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## **The Chemical Basis of Sea Urchin Embryogenesis**

SØREN LØVTRUP

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## I. Introduction

Embryogenesis may for convenience be resolved into two separate, although not independent components, viz., morphogenesis proper, and the various chemical processes which occur simultaneously.

As to the former, it may be characterized as a spatio-temporal process, in which cells and supracellular structures are involved. The mechanisms involved in sea urchin morphogenesis are known in great detail, not the least due to the recent work of GUSTAFSON and his collaborators (cf. reviews HÖRSTADIUS 1939; DAN 1960; GUSTAFSON 1961; GUSTAFSON and WOLPERT 1963).

The chemical activities in the egg can be regarded from two different angles, each presenting problems of great interest. First, compared with other cells, the oocyte is a very large cell in which most of the substances present represent inert reserves to be used for sustaining the embryonic development up to the time when the larva can begin to take up food from the environment. Among the reserves in the egg one may distinguish two fractions, one which is degraded in order to supply the energy necessary for the embryogenetic processes, and one which is transformed from inert substances to integral parts of the embryo. This differential utilization of the reserve materials will be discussed in the present paper.

However, the chemical changes may also be regarded from another point of view. The chemical activity leading to changes in the embryonic body comprises two different processes, growth and differentiation. The former leads to the increase in cell number, as well as to increase in the contents of the individual cells. The second, and more interesting process is represented by the sorting out of the total cell population into various groups of cells with different properties. Since the latter must depend upon the constituents of the cells, it follows that differentiation is associated with the acquisition of new synthetic capacities, different for each of the new kinds of cells which arise.

Correlation between morphogenesis and chemical activity may thus give information about the chemical nature of the differentiation processes on the cellular level. In a recent review of the biochemistry of morphogenesis, WRIGHT (1964) expressed the view that as to the interpretation of this correlation "a consistent picture is not yet obvious" (l.c. p. 59). It is my belief that this shortcoming in part depends upon the fact that too little attention has been focused upon the cell transformations and differentiations occurring at the cellular level. Since the chemical differentiation is directly dependent upon differential cellular activity it is necessary to embark upon the ana-

lysis of the chemical patterns with very clear notions about the pattern of cell differentiation. Before we discuss the chemistry of the developing sea urchin embryo we shall therefore devote some attention to this question.

## II. Cell differentiation

WILLMER (1960) has pointed out that all cells may be referred to a few basic cell types, and has emphasized the importance of these cells for ontogenesis and phylogenesis. If WILLMER's views are accepted then it follows that cell differentiation may be resolved into two phases, first a segregation with respect to the basic cell classes, and subsequently differentiation of the cells within each class along separate lines, to give rise to various types of tissue cells. I have recently shown that in the amphibian embryo the first differentiation process, called cell transformation, corresponds to the phase of determination or primary differentiation, whereas the second stage is that of functional or tissue differentiation. It could furthermore be shown that the pattern of cell segregation is a function of the polarities in the egg (1966).

I shall not here enter upon a similar discussion pertaining to the sea urchin embryo, but for the subsequent discussion it will be necessary to outline the cell class concept.

If the properties of a certain cell type, including its potential transformation into other cell types, has formed the basis of phylogenetic evolution, then these same properties must be responsible for ontogenetic development. In other words, the archaic cell type which once gave rise to metazoan evolution, must be represented today by the egg cell. I have previously suggested that the egg is an amoebocyte, supporting this view on recorded observations on eggs and isolated blastomeres (for references cf. 1965b). The mobility of certain oocytes, the separation of the early blastomeres in many cases, and the absence of desmosomes during the first hours of development (WOLPERT and MERCER 1963) are other traits favouring this suggestion. Further support of the view that the amoebocyte represents the basic omnipotent cell type, which through transformations, reversible as well as irreversible, may be changed into various differentiated cell forms, can be found in textbooks of zoology, very well known cases are regeneration in coelenterates and planaria. It should just be mentioned that the typical traits of an amoebocyte is that it is a solitary cell, and that it forms lobopod pseudopodia. The shape is very varying, but it may be postulated that the fundamental cell shape is spherical.

The arguments advanced here pertain only to the egg cell, not to the spermatozoon, which is an epitheliocyte (flagellate form). Although the reversible transformation amoebocyte  $\rightarrow$  epitheliocyte is known to occur in protozoa (cf. WILLMER 1960), there is reason to believe that a similar, but irreversible transformation occurs during ontogenesis. Whether or not the transformation leading to the formation of spermatozoa is reversible remains

to be seen, but it is interesting to note that chemically the spermatozoan seems to represent a higher level of development than the egg cell (cf. below).

The typical epitheliocyte is basically a spherical cell, like the amoebocyte, and is furthermore characterized by at least one of the following properties: high intercellular adhesion, formation of cilia or one to several flagella. Epitheliocytes may exist in three different forms: non-adhesive forms may be solitary ciliated or flagellated cells (e.g. protozoa and spermatozoa), whereas the adhesive cells may either be attached to a basement membrane and form a two-dimensional organization, or exhibit a three-dimensional organization, in which the cells are exposed to other cells on all sides. In the latter case no cilia are formed, in the former cilia formation may occur at the free side opposite to the basement membrane (cf. Fig. 1). It should perhaps be mentioned that these are basic organization forms, which during development may be modified in numerous ways.

The amoebocyte may also transform into a mechanocyte or fibrocyte, a solitary cell characterized by the formation of filiform, contractile pseudopodia, in contrast to the loboform pseudopodia of the amoebocyte. The mechanocyte is often oblong whereas the amoebocyte, as mentioned, may be considered to be basically spherical. The cell shape is not a very distinct criterion, being subject to great variations, thus a mechanocyte may very often be stellate, and an amoebocyte oblong.

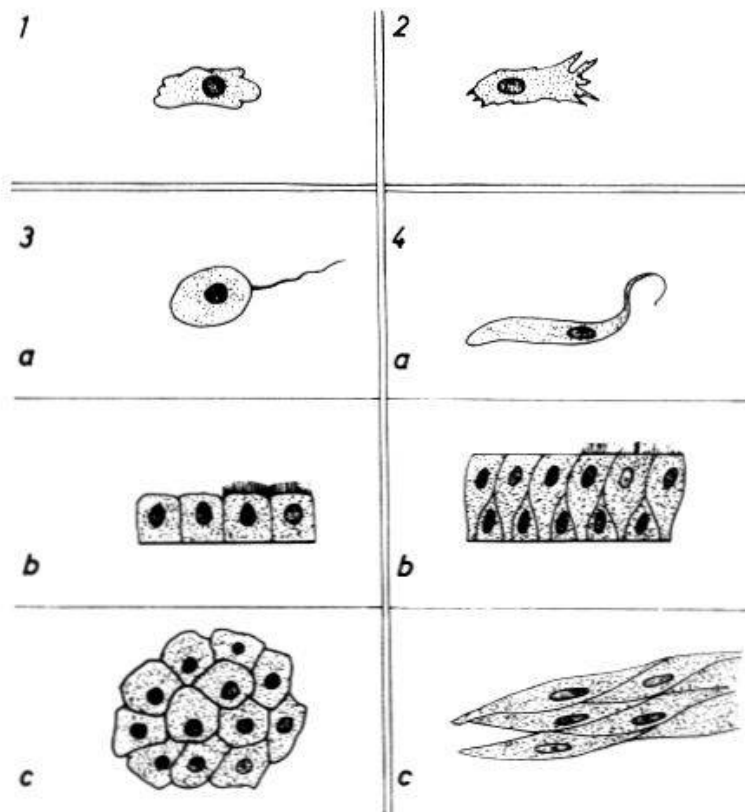


Fig. 1. Schematic illustration of the four cell classes.—1 = amoebocyte (sl-cell), 2 = mechanocyte (sf-cell), 3 = epitheliocytes (cl-cells), 4 = mechano-epitheliocytes (cf-cells); a = flagellate form, b = two-dimensional organization, c = three-dimensional organization. The name "mechanocyte" is used here in a meaning different from the one originally suggested by Willmer (1960).

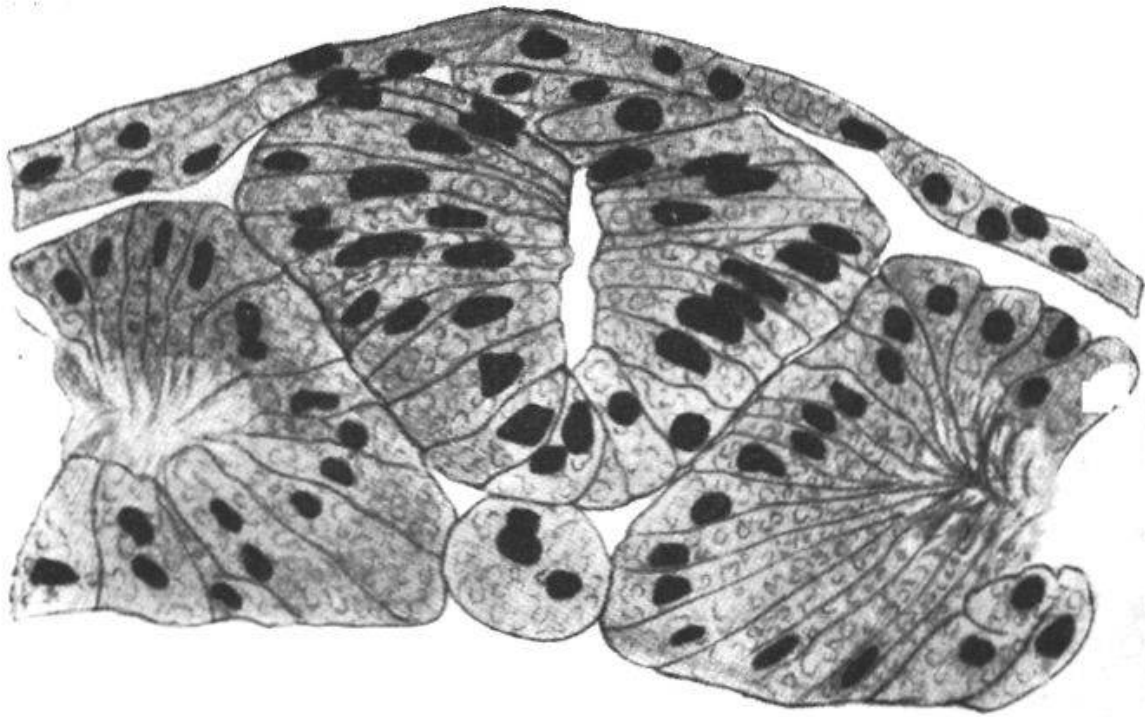


Fig. 2. Transversal section through a tail-bud embryo of Triton sp. showing that the myotome cells are flagellated (from LEHMANN: Arch. Entwickl.-mech. Org. 113 [1928]).

If both transformations occur in the same cell we shall get a spindle shaped or at least oblong cells with high intercellular adhesion and (or) forming cilia or a flagellum. The free-living flagellate form is, I believe, found in a certain type of protozoa, viz., the trypanosoma. A flagellated, adhesive form has been observed in the myotomes during amphibian development (LEHMANN 1928; cf. Fig. 2). The two other organization forms are present in many tissues, e.g., in the intestinal wall, and in smooth muscle, respectively (cf. Fig. 1).

This cell type, a mechano-epitheliocyte, has no proper name (cf. LØVTRUP 1966). As a matter of fact one meets with great difficulties to arrive at a nomenclature which is completely unambiguous. In the paper mentioned I have suggested using solo-lobocytes (sl-cells) for the amoebocytes, colligo-lobocytes (cl-cells) for the epitheliocytes, solo-filocytes (sf-cells) for the mechanocytes, and colligo-filocytes (cf-cells) for the last cell type. This nomenclature is based on the assumption that the four cell types represent the four possible results of the two transformations to which the amoebocyte may be subjected. The first transformation amoebocyte (sl) → epitheliocyte (cl) is concerned with the intercellular adhesion, hence the change of the prefix from solo- to colligo-; the latter is derived from latin *colligere*, to collect or gather. As we have just seen, this name is not quite unambiguous, since solitary cl-cells exist, e.g., the flagellates. I think this difficulty may be circumvented by letting the "c" refer not only to the adhesive properties, but also to the ability to form cilia (or flagella). It might be mentioned that the transformation s → c obviously is concerned not only with adhesion, but also with cortical tension.

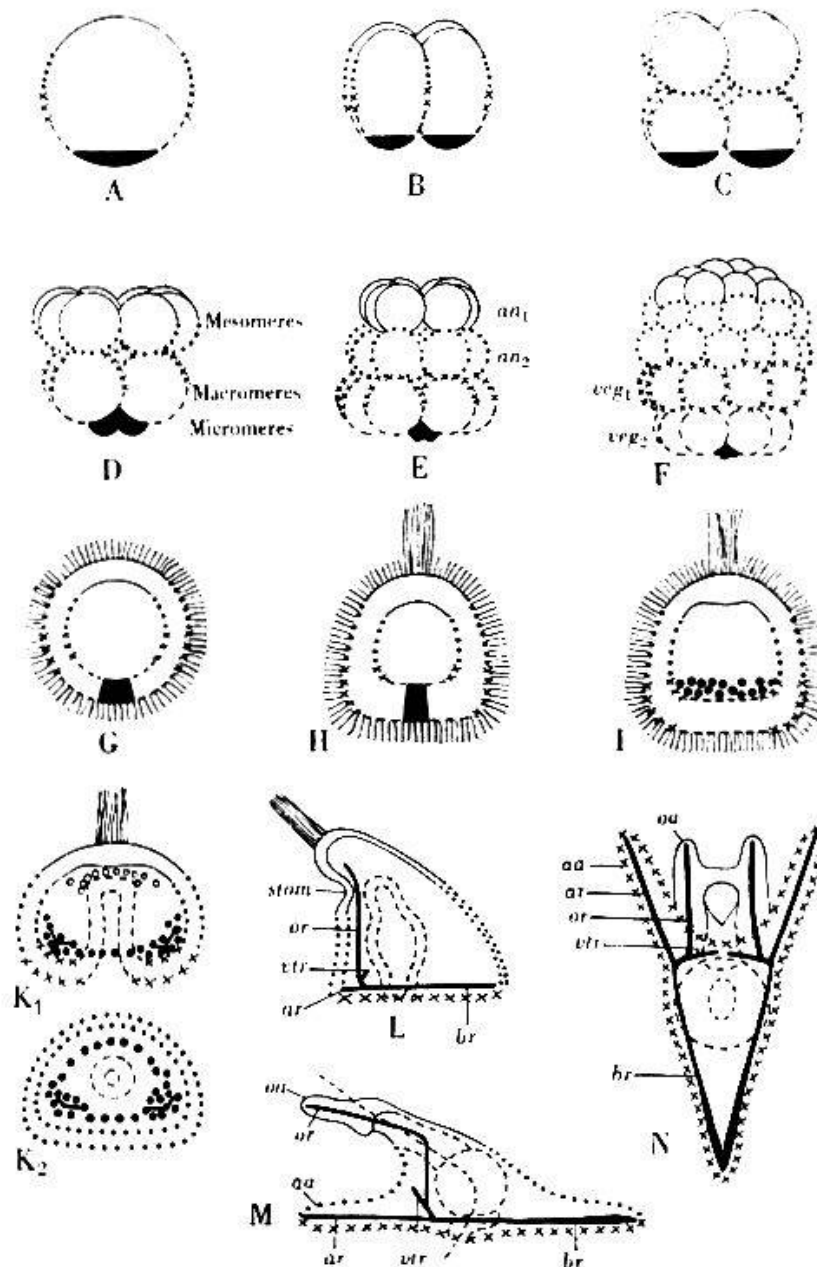


Fig. 3. Morphogenesis in the sea urchin embryo. The strongly ciliated apical cells in figs. H-L indicate that the AV-polarity represents a gradient with respect to the epitheliocyte property. The formation of mesenchyme cells at the vegetal pole shows that the VA-polarity is concerned with mechanocyte formation (from HÖRSTADIUS: *Biol. Rev.* 14 [1939]).

The second transformation amoebocytes (sl) → mechanocytes (sf) refers to the form of the pseudopodia in these solitary forms. The corresponding c-forms do not form pseudopodia, but it is supposed that they have undergone the transformation which in the s-forms influence the form of the pseudopodia.

As mentioned above the pattern of cell class distribution may be referred to the polarities of the egg; it should be emphasized, however, that the polarities of the egg have been discovered on the basis of the cell class distribution. This correlation shows that the direction of the cell transformations

is somehow determined by variations of certain properties along the egg axes. I shall not discuss this problem here but refer to the discussion of the amphibian embryo (1966).

The principal polarities in the sea urchin embryo are the animal-vegetal (AV) and the vegetal-animal (VA) gradients (cf. RUNNSTRÖM 1929; HÖRSTADIUS 1935; LINDAHL 1936). That these polarities are concerned with the cell transformation processes is directly established by the fact that the strongly ciliated apical tuft cells (cl-cells) are formed at the animal cap, whereas mesenchymal sf-cells are formed from the cells around the vegetal pole (cf. Fig. 3 and 4). Between these extremes is found one further region of cl-cells, the presumptive ectoderm, and the endodermal cells, the presumptive intestine. As we shall discuss below there is good reason to presume that the endodermal cells, situated between the two gradients, undergo both transformations and become cf-cells.

### III. Energy metabolism

#### 1. Respiration

I shall in the present discussion make certain assumptions which, although maybe obvious, should be specified at the outset. The first one is that the oxygen consumption is a direct measure of the energy consumption, which implies that there exists a respiratory control mechanism. The energy will

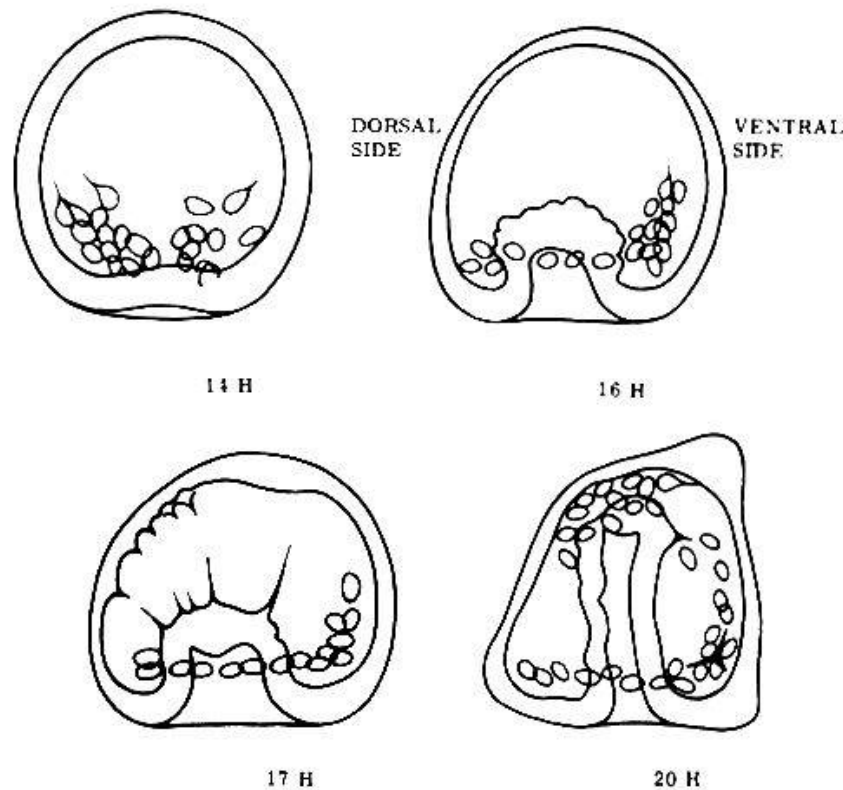


Fig. 4. Gastrulation in the sea urchin embryo. The formation of filiform pseudopodia in primary and secondary mesenchyme mechanocytes is indicated (from GUSTAFSON and WOLPERT: *Int. Rev. Cytol.* 15 [1963]).

be used partly for sustaining the cortical tension, the movements of the cells and other physical processes, and partly for the synthesis of various chemical compounds. Without trying to estimate the possible distribution of energy consumption between these two kinds of processes I shall make the further assumption that regions of the embryo which are distinguished by a lively chemical activity also consume more energy than the remaining parts.

The oxygen consumption during sea urchin development has often been studied (e.g. GRAY 1927; LINDAHL 1936, 1939 b; BOREI 1948; WHITELEY and BALTZER 1958; IMMERS and RUNNSTRÖM 1960). The curves obtained exhibit a number of typical traits (Fig. 5). Shortly after fertilization the rate of respiration commences to rise, following approximately an exponential course; around hatching this increase is interrupted by a phase of constancy lasting for some hours, but in the mesenchyme blastula the rate of oxygen consumption increases again, at first steeply, later on more slowly, after 30 hours a decline may even be observed. This latter phenomenon is probably reflecting an exhaustion of energy sources (cf. LØVTRUP 1959 d). The phase of constancy apparently indicates that the formation of new energy consuming activities suddenly comes to a standstill, a quite astonishing phenomenon which has been the subject of much speculation. It must be stressed, however, that this conclusion is valid only on the assumption that the energy consumption is uniform throughout the embryo.

There are many lines of evidence suggesting that this is not the case. The

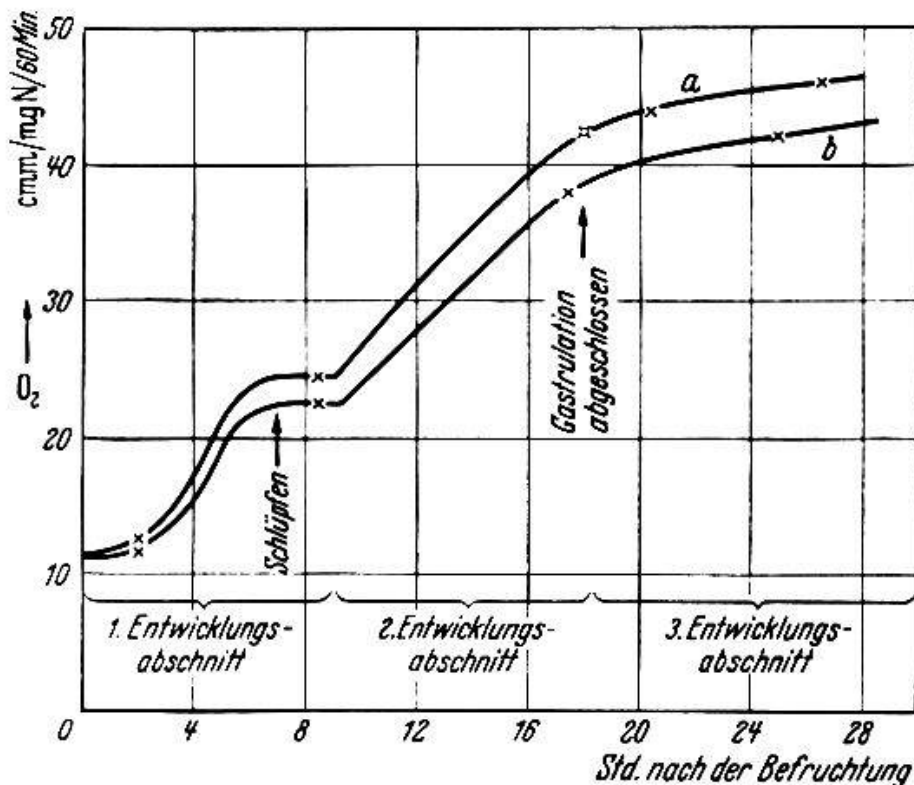


Fig. 5. Oxygen uptake during the embryonic development of *Paracentrotus lividus*. The curves represent two different batches.—Abscissa: Hours after fertilization. Ordinate: Oxygen consumption per mg N and hour (from LINDAHL: *Z. vergl. Physiol.* 27 [1939 b]).

