paupardin-Tretsch, 1974), mollusk (Cedar and Schwartz, 1972) and

cockroach (Nathanson and Greengard, 1974) ganglia, mammalian gastrointestinal tract (Gershon, 1979; Burnstock, 1981) and mammalian brain (Bennet and Snyder, 1976; Enjalbert et al., 1978a, 1978b; Quayle et al, 1978; MacDermot et al., 1979). In the green alga, Euglena gracilis (Keirns et al., 1973), and the flagellated protozoan, Tetrahymena pyriformis (Jankidevi et al., 1966), serotonin is a probable trigger for cell motility. Serotonin stimulates motility of mammalian glial cells (Hertz et al., 1979) and chemotaxis of fetal mouse palatal cells (Clark et al., 1980). In mollusks serotonin increases the beat frequency of gill cilia. Gosselin (1966) gave evidence that serotonin functions as a local hormone in the mollusk gill, not a neurotransmitter, and acts as a pacemaker through rhythmic synthesis and degradation. Serotonin is actively taken up by platelets and is involved in platelet cell shape changes and aggregation (Mester and Mester, 1978; Mester et al., 1979; Chuang et al., 1976). As a local hormone in mammalian tissues serotonin is important in the constriction of small vessels following vascular injury (Little, 1977), and blood borne serotonin acts directly on microfilament bundles of blood vessel endothelium (Buonassisi and Venter, 1976). Interestingly, the postulated functions of serotonin in preneural sea urchin embryos are often also performed in nonembryonic systems as a result of the local production of serotonin within the cell or group of cells affected and are not related to serotonergic neurotransmission.

As previously discussed, serotonin released from pigment cells was proposed to be the stimulus for ciliary activity, for cell movements that result in coelom formation and separation of certain

ciliated cells from the rest of the ectoderm, as well as for subsequent cytoplasmic movements leading to the outgrowth of nerve processes from these ciliated ectodermal cells. Ryberg, 1979, suggested that light activation of pigment granules may induce serotonin release from pigment cells. This process may be comparable with serotonin mediated photoreception in the chick retina, which involves the activation of adenyl cyclase (Hauschild and Laties, 1973). The cytoplasmic movements observed during neuron elongation in sea urchin embryos (Gustafson, 1969) were similar to serotonin induced cytoplasmic flow in nerve processes of mammalian brain cell cultures (Geiger, 1960,1963).

Mechanism of Serotonin Action in Early Sea Urchin Embryos

Conservation of the same functions for serotonin in embryonic and adult systems may also imply that the chemical mechanism of serotonin actions in early embryos and adult tissues are similar. In the adult tissues in which the mechanism of serotonin action has been examined, it has been demonstrated that serotonin must bind to a cell surface receptor molecule to exert its effect. Formation of a serotonin receptor complex triggers either a direct alteration in membrane permeability or the activation of adenyl cyclase and production of cyclic AMP. In some cases serotonin stimulated cyclic GMP production in tissue cells. Little is known about the mechanism(s) by which cyclic AMP mediates serotonin functions. It is generally assumed, however, that the mediation process is similar to that of cyclic AMP induced in several cell types by a variety of hormones. In these studies two mechanisms of cAMP action have been

uncovered. In Escherichia coli cyclic AMP regulates the expression of some genes by activating a cyclic AMP binding protein that stimulates the transciption of a defined set of genes by directly interacting with the trancriptional process (Emmer et al., 1970). In both procaryotes and eucaryotes, cyclic AMP dependent protein kinases, when activated by cyclic AMP, transfer a phosphate group from ATP to proteins, including histones and enzymes. Enzymes may be activated or inactivated (Sutherland, 1972). There is evidence that some of the effects of serotonin in brain may be modulated by cyclic AMP-stimulated protein kinase activity which phosphorylates synaptic membrane proteins (Routtenberg and Ehrlich, 1975; Bloom, 1975). Cyclic AMP modifies the permeability of a cell to inorganic ions, and inorganic ions are necessary for the generation and destruction of cyclic AMP and for the action of cyclic AMP-dependent protein kinases and phosphatases (for a review of cyclic AMP-inorganic ion interaction see McMahon, 1974).

The processes which cyclic nucleotides regulate in adult systems are numerous and include among others, RNA, protein and DNA synthesis, cell growth, ion permeability, contractility, membrane potential and metabolism (Sutherland, 1970; Butcher et al., 1968). These processes completely overlap the characteristic processes of development and differentiation. Inorganic ions have been demonstrated to be important in several developmental processes. Barth and Barth (1972) have shown that the cations Li^{+}, Na^{+} or Ca^{++} induce neuron differentiation from prospective amphibian gastrula epidermal ectoderm cells. In echinoderm gastrulae calcium is necessary for secondary mesenchyme cell pseudopodal activity (Dan

and Okazaki, 1956), an event in which serotonin has been proposed to be involved. In early embryos of sea urchin and other animals serotonin may, as in adults, exert its effects by directly modifying cell surface or intracellular membrane permeability to various inorganic ions, or by stimulating the synthesis of cyclic nucleotides which then mediate developmental processes by chemical mechanisms similar to those used in adult animals. As previously discussed, a wealth of indirect data support the intracellular location of serotonin receptors in early cleavage sea urchin embryos. Although the serotonin receptor and adenyl cyclase molecules are usually cell surface components of adult tissues, intracellular adenyl cyclase activity has been demonstrtated on intracellular membranes of rabbit skeletal (Rabinowitz et al., 1965) and dog cardiac (Entman et al., 1969) muscle and in the cytoplasm of the bacterium, Brevebacterium liquifascien (Hirata and Hayaishi, 1965). A few experiments have suggested that the effects of serotonin on early cleavage sea urchin embryos involve cyclic AMP. In the sea urchin Strongylocentrotus intermedius, cyclic AMP (Persianinov et al., 1975) and prostaglandin F_2 (Persianinov et al., 1973) were shown to have a protective action against the cytotoxic effects of serotonin antagonists on early cleavage. Prostaglandin F_2 stimulates adenyl cyclase in several mammalian tissues (Hinman, 1972). Cholerotoxin stimulates adenyl cyclase in a variety of biological systems after binding to cell membrane gangliosides (vanHeynigen, 1977). The response of rat stomach to serotonin is mediated by ganglioside serotonin receptors (vanHeynigen, 1974). As previously discussed, in early cleavage embryos a protective action of gangliosides against cytotoxic

antiserotonin effects was demonstrated. McMahon (1974) has proposed that the differentiation path of embryonic cells of most, if not all, organisms is determined by their content of inorganic ions and cyclic nucleotides which is regulated by inducer molecules. He postulated that early embryonic inducers were the same molecules which modulate adult tissue activities, hormones and neurotransmitters.

The first data obtained on the binding of [^{3}H]serotonin to late blastula, gastrula, prism and pluteus sea urchin embryo cell fractions and dissociated cells are presented in this thesis. A serotonin binding assay procedure was developed that allowed the detection of serotonin binding sites in cell fractions and dissociated cells. Additional binding studies should help determine if the binding sites described are the receptors which directly mediate serotonin effects, or if they represent uptake and/or storage sites or biologically nonfunctional sites. The cell fractionation procedure developed during the course of the experiments described may provide an initial step to the final purification of many cell organelles so that binding studies can be performed on pure cell fractions. Since chemicals generally thought of as neurohormones have been identified in preneural embryos of several animal phyla, including mammals, the sea urchin embryonic system may serve as an exceptionally convenient model for the study of universal mechanisms of neurohormone-modulated developmental processes in early animal embryos.

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