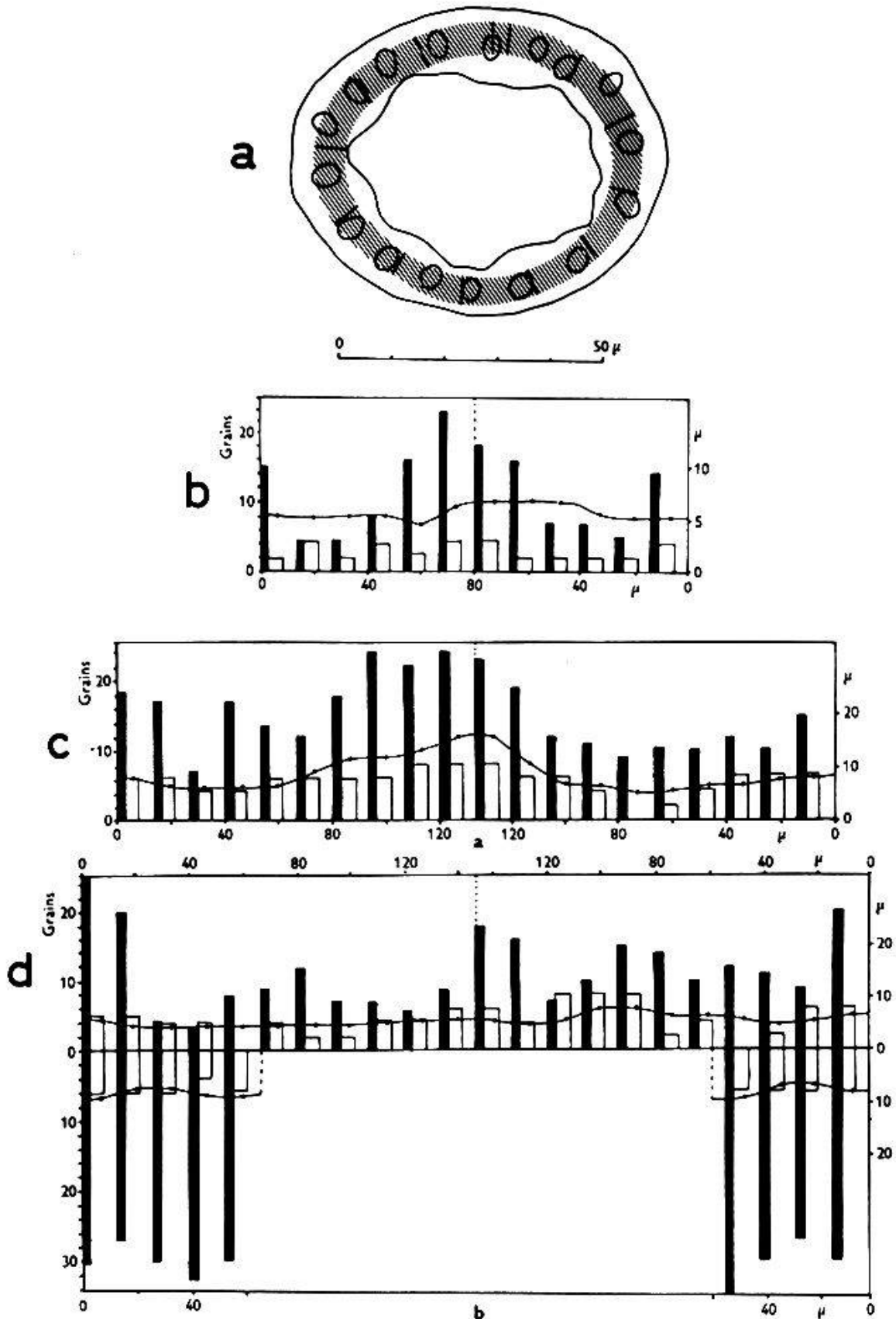


Fig. 6. Incorporation of L-leucine-<sup>14</sup>C. In the histograms are shown the regional differences in an annular section (a). The ends of the histograms represent the vegetal pole, the centre the animal pole, columns turned downwards the invaginated part of the gastrula. The filled columns represent the number of grains, the open ones the number of nuclei, and the curve the thickness of the body wall.—b = Early blastula 8 h after fertilization (*Paracentrotus lividus*). c = Mesenchyme blastula 14 h after fertilization (*Psammechinus microtuberculatus*). d = Prism 24 h after fertilization, same species as c (from MARKMAN, Exp. Cell Res. 23 [1961 a]).

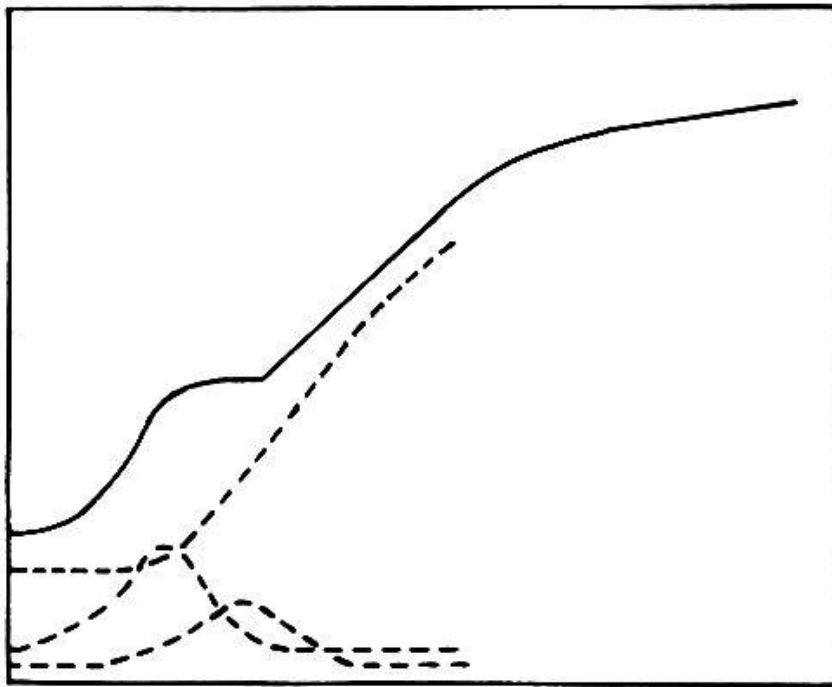


Fig. 7. Suggested resolution of curve b in Fig. 5.

work on reduction gradients (CHILD 1936; HÖRSTADIUS 1952; 1955; LALLIER 1958; BÄCKSTRÖM 1959c; CZIHAK 1962, 1963) suggests that there are two centers of high activity during early development, localized at the animal and the vegetal pole, respectively. The former begins somewhat before the latter. The mechanism underlying the appearance of the reduction gradients has recently been reviewed by GUSTAFSON (1966). I shall not here discuss this question but only, in agreement with this author, conclude that the coincidence in time between the reduction gradients and the waves of synthesis of enzymes of the pentose phosphate (PP) cycle observed by BÄCKSTRÖM (1959b, 1963) suggests that these phenomena are causally correlated. Since, as we shall discuss further in the sequel, these enzymes are part of the main source of energy supply in the early embryo, it is an obvious conclusion that two phases of energy consuming processes, spatially and temporally separated, exist in the early embryo. This point is also born out by the incorporation experiments of MARKMAN (1961a, 1961c). The results obtained show clearly that both RNA and protein synthesis (incorporation of adenine-8-<sup>14</sup>C and L-leucine-<sup>14</sup>C, respectively) during early development is particularly high in the most animal region. Gradually the rate of incorporation increases in the vegetal region; in the gastrula the highest rate is observed in the invaginated endodermal cells, whereas that obtaining around the animal pole has decreased considerably (cf. Fig. 6).

If the assumptions stated at the head of this chapter are correct it follows that the rate of respiration is not uniform in the sea urchin embryo; during the phase of constancy we may presume that two maxima of oxygen uptake occur in the animal and vegetal cells, coincident with the maxima of PP cycle enzymes observed by BÄCKSTRÖM (1959b, 1963; cf. Fig. 30). In Fig. 7

I have tried to indicate a resolution of the respiratory curves according to this suggestion; I have assumed that the volume of the animal cells involved in the first maximum comprise  $\frac{1}{5}$ , that of the vegetal cells in the second  $\frac{1}{10}$  of the total volume, and that the rate of oxygen uptake is uniform before the specific activities begin. These assumptions may surely be questioned, but I think that the general argument can be upheld. It will be realized that this suggested resolution of the curves in no way is contradicted by the failure of LINDAHL and HOLTER (1940) to find an animal-vegetal difference in oxygen uptake, since respiration is supposed to increase at both apical ends.

## 2. Energy sources

We may now turn to the problem about which substances are utilized for covering the energy needs. It seems possible to concentrate the attention on three kinds of substances, viz., carbohydrates, lipids, and proteins, since these constitute the major part of the dry material in the embryo. We shall begin with a survey of some of the analyses made on the contents of the egg and of embryos at various stages.

### a) Carbohydrates

The data compiled in Table 1 show that a considerable variation obtains with respect to the observed contents of carbohydrate. It is beyond the scope of the present paper to evaluate the methods employed, but it should be mentioned that HUTCHENS, KELTCH, KRAHL and CLOWES found the various steps involved in the glycogen determination to lead to a gradual loss of total acid-hydrolyzable carbohydrate, suggesting that not all of the polysaccharide present in the egg is insoluble in alcohol-water mixtures, or else that the part of the reducing substances is not polysaccharide. The

Table 1  
The carbohydrate content of the sea urchin egg ( $\mu\text{M}$  per embryo)\*

Authors	Total carbohydrate	Glycogen	Glucose
Paracentrotus lividus:			
EPHRUSSI and RAPKINE (1928)	47.5	45	—
ZIELINSKI (1939) . . . . .	—	56.5	5.5
ÖRSTRÖM and LINDBERG (1940)	—	29	6.5
Arbacia punctulata:			
PERLZWEIG and BARRON (1928)	10.5	—	—
HUTCHENS et al. (1942) . . . . .	22.5	10.5	—

\* Conversion factors: Paracentrotus lividus: 36 mg N per ml eggs, 0.245 g dry weight per ml eggs, 0.58 ml per  $10^6$  eggs (ÖRSTRÖM and LINDBERG 1940).—Arbacia punctulata: 25 mg protein per mg N, 5.9 mg N per  $10^5$  eggs (HUTCHENS et al. 1942).  $\mu\text{M}$ : picomole.

difference between the results of ZIELINSKI (1938) and ÖRSTRÖM and LINDBERG (1940) may probably be accounted for if the first possibility prevails.

In the second case the ribose in the soluble RNA fraction may be a possible source of error; if we assume the reserve RNA be 10 000 pg, and the average molecular weight of a nucleotide 300, then there is about 30 pM ribose in this fraction. This value corresponds pretty well to the difference between the values of ZIELINSKI and ÖRSTRÖM and LINDBERG. The glycogen isolated from the sea urchin egg is similar to usual glycogen in most respects (HUTCHENS et al. 1942), but many results would be easier to explain on the assumption that another carbohydrate reserve is present with properties different from those found in other animal tissues.

ÖRSTRÖM and LINDBERG observed that 18% of the total carbohydrate—mainly glycogen—disappears in the 10 min following fertilization. ZIELINSKI found a decline of 4% during the first 2 h of development. EPHRUSSI found a 7% decrease in the glycogen content after 12 h, and no traces left after 40 h. In contrast to that no changes were observed in total carbohydrate after 12 h, and after 40 h there was still left about 63% (20 pM) of the original content. These results show that the carbohydrate reserves are used mainly for synthetic purposes, only about one third is claimed for supply of energy. In the pluteus there is 6–7000 pg DNA, with an average molecular weight of a nucleotide of about 300 this amount corresponds to about 20 pM deoxyribose, showing that a large part of the glucose is used for DNA-synthesis.

#### *b) Lipids*

EPHRUSSI and RAPKINE (1928) have determined the lipids in eggs and embryos of *Paracentrotus lividus*. Using the same conversion factors as in Table 1 their results show that the total lipid fraction amounts to about 30 000 pg in the egg, 27 600 pg in the 12 h embryo and 24 600 after 40 h of development. According to HAYES (1938) the egg of *Arbacia punctulata* contains 5650 pg. of this about 30% is lost during the 1st h; after 43 h of development 37% of the original content still remains. The lipids disappearing seem to be confined almost exclusively to the saponifiable fraction.

#### *c) Proteins*

Innumerable Kjeldahl determinations have been made on sea urchin eggs, but most often the results have been used for reference to other parameters, whereas data referring to the nitrogen content per egg during development are rare. EPHRUSSI and RAPKINE (1928) found that the nitrogen content in the egg, calculated as protein, corresponds to about 67% of the dry weight. A decrease was found during development, but when corrected for the increased ash content, the percentage of protein rather went up. Allowing for the reductions in the contents of lipids and carbohydrate the protein content seems to be quite constant. The determinations of total N by GUSTAFSON and HASSELBERG (1951) confirm this conclusion.

It has been pointed out by BOELL (1955) that the ammonia formation demonstrated by HUTCHENS et al. (1942) implies that protein is combusted for energy supply. In contrast to ÖRSTRÖM (1941) who observed a transient ammonia formation after fertilization, these authors registered a continuous production of  $\text{NH}_3$ . According to LINDBERG (1945) this finding seems to result from a methodological error.

### *3. Oxidative metabolism in the early embryo*

In spite of the relatively large variation in the various data presented above it seems certain that only carbohydrate and lipids are used to any significant extent for supply of energy during sea urchin development, whereas the protein reserves are used almost exclusively for synthetic purposes. This result is in complete agreement with my own observations on amphibian embryos (cf. e.g. 1953a, 1959a).

Normally the final oxidation of both carbohydrate and lipids (= fatty acids) passes via the Krebs cycle in the mitochondria. As we shall presently discuss the mitochondria in the sea urchin embryo during the early stages of development are quite normal as regards the activity of various enzymes, and a rather extensive production of new mitochondria occurs. Yet, in spite of this they seem to play an insignificant role in the energy metabolism. Several lines of evidence support this point. Thus, on the basis of incorporation studies involving labelled  $\text{NH}_3$ , alanine,  $\text{CO}_2$  and acetate, HULTIN (1953c, d) was led to the conclusion that mitochondrial activity is very low before the mesenchyme blastula stage, but rises very rapidly with the beginning of this developmental phase (cf. Fig. 8). This suggests that there is a very distinct difference between the first and second generation mitochondria with respect to activity.

The slight inhibition by fluoracetate of the oxygen uptake in sea urchin eggs also suggests that acetate metabolism plays a minor role in the overall oxidative metabolism (CLELAND and ROTHSCHILD 1952b), a finding which completely confirms HULTIN's observations.

Studies of the formation of  $\text{CO}_2$  from labelled glucose has given results which suggest that during early development this substance is oxidized in the PP cycle, whereas the Krebs cycle seems not to be involved in glucose turnover (KRAHL 1956; BÄCKSTRÖM et al. 1960). This must imply that intermediates accumulate without being metabolized by the mitochondria.

As regards the apparent deficiency in the mitochondrial function it seems possible that the activity of one or more enzymes is so low as to limit mitochondrial function. It is, in this connection, interesting to note that KRAHL et al. (1941) found the succinate dehydrogenase (SDH) activity too low to be of significance for the respiration of the egg; similarly CRANE and KELTCH (1949) found that in a cell-free system the oxygen uptake in the presence of oxalacetate is only about half the respiration of intact fertilized eggs.

Another possibility has been suggested by CLELAND and ROTHSCHILD

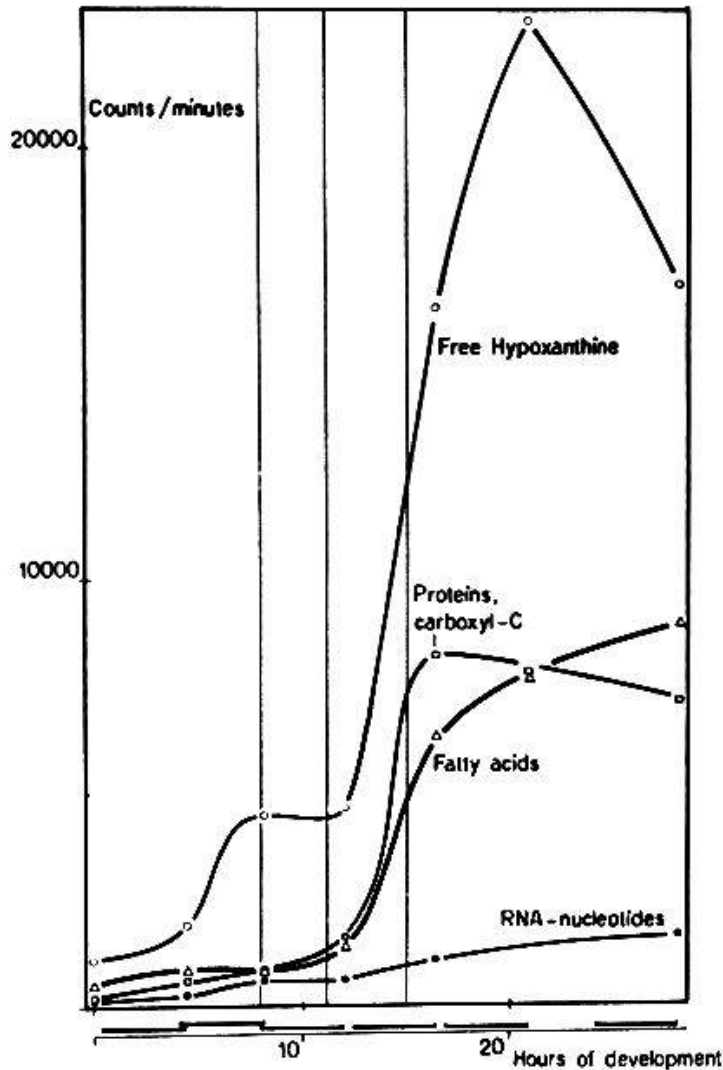


Fig. 8. Incorporation of  $1\text{-}^{14}\text{C}$ -acetate in embryo of *Psammechinus miliaris* at different stages of development. Vertical lines indicate hatching, appearance of mesenchyme cells and start of invagination, respectively. The periods of isotope treatment are indicated by horizontal lines at the bottom of the figure (from HULTIN: *Ark. Kemi* 6 [1953d]).

(1952b). On the basis of studies of the oxidation of pyruvate these authors came to the conclusion that in many enzyme preparations the activity of the condensing enzyme, catalyzing the reaction: "acetate" + oxalacetate  $\rightarrow$  citrate, is a limiting factor. If this is correct the block is to be found at the very entrance to the Krebs cycle, and thus to the mitochondria. An accumulation of acetate could possibly be anticipated as a consequence of this enzymatic deficiency.

The low mitochondrial activity seems to preclude that either of the potential energy sources to any substantial extent is completely oxidized during early development.

#### 4. Utilization of energy sources

Determinations of RQ constitute the classical approach to the analysis of the contribution of various energy sources to the total consumption of energy, and also in studies on sea urchin development has this method been

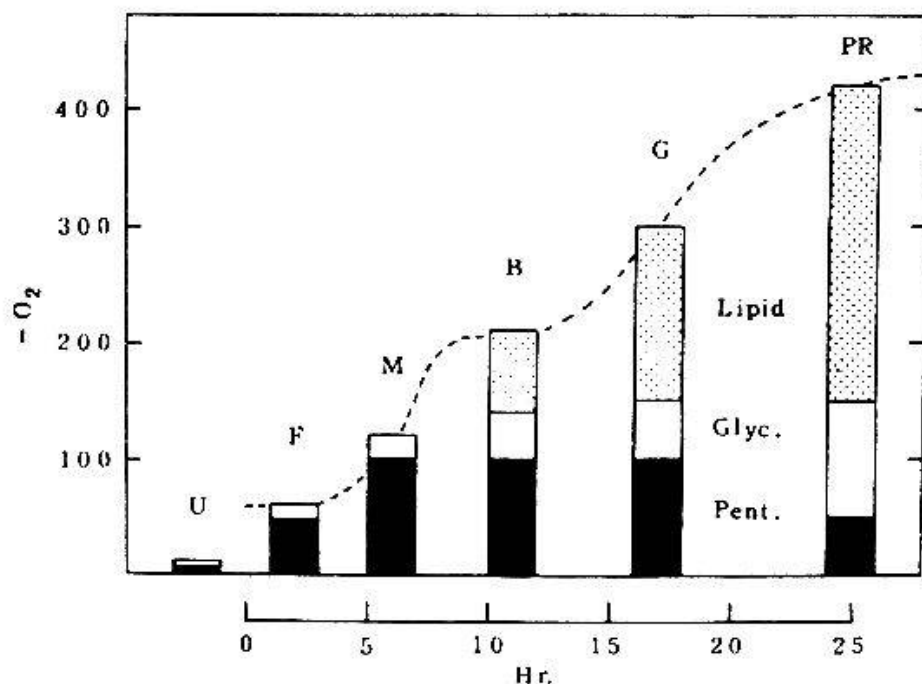


Fig. 9. Utilization of energy sources during the development of *Anthocidaris crassipina*.—Abscissa: Hours after fertilization at 20° C. Ordinate:  $\mu\text{l O}_2$  per  $10^6$  embryos and hour.—U = unfertilized eggs, F = fertilized eggs, M = morula, B = swimming blastula, G = gastrula, PR = prism.—Black zone (Pent.): Carbohydrate oxidation involving pentose phosphate cycle enzymes. White zone (Glyc.): Carbohydrate oxidation involving glycolysis and mitochondrial enzymes. Dotted zone: Oxidation of lipids. Dashed line: Changes in the rate of oxygen uptake (from ISONO, quoted by GUSTAFSON in: WEBER: *Biochemistry of animal development* [1966]).

applied (e.g. LASER and ROTHSCHILD 1939; ÖHMAN 1940; HUTCHENS et al. 1942). However, RQ determinations presuppose that the energy sources are completely oxidized, and since that precondition is not fulfilled in the early sea urchin embryo, this procedure may lead to erroneous conclusions.

The accuracy of the chemical determinations do not suffice to correlate oxygen supply and consumption of energy sources, especially in view of the incomplete oxidation of occurring glucose. Fortunately, ISONO (quoted by GUSTAFSON 1966) has investigated the relative contribution of the energy sources, also with respect to the metabolic pathways, during the first 25 h of development (Fig. 9).

The work of ISONO has not been available to me, and I have therefore had no opportunity to evaluate the results. Since furthermore the species studied by him (*Anthocidaris crassipina*) is different from the one on which the reported chemical analyses have been obtained, no direct comparison between the results is possible. All the same it seems possible to use ISONO's findings for a comparison between oxygen consumption and substrate utilization in *Paracentrotus lividus*. The area under the respiratory curve in Fig. 9 may be divided into three components, supposed to represent oxidation of carbohydrate through the pentose phosphate (PP) cycle or through the Krebs cycle, and of lipid through the latter pathway. It can be estimated that they correspond to 35, 20, and 45%, respectively, of the total oxygen consumed.

Using these values, and various data on oxygen and substrate consumption, it is possible to make a balance sheet (Table 2). It is seen that if the glucose supposed to pass through the PP cycle is not completely oxidized then the carbohydrate consumption must be much higher than anything which can be accounted for by chemical analyses. Even if an intermediate is accumulated and oxidized when the mitochondrial activity becomes more efficient, it is still impossible to account for the oxygen consumption during the development of *Paracentrotus lividus*. It seems unavoidable to conclude that there are present carbohydrate or other reserves that are not determined by the methods employed, or else that the oxygen consumption values are too high. In the American species the discrepancy is not very large, especially considering that a major part of the carbohydrate left in the embryo will represent glucose transformed into deoxyribose, a process which consumes 0.5 mole O<sub>2</sub> per mole. It should be mentioned, however, that during the early phases of development HUTCHENS et al. (1942) could not register a loss in the carbohydrate content corresponding to the oxygen consumption.

Altogether it seems that, unless the paper of ISONO has cleared up this problem conclusively, there are still several questions concerning the energy supply in sea urchin development that await their final solution.

##### *5. Metabolic pathways in the glucose metabolism during early development*

The breakdown of glucose preceding the final oxidation in the mitochondria may occur along two main metabolic pathways, either through glycolysis or through the PP cycle. Until the presence of the latter was suggested by LINDBERG (1943) glycolysis was presumed to be the major pathway for glucose breakdown, even if lack of inhibition by iodoacetate (RUNNSTRÖM 1933) suggested certain peculiarities.

Since then much study has been centered around the question whether both pathways exist in the embryo, or whether only the PP cycle is present. Various experimental approaches have been employed in this connection, mainly studies of isolated enzyme reactions and of the effect of inhibitors. Since the latter method may give the most ambiguous results, I shall deal first with those in the former group.

KRAHL et al. (1954) have studied the hexokinase activity in *Arbacia punctulata*. As appears from Table 3, glucose, 2-deoxyglucose, mannose and fructose are phosphorylated at appreciable rates, but glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P) are hardly attacked. This very low phosphofructokinase activity does not support the contention that there is an active glycolytic pathway.

Studying the NADP reduction with various substrates KRAHL et al. (1955) could establish the presence of glucose-6-phosphate dehydrogenase (G-6-PDH), 6-phosphogluconate dehydrogenase (6-PGDH), phosphoglucomutase, hexose isomerase, and fructose-1,6-diphosphatase. The two first are

Table 2  
Energy metabolism during the first 25 hours of sea urchin development\*

	Oxygen consumption per embryo (pM)			Substrate consumption per embryo							
	Total	Lipids (45%) <sup>1</sup>	Glucose in Krebs cycle (20%) <sup>1</sup>	Lipids (pg)		Glucose (pM)		PP cycle with complete oxidation <sup>3</sup>	Total		
				calculated <sup>2</sup>	found	Krebs cycle <sup>3</sup>	PP cycle <sup>4</sup>			calculated	found
<i>Paracentrotus lividus</i>	670 <sup>5</sup>	300	135	235	3350	5400 <sup>6</sup> (40 h)	22	115	39	61	18 <sup>6</sup>
<i>Arbacia punctulata</i>	130 <sup>7</sup>	59	26	45	655	0 <sup>7</sup> 2000 <sup>7</sup>	4.3	22.5	8.3	12.6	8.5 <sup>8</sup>

\* Conversion factors: *P. lividus*: 36 mg N per ml eggs; 0.58 ml per 10<sup>6</sup> eggs (ÖRSTRÖM and LINDBERG 1940). *A. punctulata*: 25 mg N per g wet weight; 5.9 mg N per 10<sup>6</sup> eggs (HUTCHENS et al. 1942)

<sup>1</sup> ISONO (quoted by GUSTAFSON 1966)

<sup>2</sup> 1 g fat requires 2.02 l or 0.09 mole oxygen for complete oxidation

<sup>3</sup> 1 mole glucose requires 6 moles oxygen for complete oxidation

<sup>4</sup> 1 mole glucose requires 2 moles oxygen for conversion to acetate

<sup>5</sup> LINDAHL (1939b)

<sup>6</sup> EPHRUSSI and RAPKINE (1928). The values are not corrected for the increase in ash content. If that is done, the lipid consumption becomes negligible, and that of glucose 12.5 pg

<sup>7</sup> HUTCHENS et al. (1942)

<sup>8</sup> HAYES (1938)

Table 3

Relative rates of phosphorylation of various hexose compounds by hexokinase preparations from *Arbacia punctulata* (from KRAHL et al. 1954).

	Manometric method	Photometric indicator method
Glucose .....	1.0	1.0
2-Deoxyglucose .....	2.0	2.0
Glucosamine .....	0.6	-
Mannose .....	1.2	1.2
Fructose .....	1.8	1.7
Galactose.....	0.0	-
Glucose-6-phosphate	0.0	-
Fructose-6-phosphate	0.2	

normal components of the PP cycle, the third and the fourth of the glycolytic pathway. When fructose-1,6-diphosphate (HDP) was incubated with NAD, the coenzyme reduction proceeded at a rate of 1-8% of that observed with NADP in the presence of G-6-P. The HDP-NAD reaction is supposed to involve two steps, splitting of the substrate by aldolase into two triosephosphates and oxidation of the latter by triosephosphate dehydrogenase (TPDH). In this procedure the observed rate must be that of the slowest reaction; in spite of the slight activity the result must be considered quite remarkable, since JANDORF and KRAHL (1942) could observe no TPDH activity in egg extracts. These results show at best that glycolysis may occur in the sea urchin egg, but that it is of extremely low quantitative importance. However, the findings are not conclusive as to the presence of the glycolytic pathway; it cannot be excluded that the observed reduction of NAD depends upon the activity of other enzymes than TPDH.

The presence of this enzyme in sea urchin eggs was first questioned by RUNNSTRÖM (1933), when he showed that iodoacetate, an inhibitor of TPDH, did not interfere with the respiration. This observation was confirmed on homogenates by LINDBERG and ERNSTER (1948), and seems to constitute a support of the findings of JANDORF and KRAHL mentioned above. However, the use of inhibitors may be ambiguous, and opposite findings have indeed been reported (cf. below), so these results may not either be entirely conclusive. LINDBERG and ERNSTER also found that the oxygen uptake in homogenates in the presence of HDP and NAD was the same as in the absence of substrate; an increase occurred after addition of iodoacetate. These findings do not lend much support to the contention that either aldolase or TPDH are present in the sea urchin egg.

CLELAND and ROTHSCHILD (1952a) observed the formation of lactate and pyruvate under anaerobic conditions, but as this activity began only after a protracted lag period it was suggested to involve an activation of a glycolytic system through damage to the eggs. Formation of lactate or pyruvate

is not, in itself, a token of glycolytic activity, for triosephosphate may also arise through the PP cycle. The use of inhibitors do not much clarify the issue, for phloridzin, iodoacetate and fluoride all inhibit steps that are common to the two pathways, viz., phosphorylase and hexokinase, TPDH, and enolase, respectively.

YČAS (1954) has attempted to determine the activity of aldolase through the formation of triosephosphate from HDP. But as we have seen above from the work of KRAHL et al. (1955) the possibility exists that HDP may enter the PP cycle, so even in this case it is impossible to accept the findings as conclusive evidence.

In a discussion of PP cycle it has been stressed by PON (1964) how difficult it is, by metabolic experiments, to decide between the presence of either of the two metabolic pathways for glucose, particularly because several of the enzymes are the same for both. It appears from the discussion above that the experimental evidence can neither exclude, nor prove that glycolysis occurs in the sea urchin egg. The tenacious attempts to demonstrate that it is present may stem from the conviction that since glycolysis has been found in next to all animal cells, including, I believe, the adult sea urchin, it should consequently be present also in the egg.

This contention is fallacious, because it does not take into consideration the phenomenon of biochemical evolution. It is well known that during development changes occur not only on the morphological level, but also on the intracellular level, as expressed by the acquisition of new synthetic capacities, etc., and that these ontogenetic changes in many cases may be correlated with phylogenetic development (cf. WALD 1952; BALDWIN 1963).

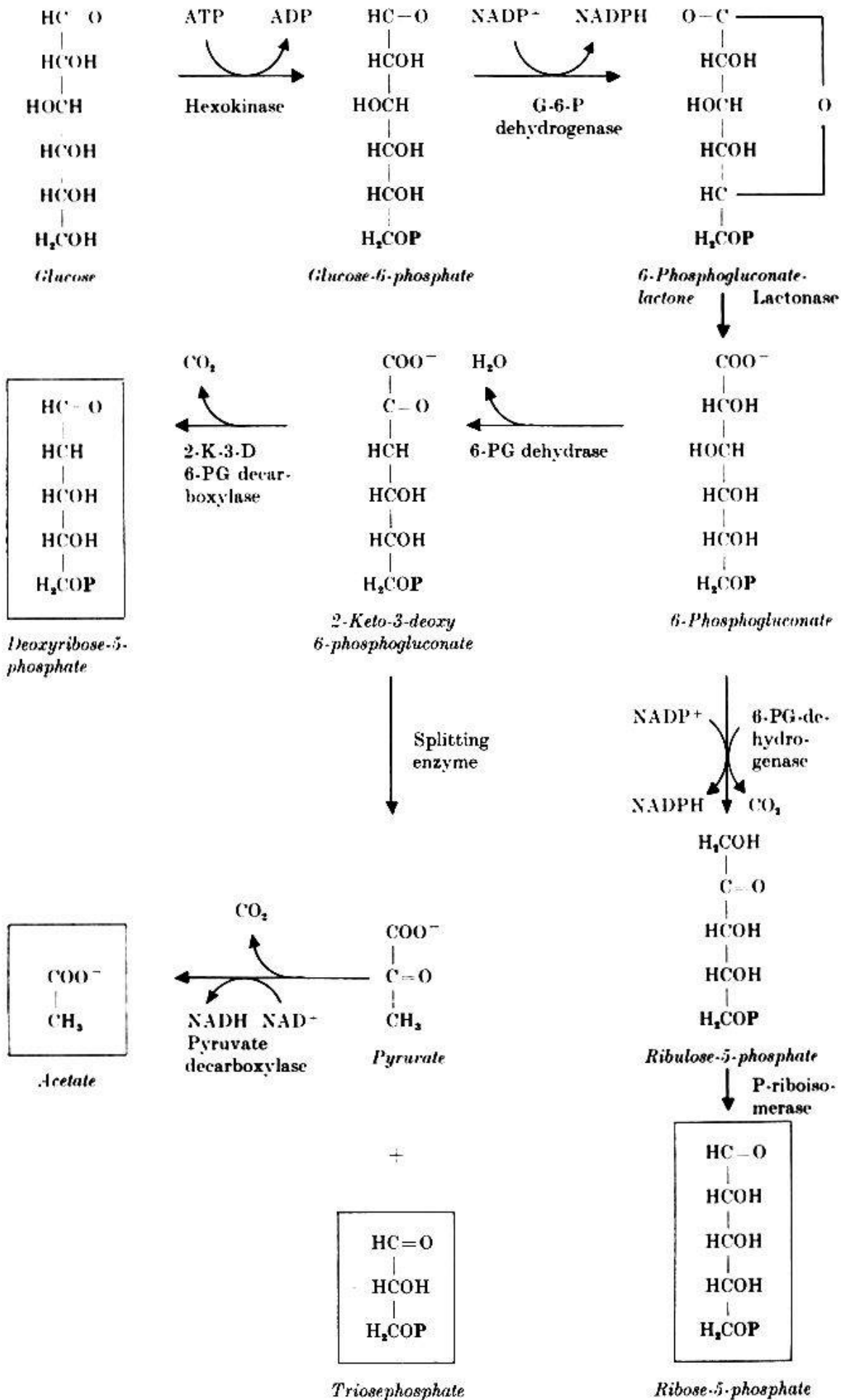
If we are allowed to extrapolate these findings back to the unicellular level, i.e., the egg, then we might expect the latter to exhibit a number of properties which today are found in unicellular organisms, i.e., in protozoa. It seems that many protozoa possess the glycolytic pathway, others have the PP cycle, some even seem to possess none of these. Thus if this phylogenetic view is valid, then there is nothing objectionable in the finding that no glycolysis occurs in the egg. It is obvious that from a practical point of view it is rather immaterial whether or not both pathways are present, because glycolysis under any circumstances is quantitatively unimportant, but theoretically it makes a great difference. It is therefore to be wished that experiments to settle definitely this question may soon be performed.

Having disposed of glycolysis for the time being, we shall turn our attention to the PP cycle. This metabolic pathway may lead to formation of ribose-5-phosphate, but it may also accomplish the formation of triosephosphate, which in turn may be oxidized to pyruvate, the first step being catalyzed by TPDH. As we have just discussed this enzyme may not be present, in which case even the PP cycle is excluded as the pathway for energy supply.

We must therefore look for a metabolic pathway leading to pyruvate without TPDH being involved. This possibility is found in the Entner-Doudoroff pathway (Table 4). The primary end products of this metabolic

Table 4

A proposal for the metabolic turnover of glucose in the sea urchin egg



sequence are as indicated triosephosphate and pyruvate, but the possibility obtains that also deoxyribose and ribose may be formed.

Pyruvate may be decarboxylated oxidatively to acetate (RUNNSTRÖM 1933; KRAHL et al. 1942; CLELAND and ROTHSCILD 1952b). As we have discussed above, it seems that the oxidation of acetate in the mitochondria proceeds at a very slow rate during early development, a certain accumulation thus probably will occur. Could not the much discussed acid, liberated at fertilization, be acetic acid? Under anaerobic conditions it seems that pyruvate may be transformed into lactate, even though the mechanism involved still is veiled by many question marks (cf. CLELAND and ROTHSCILD 1952a).

It follows from the discussion above that triosephosphate at best can be oxidized very slowly, probably a certain accumulation occurs until more efficient metabolic pathways are established as a result of the cell transformations occurring during development. The accumulation of this intermediate may possibly account for the failure of certain authors in demonstrating a loss in total (reducing) carbohydrate (EPHRUSSI and RAPKINE 1928; HUTCHENS et al. 1942).

It may seem preposterous to suggest a metabolic pathway entirely different from anything otherwise existent in animal cells, even if the available experimental evidence is compatible with this proposal. This statement may not be valid for the inhibition experiments, actually we may not know enough about the enzymes involved in the Entner-Doudoroff pathway to make any certain predictions about the effects of various inhibitors.

However, this kind of pathway is not completely unsubstantiated, if we accept the phylogenetic point of view. The parasitic protozoon, *Entamoeba histolytica*, contains G-6-PDH but not 6-PGDH. Ribose-5-phosphate is a growth requirement for this organism, and the end products of G-6-P metabolism are pyruvate and triosephosphate (HILKER and WHITE 1959; HILKER 1959 quoted by POX 1964). The presence of 6-PGDH constitutes a difference between the sea urchin egg and this amoeba, suggesting that ribose may be formed by the egg, but before conclusively proven, there seems to be no reason to postulate that other enzymes of the PP cycle are present, and if this is correct it follows that even the PP cycle is absent in the sea urchin egg.

The present discussion has not been intended to establish the mechanism of energy supply in the sea urchin embryo; that can be done only by experiments. I do hope, however, that this survey will show that this question is far from settled, and also that to solve the problem it probably must be approached without any reference to mechanism found in adult tissues.

### *6. Phosphagen*

In studies on the acid soluble phosphorous compounds LINDBERG (1943) found four different fractions in the ovary of *Brissopsis* sp., viz., inorganic

phosphate (46%), ATP (27%), a very slowly hydrolyzable compound (12%), and arginine phosphate (15%). In the eggs of this species the analyses showed the last compound to be absent, the other three were found in the concentrations 53%, 31%, and 16%, respectively. Later LINDBERG (1945) isolated the slowly hydrolyzable ester from cow brain and found it to be propanediol-1-phosphate (PDP). The compound was found to stimulate oxygen uptake and pentose formation in homogenates of sea urchin eggs. After injection to rats the specific activity of the substance was found, in the liver, to decrease slowly, whereas the activity in other, more easily hydrolyzable phosphate compounds increased, suggesting either that the organic part of the ester is transformed or that the phosphate is transferred from the PDP to other compounds. In the latter case propanediol phosphate would act as a phosphagen. The various observations made by LINDBERG do not contradict this suggestion.

As a matter of fact, the analyses reported above lead to the conclusion that either PDP is the phosphagen of the sea urchin egg, or else this cell has no phosphagen. Whatever is correct, it leads to an extension of the somewhat simplified (cf. BALDWIN, 1963) phylogenetic sequence of phosphagens:

none or PDP	→	arginine phosphate	→	creatine phosphate.
(sea urchin) eggs		invertebrates		vertebrates

The circumstance that slowly hydrolyzable phosphate esters are found in many eggs (cf. NEEDHAM, 1942) is may be a further support for the phylogenetic approach suggested here. It may be mentioned that CRANE and KELTCH (1949) could observe no phosphorylation of arginine in cell free preparation of sea urchin eggs.

In certain protozoa and lower metazoa phosphagens have been found that are slowly hydrolyzable, and distinctly different from arginine phosphate, but as far as can be judged from the rate of hydrolysis they are also different from PDP (cf. SEAMAN 1952).

It seems astonishing that this interesting compound, which apparently is found in, and metabolized by mammalian tissues, has not been subject to further studies. None of the metabolic pathways in the current biochemical repertoire can account for the formation of PDP. It should therefore be pointed out that this substance, together with triose, may be formed by the splitting of fucose, the carbohydrate so typical for eggs (cf. VASSEUR, 1952).

#### IV. Synthetic activities

The synthetic activity in the embryo involves the formation of new carbohydrates, lipids, nucleic acids and proteins etc. We have seen in the previous chapter that the two former represent the main sources of energy supply, but that only a minor part of the reserves present in the egg is used for this

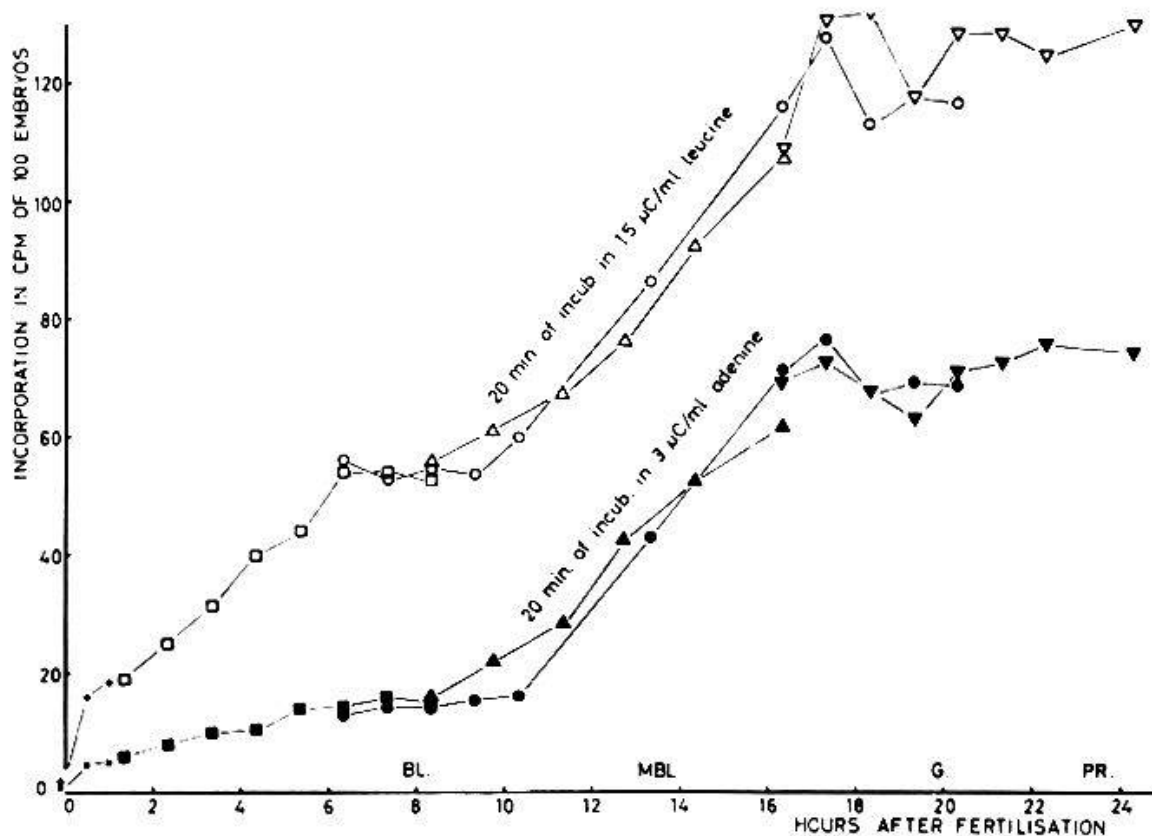


Fig. 10. Incorporation of L-leucine-<sup>14</sup>C (open symbols) and adenine-<sup>8-14</sup>C (filled symbols) into embryos of *Paracentrotus lividus* at various developmental stages.—BL = blastula, MBL = mesenchyme blastula, G = gastrula, PR = prism (from MARKMAN: Exp. Cell Res. 23 [1961 b]).

purpose. The carbohydrates and lipids present in the pluteus larva are presumably incorporated in the larval body. More than half of this carbohydrate was shown to be used for deoxyribose synthesis, most of the remaining may be used for synthesis of mucopolysaccharides (cf. IMMERS 1955, 1956). The major part of the lipid reserves is probably used for elaboration of various membrane structures. However, very little is known about the synthetic activities involving these two groups of substances, and we shall therefore concentrate our attention upon the reserves of nucleic acids and proteins, and their utilization for various synthetic purposes.

Judging from incorporation studies involving purines and amino acids, the synthesis of both types of macromolecules begins at an early stage of development. As shown by MARKMAN (1961 b) the rate of incorporation changes with time according to a curve with several phases (cf. Fig. 10).

It has been shown by KAVANAU (1954) that the protein synthesis occurs in distinctly separate stages (Fig. 11). From the time correlation these are seen to correspond to the phases of protein and RNA synthesis observed by MARKMAN, and especially to the changes in G-6-PDH and 6-PGDH activity (BÄCKSTRÖM 1959 b, 1963; cf. Fig. 30).

On the basis of his studies on the incorporation of <sup>15</sup>NH<sub>3</sub> and <sup>15</sup>N-alanine HULTIN (1953 b, c) was able to demonstrate that a fundamental change

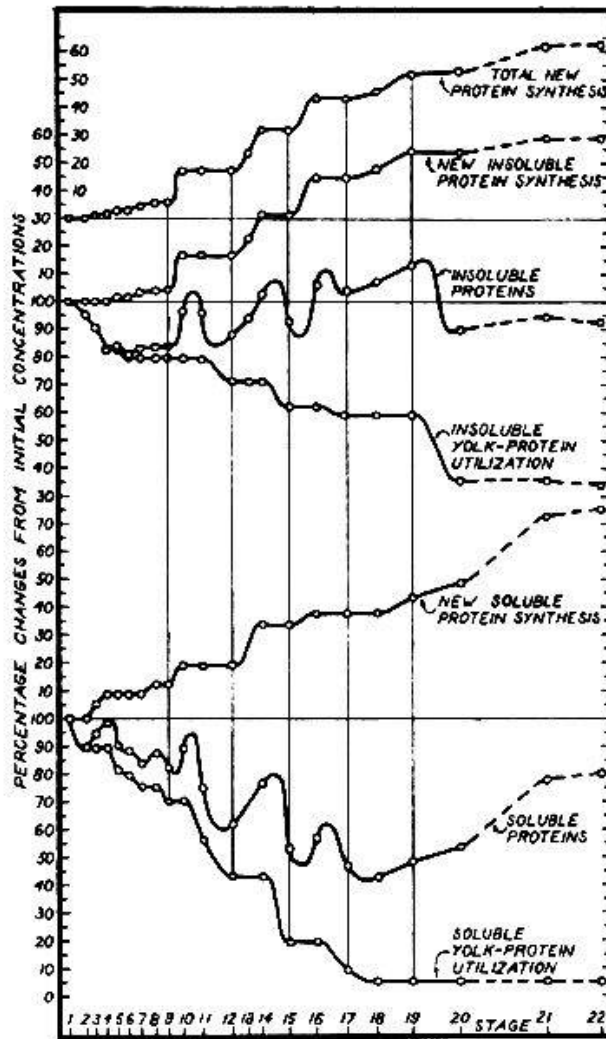


Fig. 11. Average percentage changes in various protein fractions during the development of *Paracentrotus lividus*. The curves illustrate quite clearly that protein synthesis occurs in several distinct phases (from KAVANAU: Exp. Cell Res. 7 [1954]).

occurs in the amino-acid-protein metabolism at the mesenchyme blastula stage. Before this stage "the nitrogen metabolism of amino acids is substantially restricted to exchange reactions at the amino acid level", but subsequently "the metabolic accessibility of the amino acids is considerably increased ..., the exchange reactions between amino acid nitrogen and ammonia being successively intensified" (1953c, p. 550). HULTIN considers this change to be correlated with increased mitochondrial activity.

All this synthetic activity is sustained by the conversion of inert reserves present in the egg. Since the content of amino acids decreases (e.g. GUSTAFSON and HJELTE 1951; KAVANAU 1954), and the total content of nucleic acid increases (e.g., ELSON et al., 1954) it may be inferred that there is a synthesis of nucleotides from amino acids. Determinations of the nucleic acids show that there is a substantial increase in the DNA content, whereas the RNA content remains constant, or declines slightly. However, as there is a quite extensive incorporation of isotopes into the RNA fraction throughout development, it follows that the RNA present at the outset must re-

Table 5

DNA content in sea urchin gametes and embryos (pg per gamete or embryo)\*

	CRANE (1947)	SCHMIDT et al. (1948)	VILLEE et al. (1949)	VENDRELY and VENDRELY (1949)
<i>Arbacia punctulata</i>				
sperm				0.7**
egg	84	700-1000	120 (3 h)	220**
pluteus		10 700		
	Separation, P determination	Separation, P determination	Separation, P determination	Colorimetric deoxyribose determination
<i>Paracentrotus lividus</i>				
	BRACHET (1933)	ELSON and CHARGAFF (1952); ELSON et al. (1952)	HOPF-JØRGENSEN (1954)	WHITELEY and BALTZER (1958)
			AGERL and PERSSON (1956)	BALTZER and CHEN (1960)
sperm	12	20-30	0.7	40 ⑥
egg	2000***	8000	18	4-5000
pluteus	Colorimetric deoxyribose determination	Separation, microbiol. thymine determination	Microbiol. deoxyriboside determination	Colorimetric deoxyribose determination
			Separation, UV absorb.	Separation, UV absorption

\* Conversion factors: *Arbacia punctulata*: 5.9 mg N per 10<sup>6</sup> eggs, 0.025 mg N per mg wet weight (HUTCHENS et al. 1942). *Paracentrotus lividus*: 4.07 ml eggs per g dry weight, 1.7 × 10<sup>6</sup> eggs per ml (ÖRSTRÖM and LINDBERG, 1940).

\*\* *Arbacia equituberculata*.

\*\*\* No correction for change in dry weight.

⑥ 16-20 cells.

present reserves which are gradually transformed during development. The question about the utilization of these for synthesis of either nucleic acid, as well as the possible presence of a DNA reserve, will be discussed on the following pages.

## V. Nucleic acid synthesis

### 1. DNA

Many reports have been published dealing with the DNA content of sea urchin gametes and embryos. Data from several of these have been compiled in Table 5. It is seen that with respect to the DNA content of the sperm there is good agreement, showing that the haploid amount of DNA is somewhat below 1 pg. The values obtained at the end of development, in the pluteus stage, are also essentially in accord, showing that *Paracentrotus* larva at this time contains around 0.005 mg DNA corresponding to about 3600 cells. The *Arbacia* pluteus seems to have a somewhat higher DNA content. With respect to the unfertilized egg the agreement is less striking, the recorded values ranging between 12 and 1000 pg. In order to clear up this point it is necessary to consider the analytical approach.

In principle the various methods employed can be divided into three groups. In the first one no separation between RNA and DNA is made, the latter is determined directly by a method specific for DNA (BRACHET 1933; VENDRELY and VENDRELY 1949; HOFF-JØRGENSEN 1954; WHITELEY and BALTZER 1958). In the second and third groups the nucleic acids are separated, in the former DNA is determined by a specific method (ELSON and CHARGAFF 1952), in the latter by an unspecific method (CRANE 1947; SCHMIDT et al. 1948; VILLEE et al. 1949; AGRELL and PERSSON 1956; BALTZER and CHEN 1960). The results in the third group are substantially higher (3-50 times) than those in the two first groups, with the exception of the values published by VENDRELY and VENDRELY. It is obvious that the difference cannot be blamed on the separation step (cf. groups 1-2 and 2-3); consequently either the specific methods give too low, or the unspecific too high values.

CHEN et al. (1961) propose that microbiological methods give too low results, but those obtained at the pluteus stage by ELSON et al. (1954) and by BALTZER and CHEN (1960) are almost identical, and the early values obtained by WHITELEY and BALTZER (1958) by a colorimetric method are pretty close to those obtained with the microbiological methods. GREGG (1957) has objected to the microbiological method of HOFF-JØRGENSEN that since no separation of the nucleic acids is involved, even free deoxynucleotides (or -sides) may be determined. However, these substances dissolve easily in 90-95% acetone, so if acetone-dried material is employed, any free nucleotides will be removed. Furthermore, HOFF-JØRGENSEN got very closely the same result as ELSON and CHARGAFF who separated the nucleic acids.

Recent comparisons made between SCHNEIDER's colorimetric and HOFF-JØRGENSEN's microbiological methods have shown that, unless special precautions are taken, the latter easily is the more reliable assay for DNA (LØVTRUP and ROOS 1961, 1963 a, b), and it therefore seems justified to scrutinize the methods employed in the third group. There can be no doubt, according to the very careful investigations of HUTCHISON and MUNRO (1961), that methods based upon the original SCHMIDT-THANNHAUSER method can give satisfactory results for both nucleic acids. However, this method and other methods involving separation of DNA and RNA, have been worked out on tissues where the contents of the two nucleic acids are of the same order of magnitude. In the sea urchin egg the RNA content is 6-20 000 pg (e.g. SCHMIDT et al. 1948; ELSON et al. 1954; AGRELL and PERSSON, 1956); if thus the results of groups 1 and 2 are correct, the ratio DNA/RNA will lie in the range  $1/150-1/1000$ . If this is true, only the greatest optimist could expect to accomplish complete separation of the two compounds by chemical or physical means (cf. ABRAMS 1951).

It therefore seems that the results of BRACHET (1933), ELSON et al. (1952) and of WHITELEY and BALTZER (1958) give the most correct picture of the course of DNA changes in the sea urchin embryo, but it should be emphasized that the disagreement between these and the remaining results pertain mainly to the first part of the curve. The colorimetric methods seem to give slightly

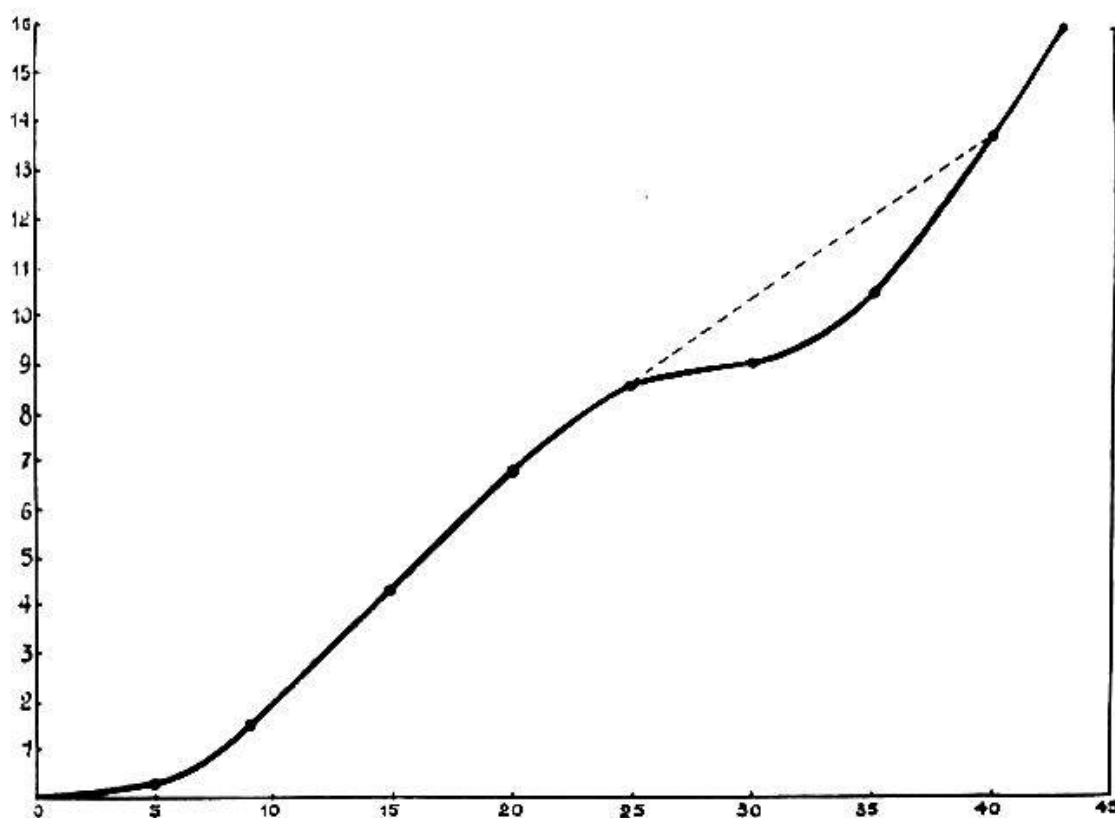


Fig. 12. Colorimetric determination of DNA in *Paracentrotus lividus*.—Abscissa: Hours of development. Ordinate: DNA, mg per g dry weight (from BRACHET: Arch. Biol. [Liège] 44 [1933]).

lower values than the other methods employed, but it should be noticed that the last value recorded by BRACHET represents a somewhat earlier stage than the ones listed for the other authors.

For *Paracentrotus lividus* BRACHET (1933; cf. Fig. 12), ELSON et. al. (1952), BALTZER and CHEN (1960) find a plateau or at least a hump on the curve around 20–30 h (the last mentioned authors have chosen to neglect this in drawing their curves). The phenomenon was not observed in this species by WHITELEY and BALTZER (1958), but in *Arbacia lixula* a very distinct phase of constancy was observed. This curve form indicates that the DNA synthesis in the sea urchin embryo occurs in two distinct phases; a similar phenomenon is well known from the amphibian embryo (cf. 1959c).

## 2. Reserve DNA

The haploid amount of DNA being around 0.7 pg it appears from the results discussed above that the quantity of DNA in the oocyte (18–40 pg) suffices to supply 13–28 nuclei.

Discussing the question whether this DNA is transformed into chromosomal DNA (in amphibia), BRACHET (1964) advances various arguments against this possibility. Among these are that no genetic meaning can be attached to it, that simple precursors (nucleotides) may be used for DNA-synthesis, and that certain forms of reserve DNA has a base composition deviating from chromosomal DNA in the same species (DURAND 1961). As far as I can see, all these arguments are equally valid for the transformation of RNA to DNA suggested by BRACHET (1933, 1964). This process is supposed to occur at the nucleoside (tide) level, but the same may happen with a DNA reserve.

If the reserve DNA is utilized for synthesis of chromosomal DNA it should become exhausted some time between the third and the fifth cleavage, according to the calculations made above. However, it is exactly at this time that DNA synthesis begins according to HOFF-JØRGENSEN (1954) and AGRELL and PERSSON (1956) (cf. Fig. 13). The latter authors observed that at the same time the synchronous cell divisions come to an end. The mentioned observations were made on *Paracentrotus lividus*, in two other species, *Psammechinus miliaris* and *Echinus esculentus*, the synchronicity lasts until the cell number is 100 and 400, respectively (AGRELL and PERSSON 1956). It would be very interesting to carry out microbiological DNA determinations on these species. It may be mentioned that the transition from synchronicity to asynchronicity in the animal region of the amphibian embryo occurs at an early blastula stage (SCHÖNMANN 1938), thus at a time which corresponds pretty well to the size of the DNA reserve (cf. the discussion in LØVTRUP 1959c).

The transition between the two types of cell division is characterized by an extension of the interphase and the formation of nucleoli (cf. DETLAFF 1964), the latter suggesting increased synthetic activity in the cell, and the

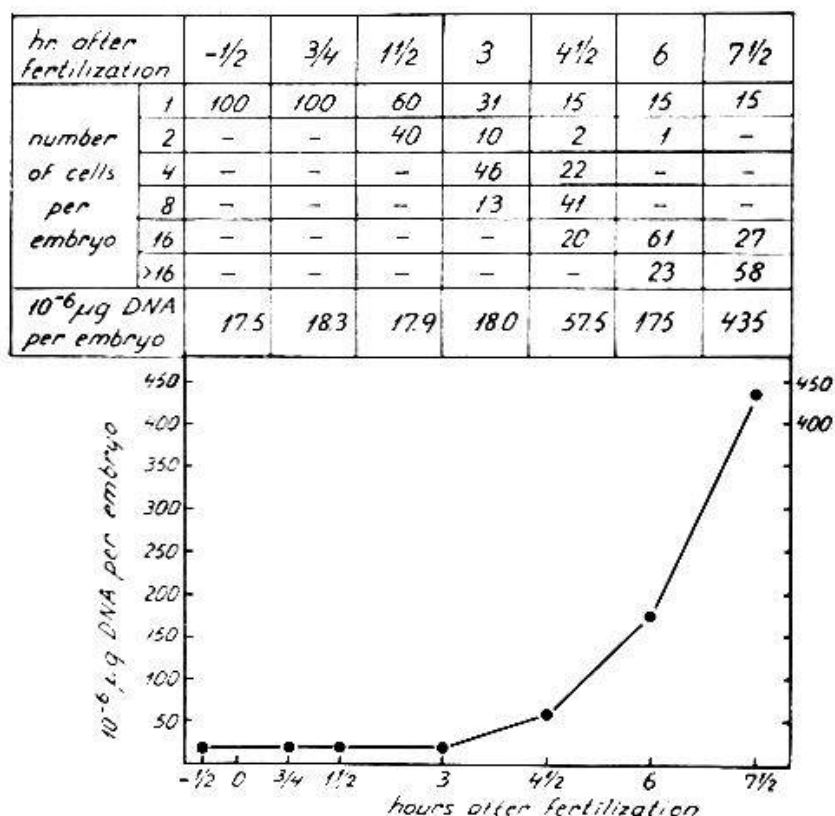


Fig. 13. Microbiological determination of DNA in *Paracentrotus lividus*. In the table is shown the percentage of embryos at different stages of development (from HOFF-JØRGENSEN: Colston Papers 7 [1954]).

former that the synthesis of some product has become the limiting factor for the rate of cell division.

The agreement between DNA analyses and morphological observations as to the time transition suggests that the DNA present in the unfertilized egg is a reserve which can be and is utilized for the first cell divisions, and that only when these reserves are used up does the synthesis of new DNA begin. The reserves may of course comprise even proteins, as suggested by MAZIA (1961). Interpreted in this way the results of the DNA analyses get a biological meaning and importance.

GRANT (1958) has, in the amphibian egg, tried to study the nature of the DNA reserve by extraction with cold PCA (perchloric acid) before microbiological determination. He found that about 50% of the DNA disappears, suggesting that half of the reserve is of relatively low molecular weight. We have repeated GRANT's experiments; in a number of cases we could confirm his results, in other cases PCA extraction was found to be without any effect. The analysis of the data is not yet completed.

### 3. RNA

Total RNA remains constant or decreases slightly during development (e.g. ELSON et al. 1954; BÄCKSTRÖM 1959 a; Tocco et al. 1963). In the two

former papers one or more oscillations were observed in the curves. As we shall discuss below isotope experiments have shown a lively incorporation into RNA during all phases of development, indicating a changeover between different RNA fractions, one of which may constitute a reserve. Because of this circumstance, determinations of total RNA cannot give any information about the RNA-synthesis in the embryo.

This problem has been attacked by Tocco et al. (1963) by fractionation of egg homogenates. As shown in Fig. 14 these authors found a decrease in the fraction containing microsomes + non-sedimentable RNA, whereas that contained in nuclei, mitochondria, and heavy microsomes increased during development. These three fractions amount to 5% of total RNA at the beginning, and to 25% at the end of development. The obvious source of this RNA is the former fraction, but as mentioned by Tocco et al. (1963), the various incorporation experiments show that *de novo* synthesis of RNA nucleotides also occurs, there are thus two potential sources of RNA.

BRACHET and JEENER (1944) observed a conspicuous difference between the RNA in amphibian eggs and various adult tissues; whereas in the latter only traces of RNA could be found in the supernatant after high speed centrifugation, 60–80% of the total RNA was recovered in this fraction in the former case. It has been surmised that this non-sedimentable type of RNA may represent a cytoplasmic reserve. It would have been very interesting to know the changes in this fraction during development, but in the result presented in Fig. 14 this fraction is combined with a microsomal fraction. In some  $^{32}\text{P}$  incorporation experiments a further separation was accomplished; the results in Fig. 15b show clearly that the rate of incorporation in the microsomal fraction increases—two phases are indicated—whereas that of the supernatant fraction remains almost constant, a finding compatible with the role ascribed to the non-sedimentable RNA, considering that it does not contain only reserve RNA.

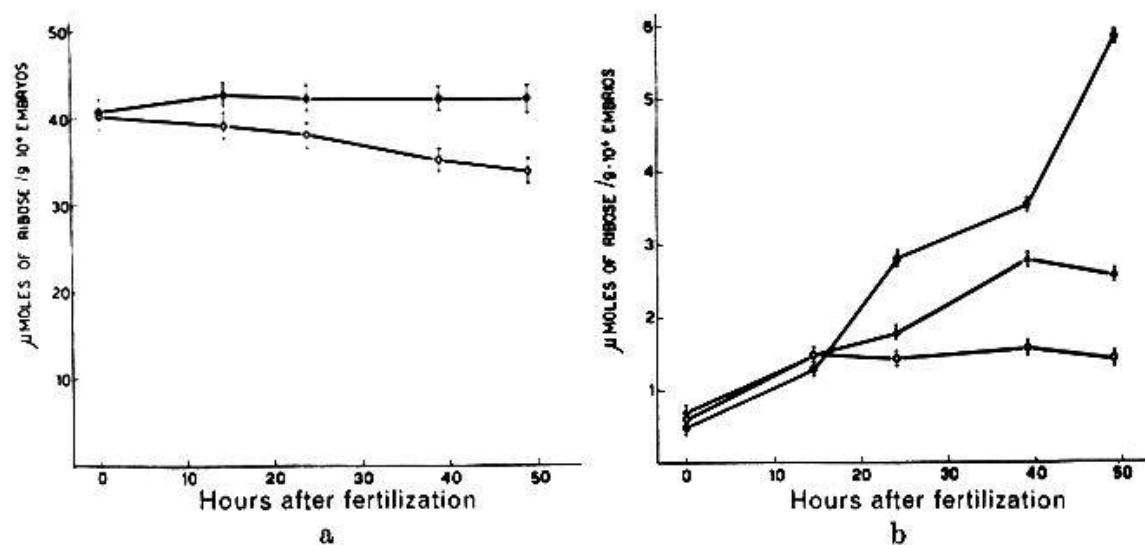


Fig. 14. RNA of subcellular fractions during the development of *Paracentrotus lividus*.— a: • = total content, ○ = microsomes + nonsedimentable fraction. b: • = nuclei, ○ = mitochondria, ▲ = heavy microsomes (from Tocco et al.: *Exp. Cell Res.* 31 [1963]).

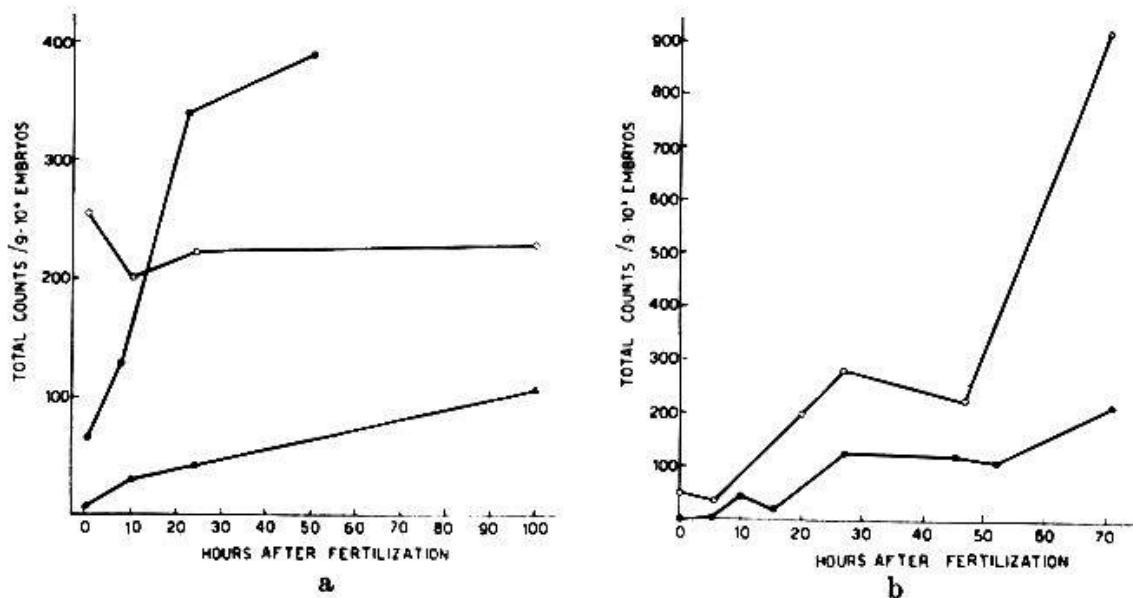


Fig. 15.  $^{32}\text{P}$ -incorporation into RNA of subcellular fractions during the development of *Sphaerechinus granularis*.—a: • = nuclei, ○ = mitochondria; ▲ = heavy microsomes. b: • = non-sedimentable fraction, ○ = microsomes (from Tocco et al.: *Exp. Cell Res.* 31 [1963]).

As suggested originally by BRACHET (1933), and recently reiterated by Tocco et al. (1963) there is no evidence speaking against the assumption that during embryogenesis nuclear DNA is formed partly from the RNA reserve present. This nucleic acid may thus be formed from stores of DNA and of RNA, and by de novo synthesis, whereas for RNA synthesis only the two latter possibilities are involved. Various isotope experiments discussed above show that amino acids are precursors for the bases. As discussed in the previous chapter both ribose-5-phosphate and deoxyribose-5-phosphate may be formed from glucose. It would be interesting to know whether the enzymes catalyzing the conversion of ribonucleotides to deoxyribonucleotides (cf. REICHARD 1961) are present in the embryo, and if so, at which stage they make their appearance. The possible formation of deoxyribose-5-phosphate suggests that enzymes of this type may not be necessary for the conversion between the two types of nucleotides.

#### 4. Isotope incorporation studies

The question whether the RNA reserves may be used indiscriminately for synthesis of either nucleic acid seems to be of great theoretical interest, and several authors have tried to study this problem. The tracer experiments of VILLEE et al. (1949) with  $^{32}\text{P}$ , of ABRAMS (1951), and of SCARANO and KALCKAR (1953) with glycine-1- $^{14}\text{C}$  and adenine-8- $^{14}\text{C}$  all show that the specific activity is much higher for DNA than for RNA. This has been used as an argument against RNA as a DNA precursor, but this reasoning is only valid if all the RNA present is actively participating in the turnover. If a major part is an RNA reserve, then the determination of specific activities may lead to erroneous conclusions; both VILLEE et al. and ABRAMS admit

that their results do not contradict the possibility that a small fraction of the total RNA can be a DNA precursor.

From the isotope dilutions found in his experiments ABRAMS (1951) calculated that some of the purines in both nucleic acids are derived from de novo synthesis with glycine as precursor but that the major part stems from an endogenous source. This source might be RNA if it were not for the fact that the value of the ratio of the specific ratio of guanine/adenine was different for DNA and RNA, being about two in the former, and one in the latter case. This finding led to the conclusion that "any simple conversion mechanism of RNA or its hydrolysis products to DNA is ruled out" (loc. p. 241).

However, if this is the only hindrance for the acceptance of a both chemically and biologically plausible mechanism the situation seems hopeful, for it appears from the results of HULTIN's  $^{15}\text{NH}_3$ -incorporation experiments (1953a) that during an essential part of the developmental period the incorporation in RNA guanine is about twice as high as in RNA adenine (Fig. 16).

As to the high ratio of the specific activities for guanine/adenine a few comments may be warranted. This has been observed, directly or indirectly, with both glycine- $1\text{-}^{14}\text{C}$  (ABRAMS 1951),  $^{15}\text{NH}_3$  (HULTIN 1953a) and adenine- $8\text{-}^{14}\text{C}$  (SCARANO and KALCKAR 1953). In the two latter cases a decrease in the ratio was observed towards the end of development. As stated by ABRAMS, this result seems to exclude that adenine is a precursor for guanine. If not, the quantities of guanine and adenine precursors in the pool must be of decisive consequence for the incorporation of labelled compounds, thus if

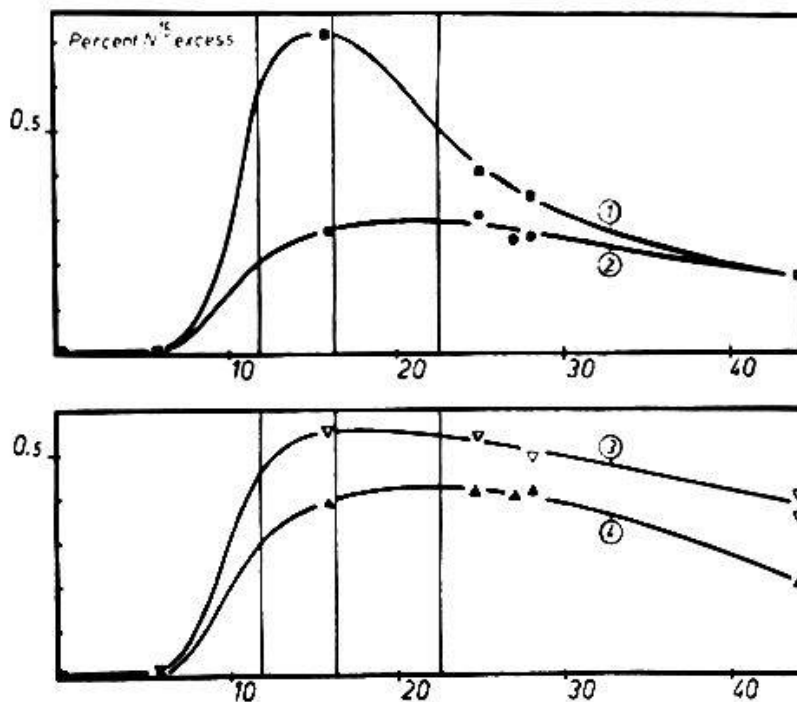


Fig. 16. Incorporation of  $^{15}\text{NH}_3$  into purines and pyrimidines of RNA in embryos of *Paracentrotus lividus* at different stages of development.—1 = guanine, 2 = adenine, 3 = uracil, 4 = cytosine (from HULTIN: *Arch. néerl. Zool.* 10, 1. Suppl [1953e]).

the guanine is present in limiting amounts, synthesis of this base from simpler precursors may be required to cover the needs. It may or may not be relevant in this connection to mention that the ratios between the various free adenine and guanine nucleotides are very large in the unfertilized eggs and that, for ATP at least, a drastic decrease occurs during development (HULTIN 1957). Here seems to be another possibility for explaining the change in the guanine/adenine ratio. The pool might also be influenced by the composition of the nucleic acid reserves, cf. DURAND (1961) who has demonstrated a DNA store of abnormal composition in the insect *Grylla bimaculatus*.

However, the adenine/guanine conversion is not completely blocked, for SCARANO and KALCKAR (1953) observed a labelling in guanine after addition of adenine-8-<sup>14</sup>C. These authors observed an enhanced relative incorporation in guanine with developmental age. The findings of HULTIN (Fig. 16) may thus also be interpreted in the way that the enzymes required for the purine interconversion are absent or of very low activity during the first 10–15 h of development, but not thereafter. The question whether the enzymes are

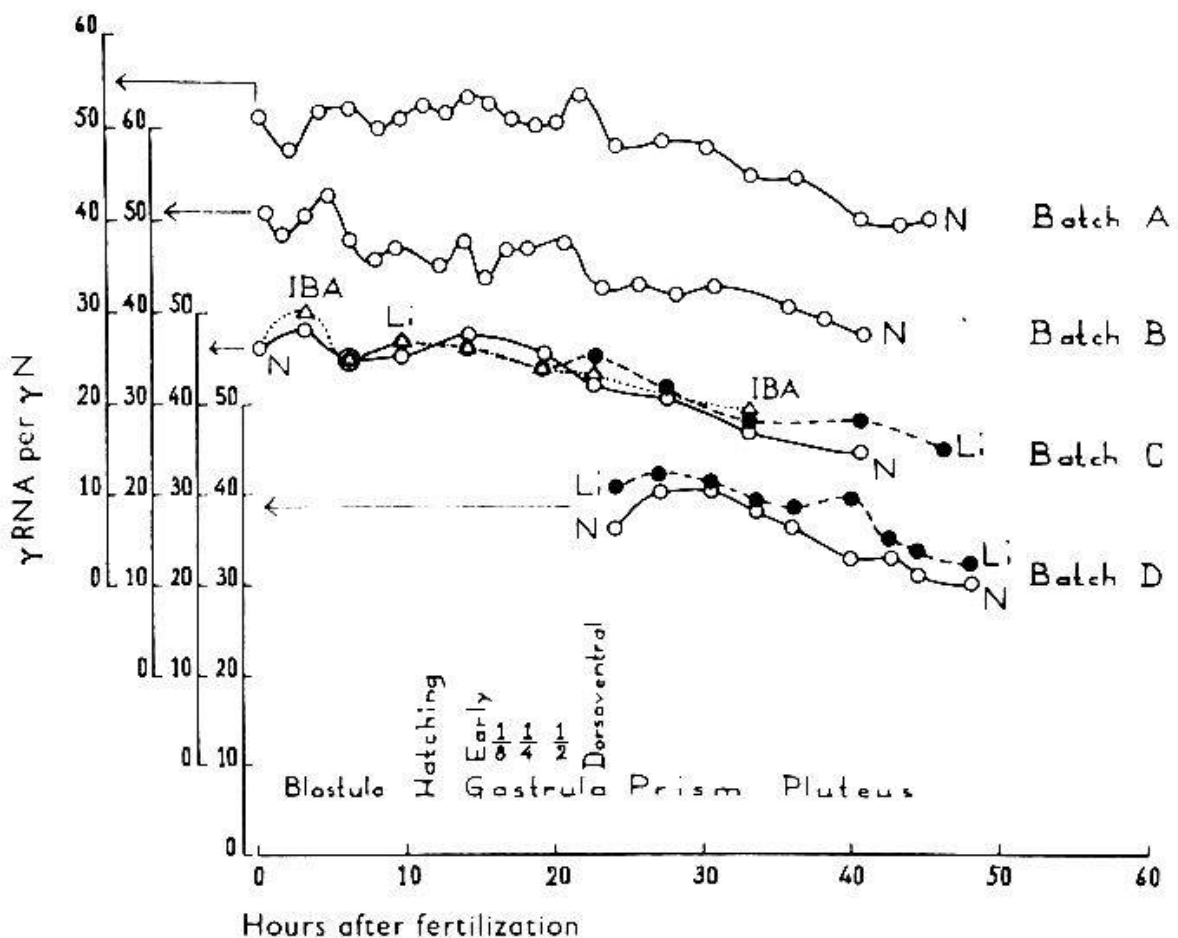


Fig. 17. Changes in the RNA content during the development of *Paracentrotus lividus*.—N = normal embryos, Li = vegetalized embryos, IBA (o-iodosobenzoic acid) = animalized embryos.—The arrows point to the ordinate for each group of curves (from BÄCKSTRÖM: *Ark. Zool. 12* [1959a]).

completely absent might be decided by incorporation experiments with labelled adenine. Unfortunately the incubation periods employed by SCARANO and KALCKAR (0-22, 12-30, and 12-48 h) were too long to settle this problem.

The slight, but significant decrease in RNA during development found by ELSON et al. (1952) and by BÄCKSTRÖM (1959a), and particularly the oscillations found by the latter author (Fig. 17) would seem incompatible with the wisdom of the workings of Nature, were it not for the possibility that the RNA represents a reserve that may be utilized for synthesis of both DNA and RNA. In this perspective BÄCKSTRÖM's findings suggest that in periods of rapid DNA synthesis the RNA reserve is tolled, causing a decrease in total RNA, whereas in periods of extensive RNA formation the content is replaced by *de novo* synthesis. BÄCKSTRÖM's interesting experiments ought to be repeated with due regard to the possible peculiar physical properties of the embryonic RNA, and under such conditions that statistical evaluation of the data were possible.

### 5. Correlation between DNA and respiration

COMITA and WHITELEY (1953) and WHITELEY and BALTZER (1958) have tried to correlate DNA content and rate of oxygen consumption (cf. also TYLER 1942 and ZEUTHEN 1952). It is a reasonable contention that respiration increases with the number of cells, even if the size of each cell decreases continuously. However, in order to establish such a correlation it appears insufficient to demonstrate that a mathematical regression can be established, that is generally possible for two gradually increasing phenomena. Agreement between the finer details of the curves should also prevail, and such accord seems to be missing on two points. The first one is that the DNA-curves show no constancy from about 7-10 h, as do the respiratory curves (cf. Fig. 5). This lack of agreement may easily be referred to the few points used to establish the DNA curves. The second point is that after about 20 h there is only a very slight increase in O<sub>2</sub>-consumption, after 35-40 h the rate even decreases (LINDAHL 1939b; COMITA and WHITELEY 1953; WHITELEY and BALTZER 1958), but during this phase the DNA-synthesis continues at a constant rate (cf. Fig. 12).

MARKMAN (1961b) has followed very closely the rates of incorporation of adenine-8-<sup>14</sup>C and <sup>14</sup>C-leucine (i.e., the rates of nucleic acid and protein synthesis) during the first 24 h of development. The curves obtained by him (Fig. 10) are almost identical with the respiratory curves except that after 20 h the rate of incorporation seems to be constant. Now, the incorporation of adenine does not represent *only* DNA synthesis, but it represents *also* DNA synthesis (cf. ABRAMS 1951), and the shape of the cumulative "DNA" curve one may establish on the basis of MARKMAN's data actually exhibit the same features as the experimentally observed curves (cf. Fig. 12), except that the hump occurs slight earlier. It therefore seems possible to

conclude that there is a correlation between the rate of oxygen consumption (energy supply) and the rate of nucleic acid (including DNA) and protein synthesis (energy requiring processes), an altogether plausible contention.

## VI. Protein synthesis

All or most proteins synthesized during embryogenesis may be classed as either structural or catalytic proteins. The former are generally more difficult to determine quantitatively, and consequently the overwhelming part of the available data are concerned with enzymes. It should be mentioned that much work has been devoted to studies of changes in proteins characterized by their solubility or immunological properties (cf. RANZI 1962; PERLMAN 1959). In spite of their importance for the understanding of embryogenesis I have not included this work in the present survey.

It was emphasized by GUSTAFSON and HASSELBERG (1951) in their study of enzyme differentiation in the sea urchin embryo that the results should be interpreted with regard to their cytoplasmic localization. This suggestion can be followed rigorously only with respect to those ubiquitous enzymes, which follow a more or less similar pattern in all cells, in which case the correlation between localization and enzyme pattern is quite satisfactory. One might say that these changes pertain to the growth processes in the embryo.

However, during development extensive differentiation processes occur, leading to profound changes in the enzyme pattern. The most important differentiation processes occurring during the early development of the sea urchin are the cell transformations. Enzymes which are typical for either of the various cell types have therefore been treated in a separate section whereas the ubiquitous enzymes are to be found under the heading corresponding to their cytoplasmic localization. Since no enzymes have been studied which are exclusively located in either the nuclei or the microsomes, these two fractions are not included in the following discussion. All questions about localization have been referred to the paper by DE DUVE et al. (1962).

### 1. Ubiquitous enzymes

#### a) Mitochondria

*Changes in morphology, number, and distribution.* – The question about the changes occurring within the mitochondrial population has attracted the interest of several authors. Variations have been observed in three different parameters, viz., morphology, number, and distribution among the different prospective embryonic regions.

Various methods have been used, thus microscopical observation after vital staining by GUSTAFSON and LENIQUE (1952; 1955); this approach as well as counting in homogenates was employed by SHAVER (1956, 1957), and electron microscopy by BERG et al. (1962) and BERG and LONG (1964). Each of these methods are subject to limitations in some respect. The first one may reveal differences in stainability rather than in number, in the second

one losses may occur, and in the third one distortions of form may take place during the preparation of the material (cf. SHAVER 1957; BERG and LONG 1964; GUSTAFSON 1966).

As far as the appearance of the mitochondria is concerned it seems that a differentiation gradually occurs, this change comprises both the size, the cristal density and the shape. The arrangement of the cristae seems to be very irregular in embryonic mitochondria. Distinct differences seem to obtain between animal and vegetal cells (cf. Table 6).

The number of mitochondria also increases, already before the 32-cell stage there is a distinct rise in mitochondrial density. This phase is of short duration, during several hours there is no further change, but in the advanced blastula the mitochondrial number increases again. This rising phase lasts until about the 25th hour, from then on a decline in relative mitochondrial density (RMD) takes place (Fig. 18; GUSTAFSON and LENIQUE 1952, 1955).

Since, as we have seen, there is a change in the shape of the mitochondria during development we may presume that the mitochondria of the earlier type gradually disintegrate, and are replaced by particles of the more advanced types. This phenomenon of mitochondrial disintegration was described by GUSTAFSON and LENIQUE (1952). It therefore seems possible that the results obtained by these authors actually represent the resultant of two separate curves as suggested in Fig. 18.

The early changes could not be confirmed by SHAVER (1956), but the rapid increase in the blastula, followed in the late gastrula by a distinct decline in mitochondrial number was observed, in confirmation of the results obtained by the former authors (cf. Fig. 18 and 19). As shown in the latter figure there is a further rise in the mitochondrial population in the pluteus larva.

The lack of agreement between the results obtained by the two methods might reflect an increased stainability of the early mitochondria, but this explanation does not agree with the observation that incorporation of amino

Table 6  
Differentiation of mitochondria in the sea urchin embryo

		Relative <sup>1</sup> size	Relative <sup>4</sup> cristal density	Shape <sup>1-4</sup>	RMD <sup>1,2</sup>
Cleavage		1.00	1.00	spherical or oval	low
Gastrula	animal	1.24	1.15	spherical, rod-shaped?	high
	vegetal <sup>5</sup>	~1.7-2.5	1.25	rod-shaped	medium

<sup>1</sup> GUSTAFSON and LENIQUE (1952); <sup>2</sup> GUSTAFSON and LENIQUE (1955); <sup>3</sup> SHAVER (1956); <sup>4</sup> BERG and LONG (1964); <sup>5</sup> Includes primary mesenchyme and invaginated cells.

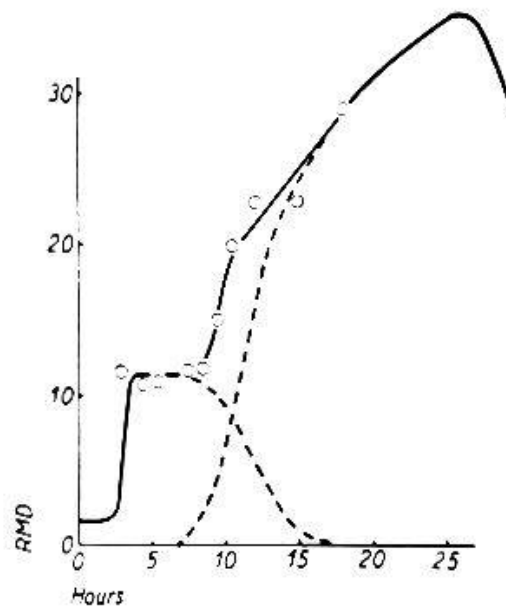


Fig. 18. Changes in relative mitochondrial density (RMD) during embryonic development of the sea urchin. The solid curve is composed from results published by GUSTAFSON and LENIQUE: *Exp. Cell Res.* 3 (1952); 8 (1955). The stippled curves represent a suggested resolution of the observed curve.

acids into the mitochondrial fraction occurs to a considerable extent even during the very early stages of development (HULTIN 1953e; NAKANO and MONROY 1958; cf. Table 7). The results of the latter (Fig. 20) show close agreement with the results of GUSTAFSON and LENIQUE (1955), especially if correlation is made for the decline in the activity in the acid-soluble fraction, which represents the most likely source of the  $^{35}\text{S}$ -methionine incorporated in the mitochondria.

These incorporation experiments, as well as the agreement with the results of SHAVER concerning the later stages, suggest that the approach used

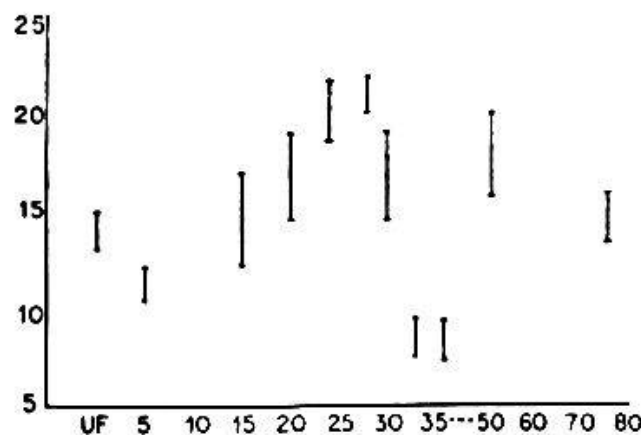


Fig. 19. Changes in the total number of mitochondria during the development of *Strongylocentrotus purpuratus* at 17° C. The vertical lines indicate the confidence limits (from SHAVER: *Exp. Cell Res.* 11 [1956]).

by GUSTAFSON and LENIQUE gives quite consistent results. This conclusion is important when we approach the problem of mitochondrial distribution. GUSTAFSON and LENIQUE (1952) reported a gradient in relative mitochondrial density (RMD) from a maximum at the animal to a minimum at the vegetal pole in the blastula gastrula stages (cf. also CZIHAK 1962). Thus the apical tuft cells are very rich in mitochondria, the mesenchyme cells correspondingly poor. These observations were substantiated by results on animalized and vegetalized embryos. For later stages two other regions of high RMD were described, the cells forming the ventral ciliated bands, and the intestinal cells. This observation was confirmed by SHAVER, and it is therefore extremely difficult to understand that he was unable to confirm the first AV-gradient (cf. 1956, 1957). BERG et al. (1962) could not either detect any AV-gradient; in a more recent paper it seems that some mesenchyme cells poor in mitochondria have been observed (BERG and LONG 1964).

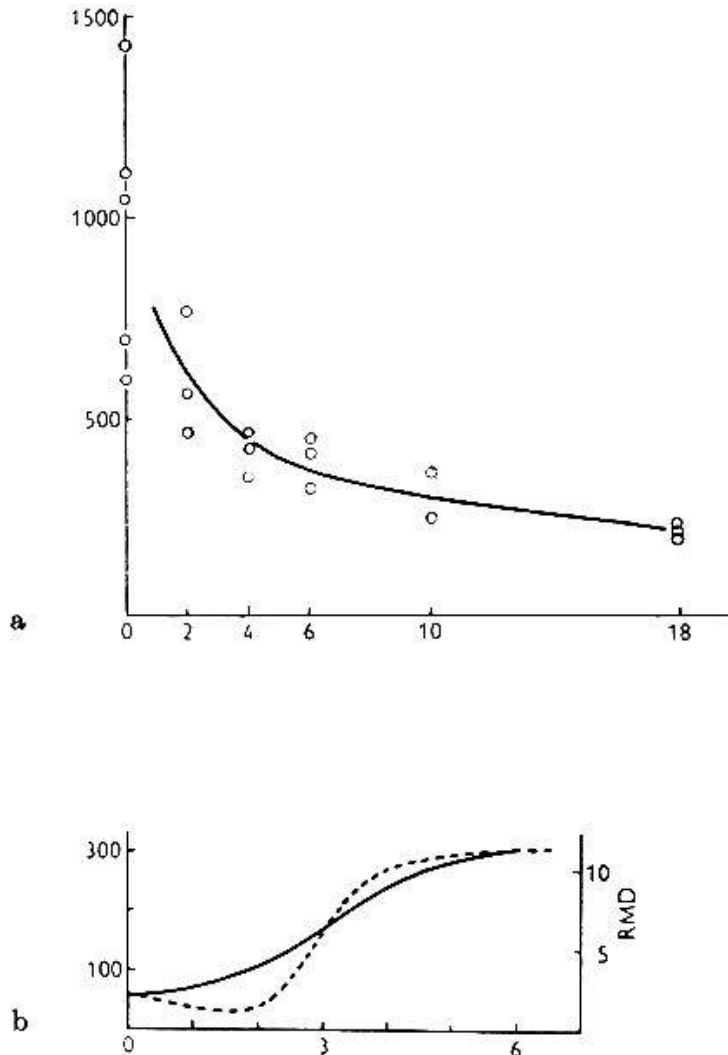


Fig. 20.—a) Isotope content ( $^{35}\text{S}$ -methionine) of the acid-soluble fraction during the development of *Paracentrotus lividus*.—b) Comparison of the curves for relative mitochondrial density (RMD) and the isotope incorporation by the particulate fraction during the first 6 h of development (from NAKANO and MONROY: *Exp. Cell Res.* 14 [1958]).

Table 7  
Incorporation of  $^{15}\text{N}$ -adenine into various cellular fractions in embryos of *Psammechinus miliaris*. Per cent  $^{15}\text{N}$  excess in purified proteins (from HULTIN [1953e]).

	Cleavage stages	Early mesenchyme blastula
Mitochondria	0.11	0.44
Microsomes	0.23	0.42
Supernatant 1	0.14	0.41
Supernatant 2	0.11	0.25

The results reported may apparently be generalized into the statement the acquisition of the c-property (ciliated cl-cells, intestinal cf-cells) entails a large increase in RMD; a corresponding, but smaller increase seems to be associated with the f-property.

Some of the observations made by GUSTAFSON and LENIQUE (1952) suggest that the characteristically large mitochondria of vegetal cells (presumably part of the f-properties) are present in the intestine.

*Enzymes.* – Several mitochondrial enzymes have been studied with regard to their changes during sea urchin development, viz., malate and succinate dehydrogenases (GUSTAFSON and HASSELBERG 1951), cytochrome oxidase (DEUTSCH and GUSTAFSON 1952), aspartate and alanine transaminases (BLACK 1964), cathepsin B, glutaminase I and ATPase, the latter three by GUSTAFSON and HASSELBERG (1951). The transaminase and the ATPase activities are presumed to represent two or more enzymes, out of which one is bound to the mitochondria (DE DUVE et al. 1962).

It was shown by GUSTAFSON and HASSELBERG (1951) that the activity of most mitochondrial enzymes show a similar pattern of change, involving a large increase in activity during the mesenchyme blastula stage. These changes could neatly be correlated with the increase in mitochondrial number subsequently established. However, as we shall presently discuss, certain irregularities obtain, suggesting that the mitochondrial population is subjected not only to growth, but also to differentiation, a conclusion in excellent agreement with the morphological observations discussed in the preceding section.

It was inferred that during embryogenesis different mitochondrial populations successively replace each other. It might be presumed that this occurrence would be reflected in the enzyme activity curves, at least in the form of periods with constant activity. The determinations on mitochondrial enzymes do not include points enough to reveal finer details in the curves, but it is interesting to note that one curve obtained by GUSTAFSON and

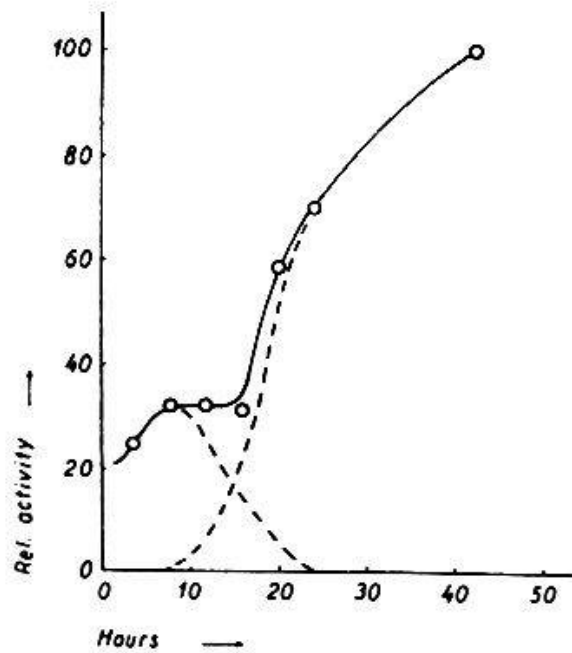


Fig. 21. Changes in succinate dehydrogenase activity during the development of *Psammochinus miliaris* (redrawn from GUSTAFSON and HASSELBERG: *Exp. Cell Res.* 2 [1951]). The stippled lines represent a suggested resolution of the observed curve.

HASSELBERG for SDH (Fig. 21) actually shows constant activity for several hours. As I have indicated in the figure, this part of the curve may indeed be composed of a declining and an increasing component, representing the two first mitochondrial populations, respectively.

Considering the large increase in RMD occurring after hatching it appears that the early mitochondria have a large SDH content, as compared with those formed later.

The changes in cytochrome c oxidase (DEUTSCH and GUSTAFSON 1952) present a very complicated picture (Fig. 22). If we resolve curve 1 into two components, we get two curves which, with respect to time, correspond reasonably well to the curves established for the changes in mitochondrial number and SDH activity (Fig. 18 and 21). A very conspicuous difference obtains with respect to activity, however, the cytochrome oxidase being very high compared to that of SDH during the early stages.

From the work of MAGGIO (1959) it is known that in isolated mitochondria there is an activation of the cytochrome oxidase activity—amounting to 20–35%—immediately after fertilization, but no further changes occur in the specific activity during the following 3 h. This implies that no mitochondrial differentiation takes place during this period. It seems that the very steep increase in enzyme activity (cf. Fig. 22) at a time when there is no increase in mitochondrial number according to Fig. 18 might be accounted for by the findings of MAGGIO. However, if the observed enzyme activity were bound to the mitochondria and if, as suggested by MAGGIO's results, no change in specific activity occurs, then the increase in mitochondrial number and in cytochrome oxidase activity should be proportional. This

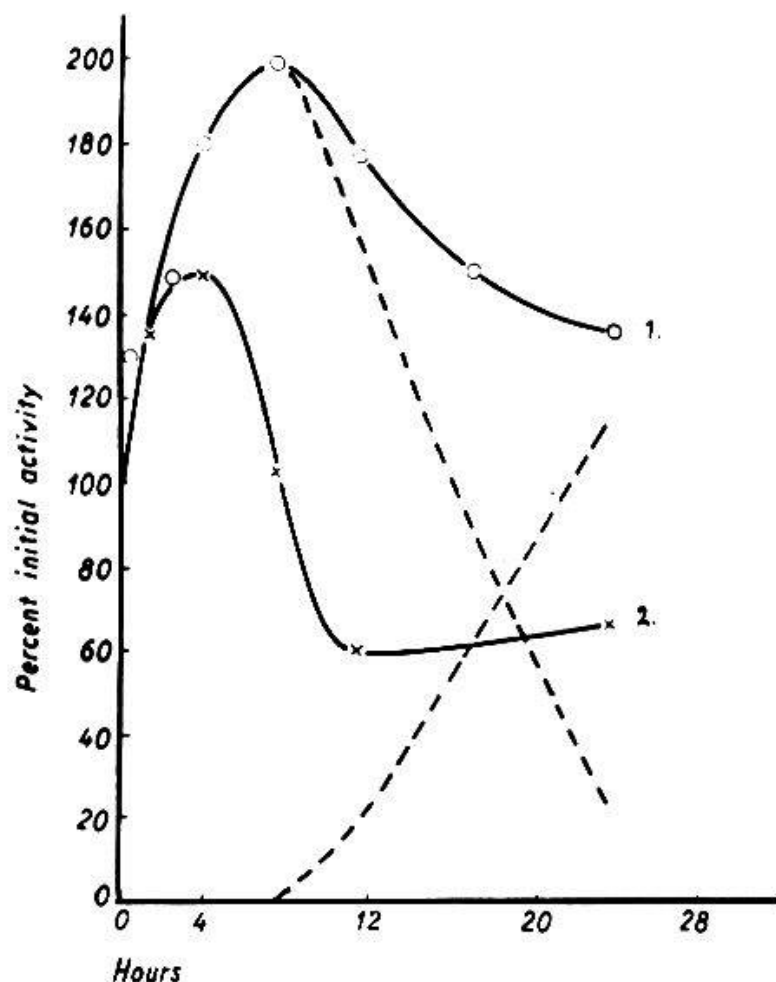


Fig. 22. Changes in cytochrome c oxidase activity during the development of *Psammochinus miliaris* (redrawn from DEUTSCH and GUSTAFSON: *Ark. Kemi* 4 [1952]).—The stippled lines represent a suggested resolution of the observed curve.—It should be emphasized that all the curve resolutions suggested in the present paper (cf. Fig. 7, 18, 21, and 22) at best are very crude approximations. If it be possible to make similar resolutions on the basis of experiments, a number of interesting details in the curves may be anticipated.

expectation is not fulfilled; thus, although the mitochondrial density increases at least five-fold, the rise in enzyme activity is only about 50%, after correction for the fertilization activation. This disagreement suggests that a considerable part of the activity represents an extramitochondrial cytochrome oxidase. This question was investigated by HUTCHENS et al. (1942), who found that 67% of the total activity in an egg homogenate is present in a supernatant fraction, and only 1% in the mitochondria. However, as they could account only for 80% of the total activity, it cannot be excluded that the mitochondrial enzyme is more active in the unfractionated homogenate. These authors employed a manometric method, whereas DEUTSCH and GUSTAFSON (1952) as well as MAGGIO (1959) used spectrophotometry. It is not impossible that different methods may give somewhat divergent results, but all the same it seems warranted to conclude that there are two different cytochrome oxidase fractions in the early embryo, both of which decline rapidly during early development.

It might be possible by calculations to get an approximate estimation of the contents in each of these fractions, but a renewed experimental attack ought to give more satisfactory information. The differences between curves 1 and 2 in Fig. 22 suggest that in the latter case the enzyme activity in the second generation of mitochondria is relatively lower than in the first batch.

None of the curve resolutions performed above (Fig. 7, 18, 21, and 22) have been suggested directly by the observed results, the justification for this expedient is mainly to be found in indirect evidence, first of all through the agreement between different lines of approach thus established. However, the results of IMMERS and RUNNSTRÖM (1960) on the DNP stimulation of the respiration (Fig. 23) show two clearly separated curves; the minimum uniting them is located at the end of the phase of constant respiration. If, as these authors suggest, the curve of DNP-stimulated oxygen uptake represents the maximum oxidative capacity in the embryo, then it seems absolutely unavoidable to conclude that two different mitochondrial (or extra-mitochondrial) oxidative systems are present in the sea urchin embryo, the first one being gradually replaced by the second one during gastrulation.

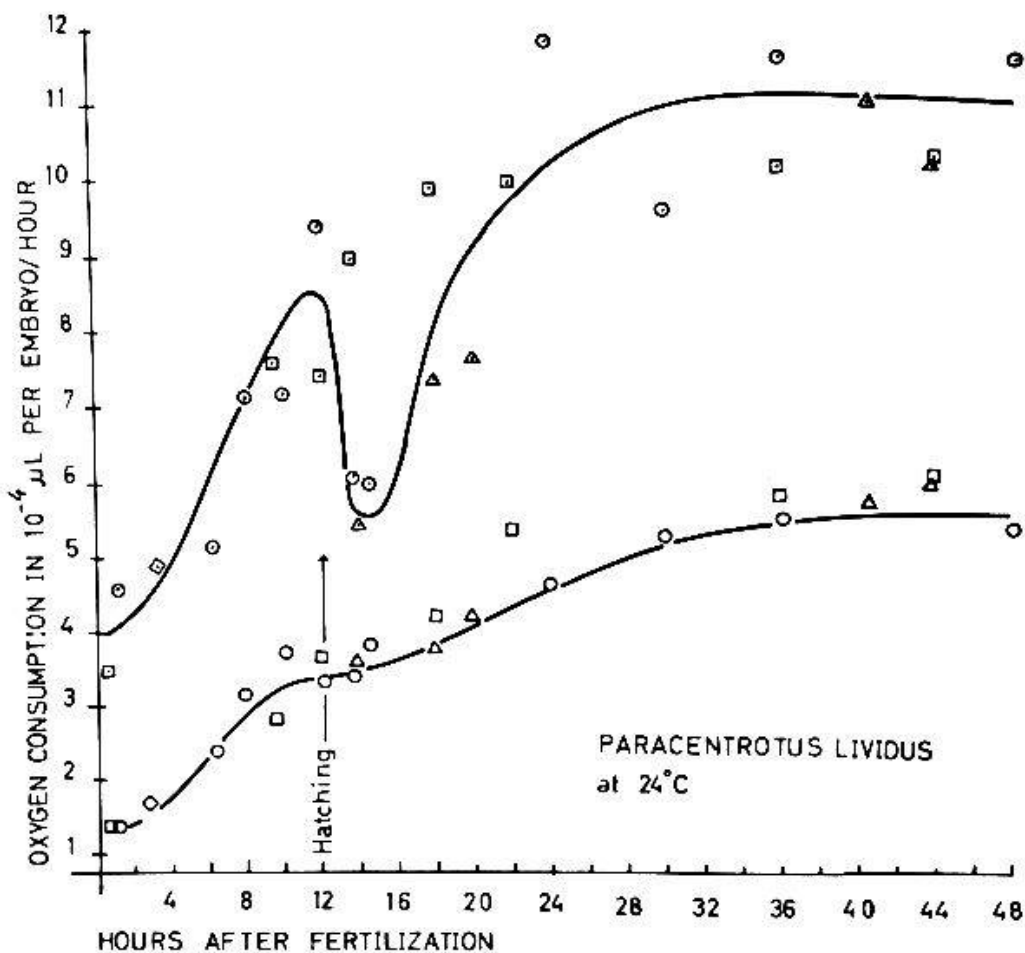


Fig. 23. Lower curve: Normal respiratory rates at different stages of development. Upper curve: Oxygen consumption after addition of 2,4-dinitrophenol ( $5 \times 10^{-5}\text{M}$ ) (from IMMERS and RUNNSTRÖM: *Develop. Biol.* 2 [1960]).

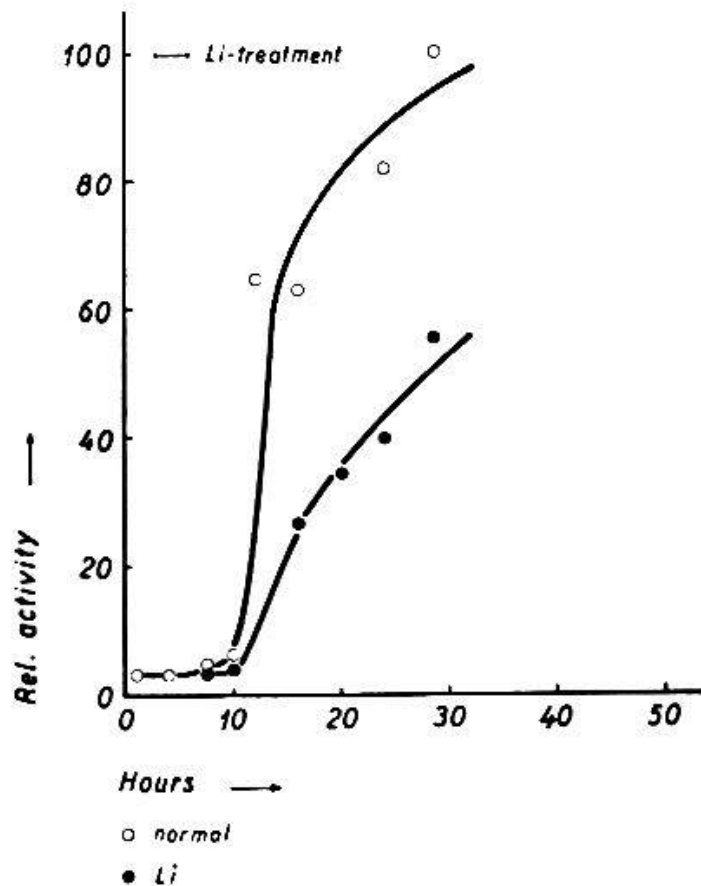


Fig. 24. Changes in cathepsin B activity during the development of *Psammechinus miliaris* (from GUSTAFSON and HASSELBERG: *Exp. Cell Res.* 2 [1951]).

Among the remaining enzymes the increase in activity for cathepsin B, glutaminase I and ATPase begins at a time corresponding to the second phase of mitochondrial formation. For the two former enzymes the relative increase seems to be much higher than for the dehydrogenases, suggesting that the early mitochondria are very poor in these enzymes (Fig. 24). Conversely, the relative increase in ATPase activity is quite low (Fig. 25), a phenomenon which of course also might be related to the enzyme content of the first mitochondrial population. It is more likely, however, that this phenomenon should be referred to the fact that, as mentioned above, this enzyme is not located exclusively in the mitochondria. The observed activity may be the sum of a constant, extramitochondrial component, and a mitochondrial enzyme which increases together with the other mitochondrial enzymes.

Similar results were obtained with respect to the transaminases studied by BLACK (1964), two other enzymes known to be localized both in and outside the mitochondria (Fig. 26). Because of the few points, and the low relative increase, it is difficult to draw any definite conclusions, but it seems that the alanine transaminase begins to increase in the late blastula stage, suggesting that it is synthesized together with the other mitochondrial enzymes during the second phase of mitochondria formation. The aspartate transaminase activity, on the contrary, decreases until the pluteus stage. This

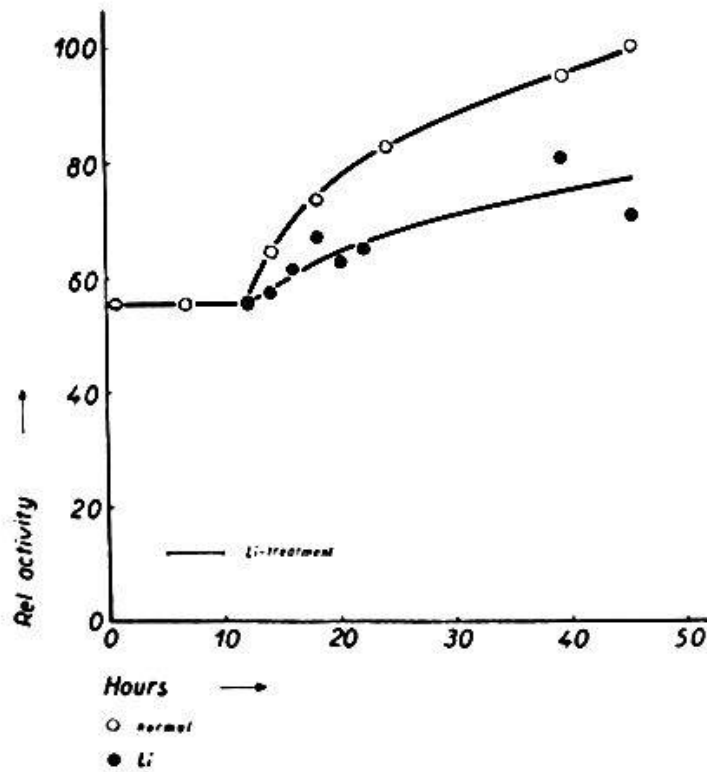


Fig. 25. Changes in ATPase activity during the development of *Paracentrotus lividus* (from GUSTAFSON and HASSELBERG: *Exp. Cell Res.* 2 [1951]).

might indicate that this enzyme is synthesized only in association with the third phase of mitochondria formation, the one described by SHAVER (1956). If this interpretation is correct, it is obviously possible to conclude that the particles synthesized in each phase are chemically distinct.

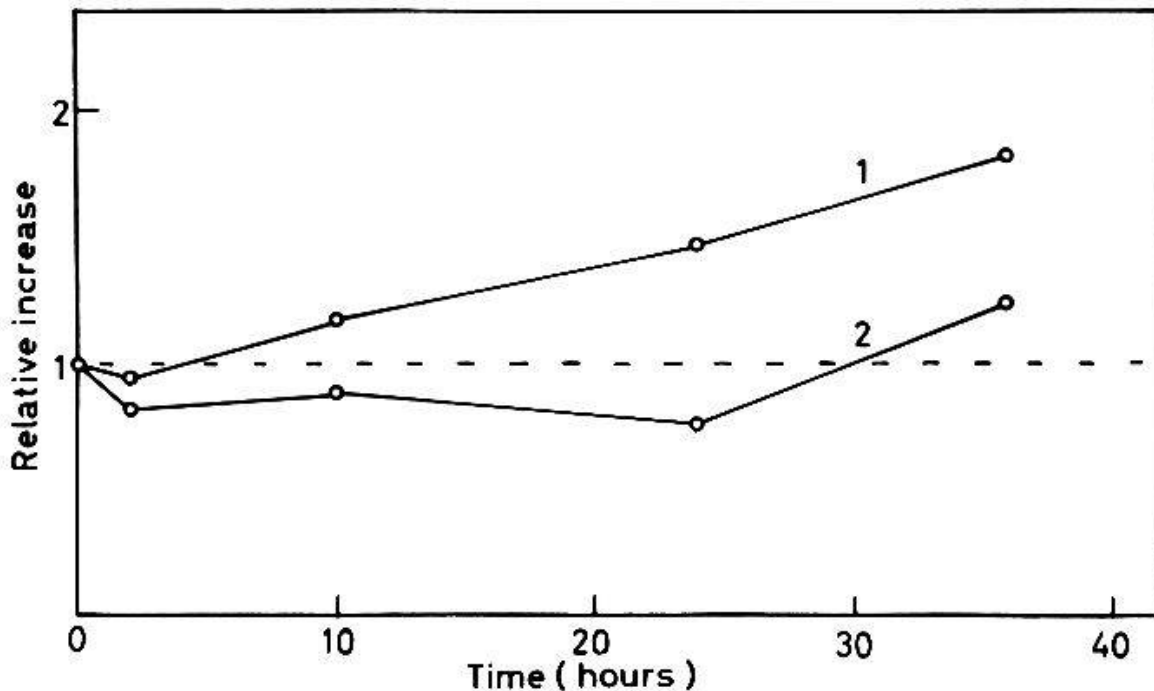


Fig. 26. Changes in transaminase activity during the development of *Lytechinus variegatus*.—1 = alanine transaminase, 2 = aspartic transaminase (the figure has been drawn on the basis of results published by BLACK [1964]).

It is remarkable that the transition between the second and third phase has not been reflected in any of the enzyme activity curves. This may in some cases depend on the fact that the determinations were discontinued too early, but in other cases this explanation cannot account for the observations. One possibility which might be invoked is that the enzymes are preserved when the mitochondria disintegrate, but this explanation seems quite unlikely. Another possibility, which can partly explain the findings, is that the enzyme activity in the third generation of mitochondria is higher than that of the second one.

The results discussed in this section show that both growth and differentiation occur with respect to the mitochondrial population. It is remarkable to note that these changes occur at a time when no enzyme changes can be recorded in other cytoplasmic fractions (cf. below). This autonomy certainly suggests that mitochondria form by self reproduction (cf. the discussion by LEHNINGER 1964).

The differentiation of the mitochondria is not a gradual process, rather one population of particles is, in a relatively short period of time, replaced by a new one, which may be different with respect to both morphological and chemical traits. I believe that this phenomenon may be best understood from a phylogenetic point of view (cf. below).

It seems possible to correlate the transfer from the first to the second generation of mitochondria with the transformation of the amoebocytes and mechanocytes, respectively. One might even anticipate two different populations of mitochondria, specific for each of the cell types. There are some indications in support of this contention, thus it was observed by BERG and LONG (1964) that the increase in cytochrome oxidase is higher in vegetal than in animal halves. As the latter contain more mitochondria (GUSTAFSON and LENIQUE 1952) one may presume that they have a low relative content of this enzyme. Likewise, histochemical determination of NAD-cytochrome c reductase shows that this enzyme is mainly confined to the mesenchyme cells (CZIHAK 1962). In contrast the indophenol oxidase activity, up to the mesenchyme blastula at least, seems to be localized in animal-ventral cells (CZIHAK 1963). Whether this activity represents a mitochondrial or an extramitochondrial enzyme may be difficult to decide.

The transition from the second to the third generation apparently cannot be correlated with any morphological event, as mentioned it is not even reflected in the enzyme curves.

I shall not begin a discussion about the possible mechanisms involved in the differentiation of the mitochondria, but only mention that a mechanism like the nuclear-cytoplasmic interaction believed to be responsible for cellular differentiation might be at work in this case. This is particularly suggestive if, as proposed, mitochondria contain DNA (CHÈVREMONT et al. 1959; NASS and NASS 1962).

More mitochondrial enzymes than those treated here are known to be present in the sea urchin embryo, but none of these have been studied with

regard to the changes in activity during development. Some of them have been dealt with in the previous chapter.

*Cytochromes and oxidation in the early embryo.* — As we have seen, the sea urchin egg contains a very powerful cytochrome c oxidase. It would as a consequence be expected to contain cytochrome c, but in spite of numerous attempts (BALL and MEYERHOF 1940; KRAHL et al. 1941; ROTHSCHILD 1949; BOREI 1951; YCAS 1954), only two cytochromes have been detected spectrophotometrically, belonging to the groups a and b, respectively. MAGGIO and GHIRETTI-MAGALDI (1958) reported the presence of cytochromes a, a<sub>3</sub>, b, and c in sea urchin mitochondria; unfortunately the latter was lost during the preparation.

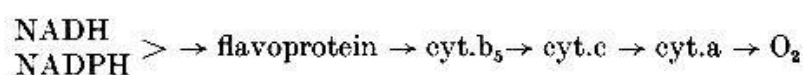
Cytochrome a<sub>3</sub> (= cytochrome oxidase or at least part of this enzyme) cannot be demonstrated spectrophotometrically, according to ROTHSCHILD (1949) because certain pigments interfere with the determination. The approach used by MAGGIO and GHIRETTI-MAGALDI should circumvent this difficulty. In support of its presence ROTHSCHILD (1956) points out that cytochrome c oxidation occurs in homogenates (KRAHL et al. 1941), and the oxidation of dimethyl-p-phenyldiamine and similar substrates has also been used as evidence (cf. RUNNSTRÖM 1956). As to substances of this type it was observed by PAPPENHEIMER and WILLIAMS (1954) that they may be oxidized through a pathway not involving cytochrome c, and that this method therefore cannot provide an unambiguous measure of cytochrome oxidase. The results with cytochrome c oxidation are of course more clearcut, but they do only prove the presence of cytochrome a<sub>3</sub> on the presumption that this is the only terminal oxidase that can react with cytochrome c.

The chemistry of cytochrome oxidase seems to be in a state of ambiguity. It was suggested by KEILIN and HARTREE (1938) that it may consist of two cytochromes, a and a<sub>3</sub>, and that it is a copper-containing protein. At present opinions seem to diverge, it may be a+a<sub>3</sub>, or a+c, or it may be only one enzyme, possibly then a<sub>3</sub> (cf. WAINIO 1961; HORIO et al. 1961, and the discussion following these papers). KRAHL et al. (1941) tested the effect of two inhibitors of copper containing enzymes, 8-hydroxyquinoline and diethyl-dithiocarbamate on the *Arbacia* cytochrome c oxidase. None of these produced any inhibition, on the contrary a considerable stimulation was observed with the latter compound. This finding does not support the contention that the terminal oxidase is the same as that found in other animal tissues, and the absence of the a<sub>3</sub> spectrum can therefore be a simple consequence of the fact that the enzyme is not there. This may not hold for the mitochondria, but they are anyhow responsible only for a small fraction of the total cytochrome oxidase activity.

It is of interest to know the activity of the various enzyme preparations in relation to the oxygen uptake of the egg. In the unfertilized *Arbacia* egg there is—assuming 0.1 mg N per mg dry weight (HUTCHENS et al. 1942)—an oxygen consumption of 4–5  $\mu$ l per hour and mg N, the corresponding value

for the fertilized egg is 20, whereas a dialyzed enzyme preparation gave a value of 100, referring to the dry weight of the eggs (KRAHL et al. 1941). The homogenates used by HUTCHENS et al. (1942) showed an oxygen consumption of 2400  $\mu$ l per hour and mg N. The enzyme activities estimated in vitro are thus in general very high compared to those in vivo.

The cytochrome b found in the sea urchin has by YČAS (1954) been suggested to be  $b_5$ , the microsomal cytochrome (cf. STRITTMATTER 1961). This, and  $a+a_3$  are the main cytochromes present in the midgut of larvae of the *Cecropia* silkworm. The enzyme activities in preparations from this source have been studied by PAPPENHEIMER and WILLIAMS (1954). These authors found that  $b_5$  is an electron donor in the reduction of cytochrome c by NADH, but  $b_5$  also reacts with NADPH (STRITTMATTER 1951), so that if indeed the cytochrome present is  $b_5$ , we may establish the following reaction sequence by addition of cytochrome c to homogenates of sea urchin eggs:



In this scheme cyt. a represents the cytochrome oxidase. This pathway is not inhibited by antimycin A, showing that the Slater factor is not involved (PAPPENHEIMER and WILLIAMS 1954). These authors also showed that cytochrome  $b_5$  is autoxidizable, in a reaction which is uninhibited by cyanide and carbon monoxide, and suggested that this compound represents the principal terminal oxidase in tissues deficient in cytochrome c in general, and in the insect larva during diapause in particular. In this way it becomes possible to account for the  $\text{CN}^-$  and CO-insensitive respiration observed in the latter case. If oxidations with  $b_5$  as the terminal oxidase can sustain the energy supply to larvae in diapause then it may be anticipated that the electron transfers in the suggested chain are associated with formation of ATP. It may be presumed that the oxidative phosphorylation observed by LINDBERG and ERNSTER (1948) in homogenates with glucose and other substrates have followed this pathway, and not the normal one present in the mitochondria. It may be relevant, in this connection, to mention the CO-insensitive oxidative phosphorylation observed by McEWEN et al. (1964) in isolated nuclei.

The terminal oxidase, cytochrome a, in the sea urchin egg, has been studied repeatedly by means of cytochrome oxidase inhibitors, both in vivo and in vitro. With enzyme preparations it has been found that the activity can be suppressed completely with cyanide and azide, and that the inhibition by CO is relieved by illumination (KRAHL et al. 1941). It thus shares a number of properties with the usual cytochrome oxidase. In studies on eggs it has been found that the inhibition by CO does not follow the rules established by WARBURG (1926) for the effect of this substance, and irregularities obtain also with respect to the effect of  $\text{CN}^-$  (cf. RUNNSTRÖM 1956; LINDAHL 1939a; ROBBIE 1946; ROTHSCHILD 1949, 1956). Apparent insensitivity was observed

in the unfertilized egg, but in the developing embryo sensitivity was gradually acquired. According to LINDAHL the paradoxical situation may be explained by the fact that CO has an activating influence upon the oxygen uptake; when this is corrected for, normal inhibition obtains; a somewhat similar mechanism can account for the results with cyanide. Other opinions have been voiced by ROBBIE (1946) and ROTHSCHILD (1949).

A final solution of this problem does not seem established, but it should be mentioned that if the cytochrome in the sea urchin egg is  $b_5$ , or at least has properties similar to those of this compound, then it may be possible to interpret the inhibition experiments along the lines suggested by PAPPENHEIMER and WILLIAMS.

It seems quite interesting, also from a phylogenetic point of view, that the microsomal cytochrome may be the only cytochrome b present in the sea urchin egg. As already mentioned, HUTCHENS et al. (1942) observed that very little cytochrome c oxidase is bound to the mitochondria, most of the activity is found in the supernatant. Whether or not the enzyme in this fraction is bound to the microsomes remains to be seen, but it is certain from the inhibition studies of KRAHL et al. (1941) that the terminal oxidase cannot be cytochrome  $b_5$ , both this and cytochrome a must therefore be present in the supernatant.

In the midgut preparations used by PAPPENHEIMER and WILLIAMS (1954) the oxidation of succinate—in the presence of cytochrome c—was very low compared to that observed with NADH, suggesting that the Slater factor limits the reaction rate. Also in this respect there is a great similarity between the silkworm larva and the sea urchin egg, because it has been observed repeatedly that succinate is not oxidized by enzyme preparations from eggs (e.g. KRAHL et al. 1941; GOLDINGER and BARRON 1946; CRANE and KELTCH 1949). In isolated mitochondria a slight succinoxidase was reported (MAGGIO and GHIRETTI-MAGALDI 1958). Most other Krebs cycle substrates, except oxalacetate which probably may form pyruvate, cannot be oxidized either (CRANE and KELTCH 1949).

These results demonstrate the inefficiency of the mitochondria already discussed in a previous section, and also that the extramitochondrial pathway has no means of dealing with intermediates in the tricarboxylic cycle.

The repeated efforts to demonstrate cytochromes c and  $a_3$  in the sea urchin egg seem to stem from the conviction that negative results must be due to experimental errors, since these compounds are found in almost all aerobic cells. Yet, there are exceptions to this rule, thus it was shown by MØLLER and PRESCOTT (1955) that in the protozoa *Amoeba proteus*, *Chaos chaos*, and *Tetrahymena geleii* only cytochrome a (or cytochrome e oxidase), cytochrome b, and cytochrome e can be found. It appears that cytochrome e = cytochrome  $c_1$  and cytochrome  $b_5$  are very closely related (PAPPENHEIMER and WILLIAMS 1954; STRITTMATTER 1961). Without entering into a discussion of cytochrome nomenclature I think it can be concluded that the content of cytochromes in the sea urchin egg is very similar to that of the investigated

protozoa. It should be mentioned that the terminal oxidase in these organisms does not react with cytochrome c (ANDRESEN et al. 1951), and that the oxygen uptake is inhibited by cyanide.

It has been argued above that the egg cell and the first blastomeres may represent a phylogenetically very primitive cell, an amoebocyte, exhibiting various archaic biochemical traits. To the list of those already established it seems that we may add lack of cytochrome c. That certain living protozoa are similar in this respect is very interesting from a comparative biochemical point of view, but it is necessary to point out that most protozoa apparently possess cytochrome systems more similar to those found in metazoa. Maybe this phenomenon reflects a series of independent mutations along a metazoan and one or several protozoan lines, originating from a cell possessing a primitive cytochrome system of the type found partly in *Chaos chaos*, partly in the sea urchin egg.

There are two points which should be emphasized. The first one is that the discussion here does not imply the absence of cytochrome c in all eggs, the formation of this substance may have become possible at various stages of the phylogenetic development, by independent mutations. A study of the pattern of distribution of cytochrome c among various eggs might indeed furnish very interesting information of importance for the problem of phylogenesis. The other point is that the presence of cytochrome c in sea urchin sperm has been demonstrated beyond doubt (ROTHSCHILD 1948; KEILIN and HARTREE 1949). This finding does not invalidate the reasoning advanced above, since the sperm cell is no amoebocyte, but belongs to the class of epitheliocytes; it may thus represent a higher phylogenetic stage. If mitochondria are self reproducing, then the mitochondrial cytochrome c containing material in the sperm (cf. AFZELIUS 1955) may be necessary for the formation of later mitochondrial generations with more developed chemical properties.

The extramitochondrial cytochrome c oxidase system as well as the inefficient mitochondria present in the egg seem to be typical for the class of amoebocytes, since they are preponderant as long as most cells belong to this cell type. As we have seen, when the amoebocytes are transformed into other cell types new kinds of mitochondria are produced, and the very peculiar extramitochondrial cytochrome c oxidase activity disappears, being replaced by the usual mitochondrial enzyme system.

#### *b) Lysosomes*

Thanks mainly to the work of DE DUVE and his collaborators (cf. review 1963) it is today possible to distinguish several fractions of large granules, previously isolated together in the mitochondrial fraction. The most important of these accessory granules is the lysosome. Among the enzymes studied in the sea urchin embryo by GUSTAFSON and HASSELBERG (1951) it is possible to distinguish a separate group of enzymes, acid phosphatase, cathepsin D and aryl- or phenylsulfatase, purported to be localized in the lysosomes.

With respect to the two first enzymes the localization seems relatively certain, but the situation is somewhat unclear concerning arylsulfatase. From rat liver three different enzymes are known, designated by the letters A, B, and C. A and B are lysosomal enzymes with a pH-optimum around 6; they have little or no activity with p-nitrophenylsulphate as substrate. C, on the contrary is a microsomal enzyme with a slightly alkaline pH-optimum, and it is active towards the mentioned sulfate ester (DODGSON et al. 1955; ROY 1958). Judging from the substrate affinity the enzyme investigated by GUSTAFSON and HASSELBERG is arylsulfatase C, but it has a pH-optimum around 6, suggesting that it is of the lysosomal type (A or B). In spite of the uncertainty concerning the classification of this particular enzyme, the experimental results establish the fact that no increase in lysosomal enzymes occurs during the investigated phase of sea urchin development. Although it cannot be excluded that lysosomal enzymes are synthesized, it seems likely that, in contrast to the mitochondria, no increase occurs in the number of these particles. The results presented in Fig. 27 are typical for the enzymes in this and the following group.

*c) Supernatant*

The following enzymes may be referred to the supernatant fraction: glutathione reductase (BÄCKSTRÖM 1959a), dipeptidase (HOLTER and LINDAHL 1940), adenosine deaminase, pyrophosphate (and hexametaphosphate?) hydrolase, and aldolase (GUSTAFSON and HASSELBERG 1951). No increase occurs in the activity of any of these enzymes suggesting that the supernatant fraction is subjected to no increase (growth) during the investigated period of development.

As already discussed in a previous chapter the presence of aldolase in the sea urchin embryo is questionable.

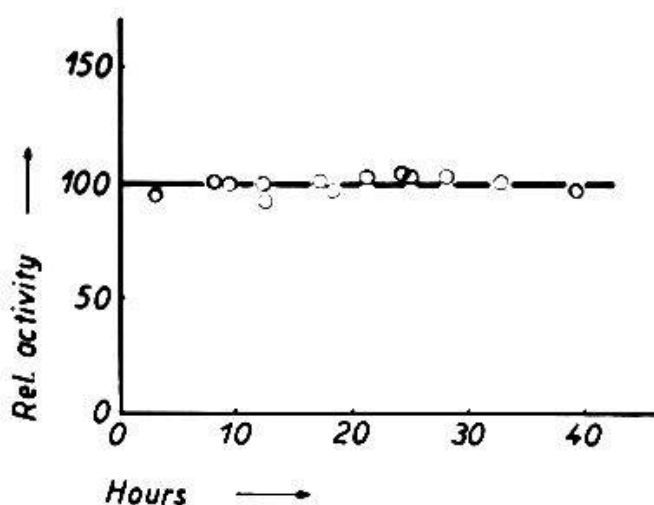


Fig. 27. Activity of cathepsin D during the development of *Psammechinus miliaris* (from GUSTAFSON and HASSELBERG: *Exp. Cell Res.* 2 [1951]).

## 2. Cell class specific enzymes

Under this heading will be treated partly certain enzymes which with some assurance can be referred to the new cell types arising during embryogenesis. There are other enzymes, for which such a correlation is not possible, but having in common that their activity decreases during development it may be suspected that they are enzymes typical for amoebocytes. I have, without further justification, classed these as amoebocyte enzymes.

### a) Amoebocyte enzymes

*Catalase.* – During development the catalase activity decreases in two phases (DEUTSCH and GUSTAFSON 1952). The first, slight decrease lasts until the mesenchyme blastula stage, the second during the remaining part of the investigated developmental period (Fig. 28). The decreasing activity may, as suggested by the authors mentioned, reflect a gradual formation of inhibitors. I may suggest that compared to the other cell types, a high content of catalase may be a typical trait of amoebocytes, and that the gradual decrease in enzyme activity represents the continuous reduction in the number of sl-cells.

According to DE DUVE et al. (1962) catalase is found in a special type of granules, containing besides uricase and D-amino acid oxidase. It would be interesting to know about the changes in the activity of the latter enzyme, if present.

*2'-deoxyribosyl 4-aminopyrimidine 5'-phosphate deaminase.* – This enzyme, dAPDase, has been discovered by SCARANO (cf. 1961). It catalyzes the conversion of deoxycytidylic acid to deoxyuridylic acid and of deoxymethylcytidylic acid to thymidylic acid. It is found in large amounts in the sea

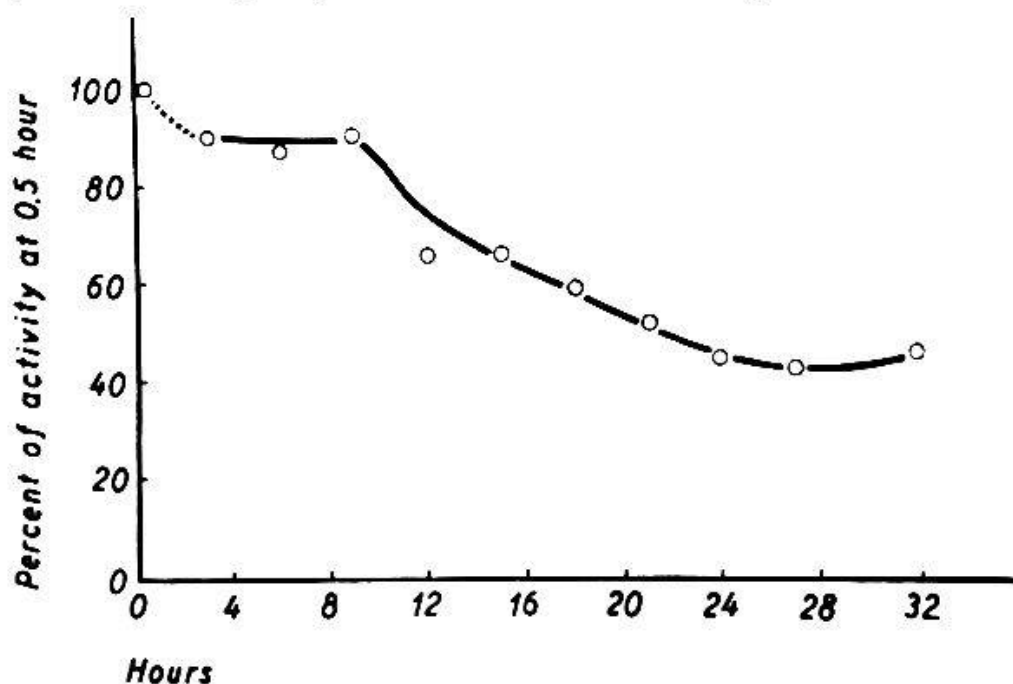


Fig. 28. Changes in catalase activity during the development of *Psammechinus miliaris* (from DEUTSCH and GUSTAFSON: *Ark. Kemi* 4 [1952]).

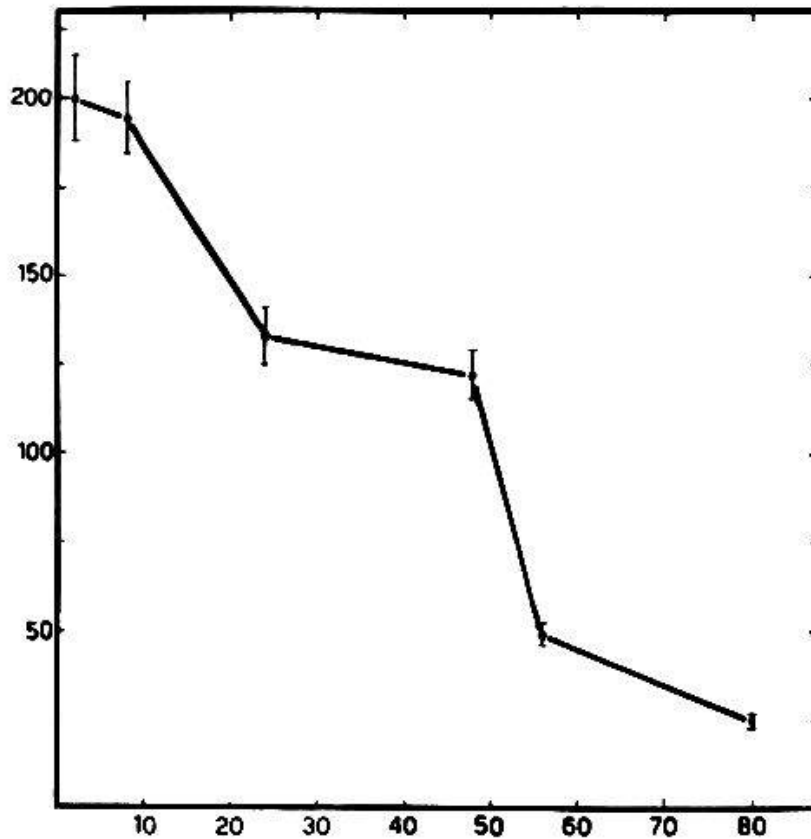


Fig. 29. Changes in 2'-deoxyribosyl 4-aminopyrimidine 5'-phosphate deaminase activity during the development of *Sphaerechinus granularis* (from SCABANO: Symp. on germ cells and development [1961]).

urchin egg, but the activity gradually diminishes during development. This decrease occurs in two phases, just as the DNA-synthesis, suggesting a correlation between these two phenomena (Fig. 29). The timing does not correspond to that shown for DNA-synthesis in *Paracentrotus lividus*, but this discrepancy may be correlated with the fact that the species in which the dAPDase has been investigated, *Sphaerechinus granularis*, apparently develops more slowly.

The pattern of change suggests that this enzyme is characteristic for the amoebocytes, disappearing gradually as these are transformed into other cell types.

*Deoxyribonuclease.* - MAZIA (1949) has followed the activity and the sub-cellular localization of deoxyribonuclease during the development of *Arbacia punctulata*. The activity remained constant throughout, but from being completely soluble at the outset the enzyme could be sedimented to 90% at the end of development. MAZIA concludes from these results that the enzyme becomes localized in the nucleus.

It seems possible to distinguish two different DNAases, one active at alkaline or neutral pH, and activated by  $Mg^{++}$ , the other with an acid pH optimum, and  $Mg^{++}$ -independent. The former is a mitochondrial enzyme, the latter is found in the lysosomal fraction (DE DUVE et al. 1962). The en-

zyme investigated by MAZIA (1949) was active at neutrality,  $Mg^{++}$  was added but it was not stated whether it was required or not.

According to these characteristics the enzyme investigated by MAZIA may be the mitochondrial enzyme, and since the centrifugal force applied (20 000 g for 15 min) should be strong enough to sediment the mitochondria it does not seem excluded that the enzyme was actually bound to these particles.

The question about the localization remains open, but I would suggest that the primitive mitochondria in the amoebocyte may be distinguished by not containing any DNase, in contrast to the more evolved mitochondria contained in the other cell types. The constant curve observed by MAZIA might thus be composed of a gradually decreasing and a gradually increasing component. It could be argued that chances for the resultant be a horizontal line would be minimal, but the number of points, and the precision of the determinations would hardly warrant the detection of smaller changes.

*Glucose-6-phosphate and 6-phosphogluconate dehydrogenase.* – BÄCKSTRÖM (1959b) has followed the changes in the G-6-PDH activity during development. This enzyme is localized in the supernatant, but the activity is not constant like that of the other enzymes in this fraction, on the contrary, there is a substantial increase in activity up to the stage of hatching, after which the enzyme activity gradually diminishes. Several peaks were observed on the curve, both during the rising and the declining phase (Fig. 30).

The absence of activity in the unfertilized egg shown in the figure can

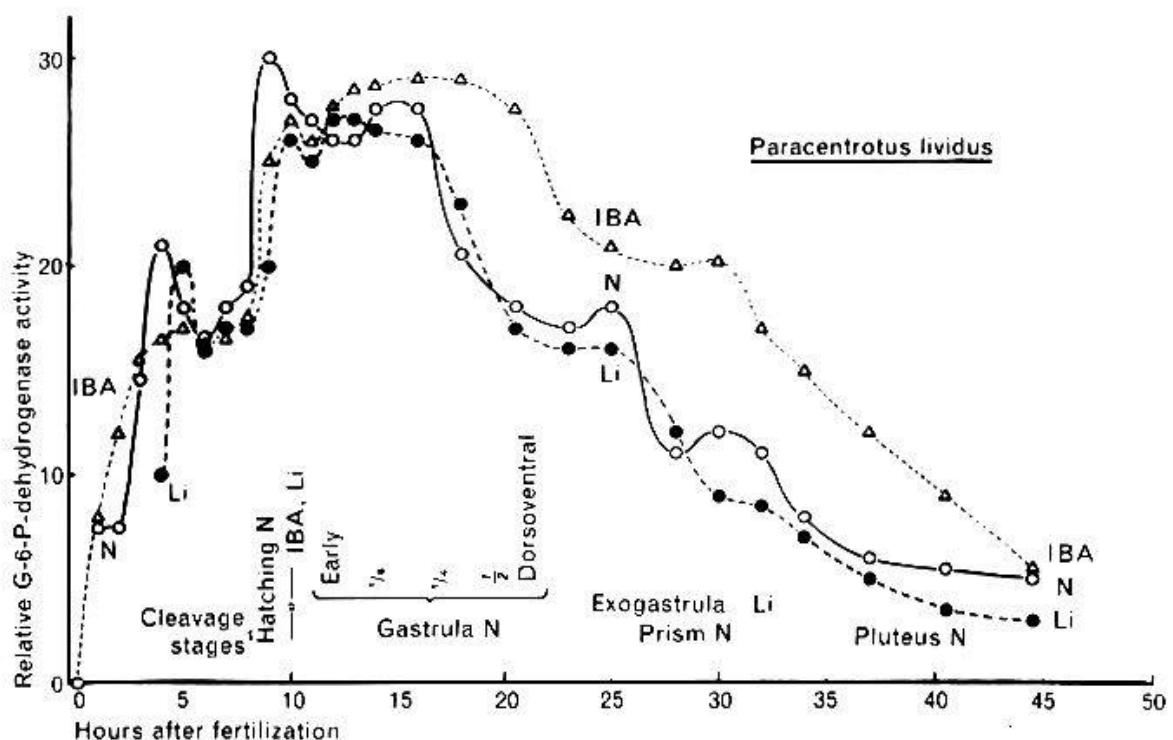


Fig. 30. Changes in glucose-6-phosphate dehydrogenase activity during the development of *Paracentrotus lividus* (N = normal embryos, Li = vegetalized embryos, IBA (o-iodobenzoic acid) = animalized embryos (from BÄCKSTRÖM: Exp. Cell Res. 18 [1959b])).

hardly be a general phenomenon, in other batches of *Paracentrotus lividus*, and in *Psammechinus miliaris* there was measurable activity at this stage. This confirms the findings of KRAHL et al. (1955); these authors furthermore did not observe any change immediately after fertilization in *Arbacia punctulata*. G-6-PDH is the first of the enzymes in the PP cycle. This metabolic pathway has been suggested to be of general occurrence in embryonic cells, but as we have discussed above the presence of the whole cycle does not seem to have been proven.

However this may be, it seems that the metabolic pathway involving G-6-PDH represents the main source of energy supply in the early embryo. Changes in the energy consumption must of course entail changes in the oxygen consumption and in the activity of the particular enzymes, but not necessarily the enzyme synthesis which apparently takes place. The curves obtained by BÄCKSTRÖM (1959b) need not, indeed, represent enzyme synthesis, they might as well represent enzyme activation. However, as appears from the discussion elsewhere in this paper, I do not think one ought to take recourse to this explanation unless definitely required, and there seems to be no reason to do it in the present case. The fact that the content of G-6-PDH is high during early development and gradually declines suggests that this enzyme is a distinguishing, but of course not a unique feature of the amoebocyte, as well as mitochondria of a morphologically distinct and chemically inefficient type.

As we have already discussed there are indications that mitochondria of this type are produced during pre-hatching development, and other enzyme systems, including G-6-PDH might be synthesized as well. There is no reason to doubt that the egg may possess the necessary apparatus for synthesis of amoebocyte proteins, and as we know from the isotope studies of HULTIN (1953c) and MARKMAN (1961b), the rate of incorporation in the protein fraction is relatively high during early development, as compared with for instance the incorporation into nucleic acids (cf. Fig. 10).

The two phases of enzyme increase observed before hatching suggest energy requiring activity occurring in two separate regions of the egg and I believe that BÄCKSTRÖM (1959b) is correct in proposing the first phase to be associated with development of the animal, the second with the development of vegetal cells. Each of these phases of enzyme increase may be associated with increased oxygen consumption, as suggested above (cf. Fig. 7). In my opinion this correlation definitely excludes that the enzyme synthesis, as well as the mechanism of glucose oxidation, can be of specific importance for either animal or vegetal determination proper. Their only role seem to be the supply of energy to both processes. The first peak occurs slightly before or simultaneously with the onset of the synthesis of acetylcholine esterase typical for epitheliocytes, and a similar correlation obtains between the second enzyme and alkaline phosphatase, the enzyme characterizing the mechanocyte (cf. the following sections). It may be envisaged that in association with their own transformation the amoebocytes respond

to the increased energy demands by synthesizing the enzymes which form part of their main source of energy supply. The decline would reflect that the transformation has occurred, that the rate at which G-6-PDH is synthesized by the new cell types is too low to keep up with the breakdown.

I would like to insist that these two peaks correspond only to the cells near the poles, the cells at the apical tuft, and the presumptive primary mesenchyme cells. Gradually even the remaining cells transform; the third peak, occurring during early gastrulation might represent the secondary mesenchyme cells, the fourth and fifth might represent phases of transformation of either endodermal or ectodermal cells (cf. BÄCKSTRÖM 1959b).

The peaks during the later part of development are quite modest, although the number of cells involved, according to the present interpretation, is much larger. This lack of correlation may be seen on the background that the cells at this stage of development are well supplied with mitochondria, direct oxidation may no longer be the main pathway for energy supply. This is revealed among other things by the plain fact that the rate of oxygen consumption after hatching is considerably higher than before, although the G-6-PDH activity is substantially reduced. The fluctuations in energy consumption purportedly demonstrated by the enzyme curves are not reflected by the oxygen consumption curves (cf. Fig. 5). This could hardly be expected, since the differential activity at any moment is concentrated in a small part of the embryo.

Studies on 6-PGDH has shown that its activity changes according to a pattern very similar to that observed for G-6-PDH, with the exception that the decrease is less distinct in the pluteus (BÄCKSTRÖM 1963).

#### *b) Epitheliocyte enzyme*

*Acetylcholine esterase.* – Among the enzymes investigated in the sea urchin embryo, only two remain, acetylcholine esterase (AChE) and alkaline phosphatase (Aph).

It holds for both that more than one enzyme is known to attack their typical substrates, and various expedients (substrate specificity, activation, etc.) are required to distinguish between the isozymes (cf. AUGUSTINSSON 1950; STADTMAN 1961). Both enzymes are relatively diffusely distributed among the cellular fractions (DE DUVE et al. 1962).

AUGUSTINSSON and GUSTAFSON (1949) have studied the content of AChE during the development of *Paracentrotus lividus*. No demonstrable enzyme activity could be recorded in the unfertilized egg, but during development a gradually increasing curve was observed (Fig. 31). Tracing this curve back to zero activity it can be estimated that the synthesis begins 4–5 h after fertilization. On the basis of their results and on various evidence from the literature the authors concluded that there is a close correlation between the development of ciliary or contractile activity and synthesis of AChE.

It was possible by treatment with  $\text{Li}^+$  to distinguish three different phases of synthetic activity (Fig. 31). The first phase begins, as mentioned, a few

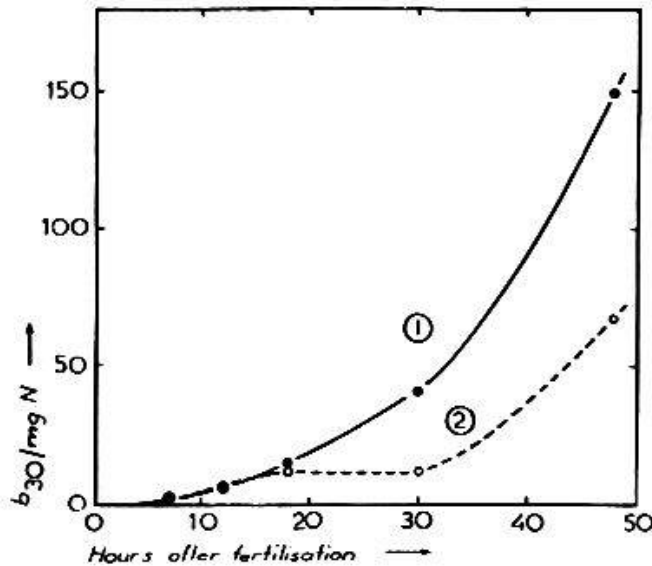


Fig. 31. Changes in acetylcholine esterase activity during the development of *Paracentrotus lividus*.—1 = normal embryos, 2 = vegetalized Li-treated embryos (from AUGUSTINSSON and GUSTAFSON: *J. cell. comp. Physiol.* 34 [1949]).

hours after fertilization and lasts to about 15–18 h, corresponding to late gastrulation. This synthesis must reflect partly the development of the apical tuft and partly the formation of ciliated cells in the remaining part of the embryo.

The second phase lasts from 15–18 h to 30–35 h and reflects the development of the ventral ciliary bands and other ciliated ectodermal structures.

The third phase beginning after 30–35 h corresponds to the development of the intestine which at this time begins to show contractility.

WILLMER (1960) has also pointed out the correlation between ciliation and AChE, and if we adopt his cell classification scheme, we might say that the appearance of AChE activity demonstrates the occurrence transformation  $sl \rightarrow cl$ . If this formulation is correct it follows that no activity should be found in the early embryo, containing only amoebocytes, and this expectation is confirmed by experimental evidence. It appears that the cell transformation with respect to the c-property occurs at a quite late stage in the endoderm and certain parts of the ectoderm. This timing was actually suggested by the results of the G-6-PDH discussed in the previous section. It should be emphasized that the time of transformation need not be correlated with the time of determination in any simple fashion.

### c) *Mechanocyte enzyme*

*Alkaline phosphatase.* – As suggested AChE may be typical of epitheliocytes, and the synthesis of this enzyme accordingly an index of the formation of cells with the c-property. A similar correlation seems to obtain between APh and the f-property. The absence of either enzyme from the amoebocytes in the early embryo would appear a necessary requirement for the acceptance of this contention, and for AChE this expectation was ful-

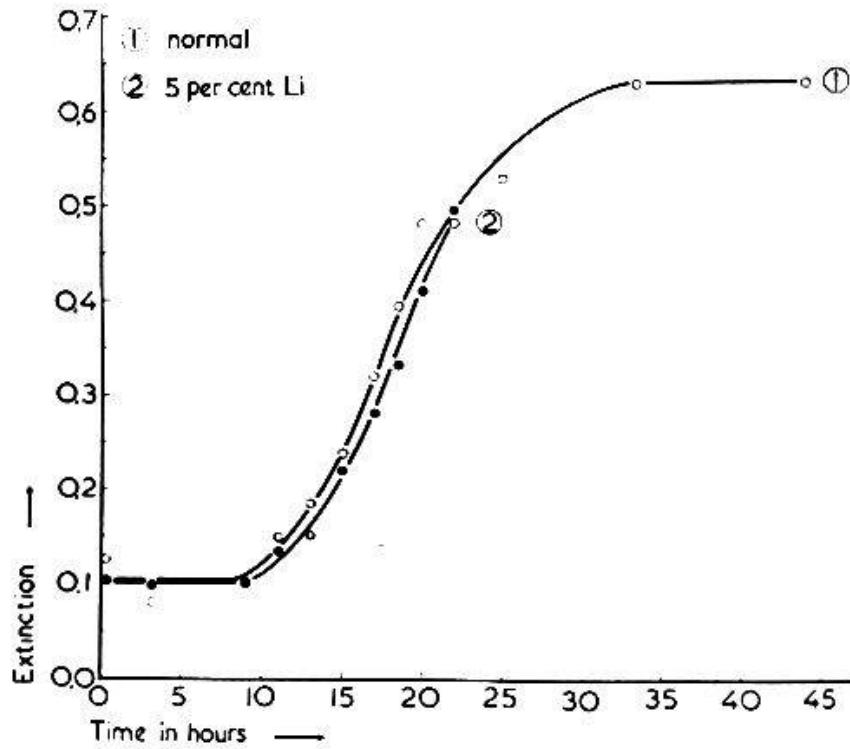


Fig. 32. Changes in alkaline phosphatase activity during the development of *Psammechinus miliaris*.—1 = normal embryos, 2 = vegetalized Li-treated embryos (from GUSTAFSON and HASSELBERG: *Exp. Cell Res.* 1 [1950]).

filled. Matters are somewhat more obscure with respect to APh, for as shown in Fig. 32 there is substantial APh activity in the unfertilized egg (GUSTAFSON and HASSELBERG 1950). The moderate increase in relative enzyme activity during development shows that the amount of enzyme present at the beginning is quite substantial. In amphibian embryos matters are simpler, for here the enzyme activity before gastrulation is hardly measurable, the presence of APh at this stage can be questioned. The very low activity at the early stages is reflected in the large relative increase (cf. KRUGELIS 1950; KRUGELIS et al. 1952; DE CESARIS COROMALDI 1955; LÖVTRUP 1955, 1958, 1959b).

There seems to be two possibilities to account for the experimental findings without violating the principles advocated above. The first one is that the activity at alkaline pH represents residual activity of the acid phosphatase. As we have seen above, the activity of this enzyme remains constant during development, and since its activity in the gastrula stage is not much lower than that of APh (Fig. 33), it follows that in the early stages it must have a much higher activity than the latter enzyme (cf. Fig. 32). The second possibility is that, as already mentioned, several alkaline phosphatases exist, one of which is specific for sf- and cf-cells, and that the activity present in the sea urchin egg represents an enzyme different from this one. The available evidence cannot decide the question but I would like to men-

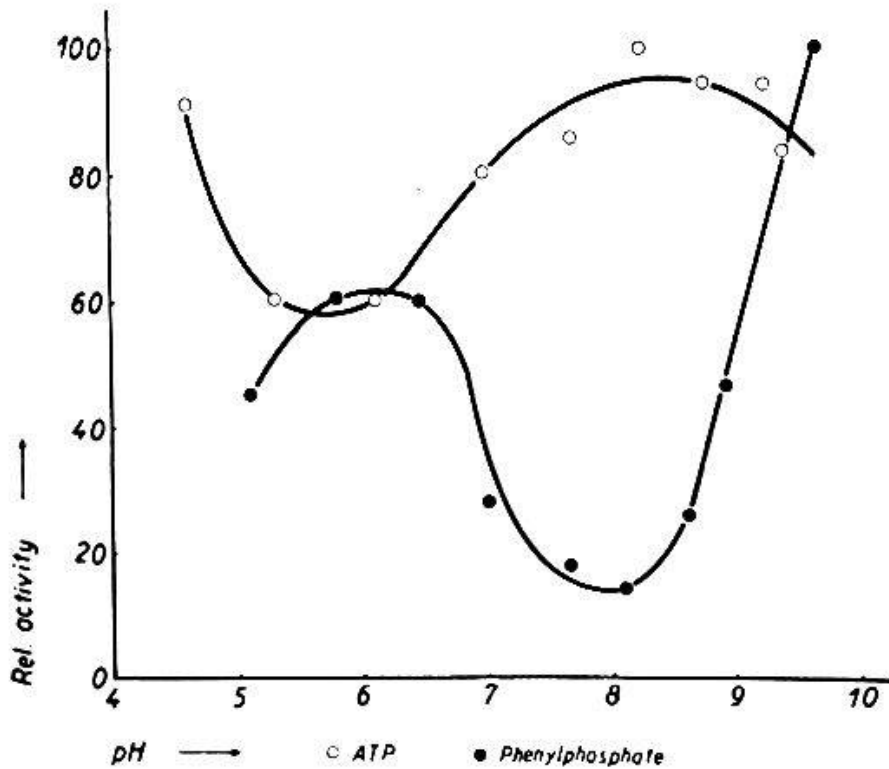


Fig. 33. Activity-pH curves for the splitting of ATP and phenylphosphate by extracts of *Paracentrotus lividus* gastrulae (from GUSTAFSON and HASSELBERG: *Exp. Cell Res.* 2 [1951]).

tion a few more facts in support of the view that APh is typical for cells possessing the f-property. Firstly, it is well known that several kinds of mechanocytes, for instance those concerned with bone formation, are rich in APh. Secondly, it has been possible to demonstrate acid, but not alkaline phosphatase activity in various protozoa (HOLTER and LOWY 1959; PIGON 1962). Since these—except probably the trypanosomas, which contain APh (HARVEY, 1949)—are either amoebocytes or epitheliocytes, this observation supports the suggestion that synthesis of APh constitutes part of the complex of properties characteristic for the f-property.

However, the strongest evidence in favour of my thesis is maybe the localization of the APh activity appearing after the mesenchyme blastula stage. By histochemical methods (Fig. 34) it has been found that all this activity is localized in the mesenchymal sf-cells and the endodermal cf-cells (EVOLA-MALTESE 1957; HSIAO and FUJII 1963). The latter authors published values suggesting that the relative increase in activity is much lower with glycerol-2-phosphate as substrate than is that found with phenylphosphate (GUSTAFSON and HASSELBERG 1950). This observation indicates that we are dealing with at least two separate enzymes with different substrate affinities, the one synthesized during development hydrolyzing phenylphosphate at a greater relative rate than the one present in the egg. Similar observations have been made with amphibian material (LØVTRUP 1955; URBANI 1962).

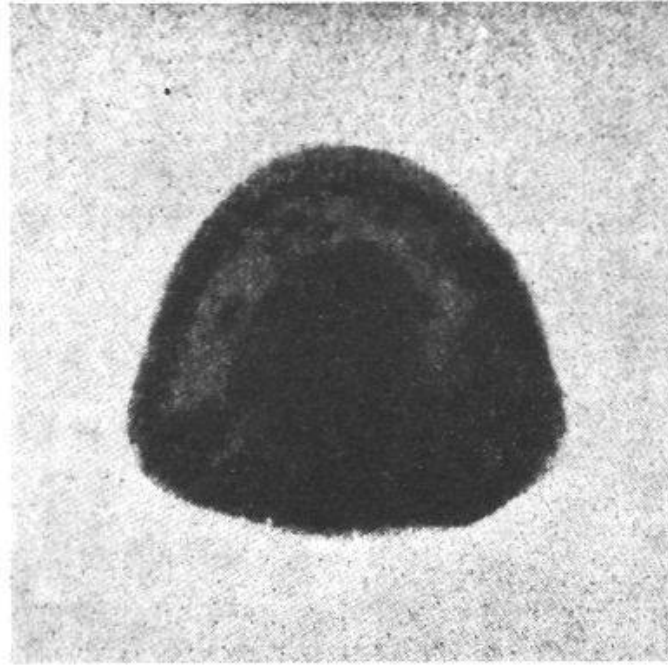


Fig. 34. Localization of alkaline phosphatase activity, as evidenced by the Gomeri method, in a gastrula of *Paracentrotus lividus* (from EVOLA-MALTESE: *Acta Embryol. Morph. exp. (Palermo) 1* [1957]).

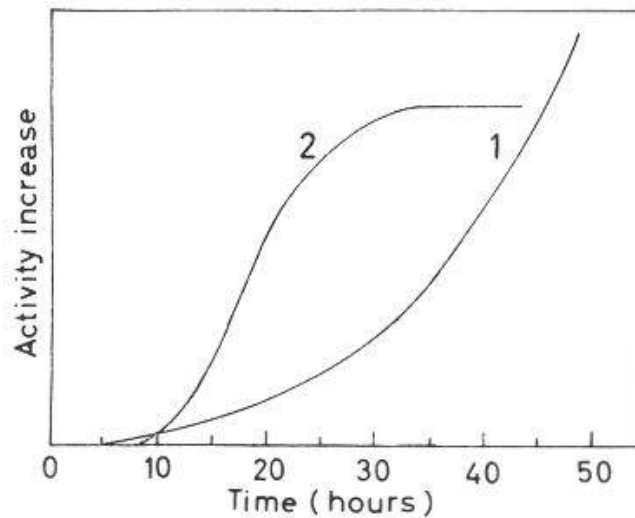


Fig. 35. Superimposition of the curves for acetylcholine esterase (Fig. 31) and alkaline phosphatase (Fig. 32).—1 = AChE, 2 = APh.—The time difference between the onset of synthesis of the two enzymes is clearly shown. In order to facilitate the comparison the early constant APh activity has been subtracted from all APh values.

The difference with respect to the time at which the synthesis of AChE and APh begins is shown in Fig. 35. The correlation with the two maxima in Fig. 30 is quite satisfactory.

#### *d) Cell transformation and cell differentiation*

At the conclusion of this section it is necessary to consider briefly the question about the separation of differentiation into two components, one concerned with cell transformation and one with the subsequent cell diffe-

rentiation. In the amphibian embryo it is easy, also with respect to enzyme synthesis, to distinguish between these two phases. Thus, the early, and very slight synthesis of AChE may be correlated with the formation of cells possessing the c-property, whereas the later and much more extensive phase of enzyme synthesis mainly corresponds to the differentiation of the nervous system (e.g. YOUNGSTROM 1938; SAWYER 1943; BOELL and SHEN 1950). A similar distinction can be made for APh activity between the formation of cells with the f-property and the differentiation of the intestine (e.g. LØVTRUP 1955). It might be argued that in the sea urchin embryo the later phases of synthesis of either of these enzymes represent cell differentiation in ectoderm and endoderm, and not cell transformation. It is very difficult to settle this matter as long as the investigations do not comprise enzymes typical for the cell differentiation process—as for instance trypsin in the amphibian embryo (cf. LØVTRUP 1955).

With this one reservation I think it can be accepted that the two enzymes, AChE and APh, can be considered as indices of the primary differentiation, the phase of cell transformations, in sea urchin development.

*e) Enzyme patterns in animalized and vegetalized embryos*

Animalization consists of a partial or complete suppression of the formation of cells with the f-property. In vegetalization a certain suppression of the acquisition of the c-property may occur, but the main factor seems to be an enhanced spreading of the f-property; the specific feature of vegetalization is the formation of cf-cells from potential cl-cells. We shall analyze the enzyme pattern in animalized and vegetalized embryos on the basis of this description of the processes, without any reference to the possible basic mechanisms involved.

Before we start this discussion be it mentioned that animalizing and vegetalizing agents are suggested to interfere with differentiation processes. As the latter are invariably associated with differential protein synthesis, it follows that we may expect to discover interference when changes in enzyme pattern occur, but in those cases where the activity remains constant there is no reason to presume any effect of the various morphogenetic agents. In all cases where this point has been tested experimentally, the expectation has been borne out (GUSTAFSON and HASSELBERG 1951).

*Mitochondrial enzymes.* – It was observed that the changes in the mitochondrial enzymes may partly represent a growth process, partly a differentiation process, since different types of mitochondria arise during development.

In embryos treated with  $\text{Li}^+$  the synthesis of three mitochondrial enzymes, cathepsin B, glutaminase and ATPase, was found to be reduced by about 50% (GUSTAFSON and HASSELBERG 1951; cf. Fig. 24 and 25). We do not know enough about the enzyme contents of the various types of mitochondria to correlate this finding with the morphological changes.

*Glucose-6-phosphate dehydrogenase.* — This enzyme was suggested to be typical for amoebocytes, and must consequently disappear as a result of the cell transformations occurring during development. Since neither animalizing nor vegetalizing agents block the transformation processes as such, but only interfere with respect to the cell types arising, one might not expect any interference with the changes in this enzyme. As far as the rise and the decline is concerned this expectation is fulfilled (cf. Fig. 30), but certain displacements of the curves are observable; in animalized embryos the activity decrease is delayed, in vegetalized embryos it is accelerated (BÄCKSTRÖM 1959b). As a matter of fact, the rate of decline is faster in this case than indicated by the figure, for the results were obtained with the «difference method», in which embryos of the same age are compared; as  $\text{Li}^-$  has a slight retarding influence (cf. Fig. 32) it follows that if the results were compared with reference to morphological development, the curve for the vegetalized embryos should be located further towards the ordinate axis. The relative position of the three curves consequently seems to demonstrate that in  $\text{Li}^+$ -treated embryos cell transformation is accelerated, in embryos treated with *o*-iodobenzoic acid it is retarded.

The smoothing of the curves, as compared with that representing normal development, suggests in agreement with morphological observations that the fine pattern in the succession of the cell transformations is partly upset by the morphogenetic agents.

*Acetylcholinesterase.* — This enzyme, representative of cl- and cf-cells, might expectedly be present in enlarged amounts in animalized, and in reduced quantities in vegetalized embryos. Of these two cases only the latter has been investigated (AUGUSTINSSON and GUSTAFSON 1949). It turned out that among the three phases of enzyme synthesis only the second one, representing the ectodermal differentiation, was suppressed. The first one, corresponding to the differentiation of the apical tuft and the ciliation of the superficial cells was not influenced, and the same holds for the third phase, related to the differentiation of the intestine. These results seem to be in conformity with morphological observations. Since the number of endodermal cells presumably is higher in a vegetalized embryo, one might expect the rate of synthesis in the third phase to be greater than in the normal embryo. The curves published by AUGUSTINSSON and GUSTAFSON (cf. Fig. 31) do not support this possibility, but the number of determinations do not suffice to decide this question. However, since  $\text{Li}^+$  interferes with the oxygen supply and the utilization of the reserve material, it is not unlikely that the synthetic activity may be interfered with. A similar situation obtains in amphibian embryos reared at high temperatures (LÖVTRUP 1953b).

*Alkaline phosphatase.* — One may expect that by sufficient strong animalization the synthesis of APh becomes completely suppressed. This question has not been investigated. Vegetalization furthers the formation of cells

possessing the f-property, and as we have seen above, it may even accelerate the rate of cell transformation. This acceleration is offset by a slight retardation of development, so no major change in the timing of the onset of synthesis may probably be anticipated, but a distinct enhancement of the rate of synthesis might be expected. Experiments show that the rate and extent of synthesis is unchanged. If one wishes to avoid any specific mechanism to be involved, I think that this result can be explained only by assuming interference with energy supply and utilization of reserve materials, as discussed above.

## VII. Conclusion

### *1. Morphogenesis and phylogenesis*

As has been discussed very briefly in the present paper morphogenesis is the outcome of the interplay, active or passive, between a limited number of different cell types. The egg cell, as well as the early blastomeres, belong to one and the same class, cells of the other types derive from the original ones through transformation processes. Of these there seems to be only two, consequently there can be only four different basic cell classes. All other kinds of cells may be regarded as further differentiations of each of these four types. It was shown above that the polarities of the sea urchin embryo determine the cell type distribution, and the same seems to hold for the amphibian embryo (LØVTRUP 1966). The primary morphogenesis can be considered the resultant of the interaction of these four cell types, and a few extracellular structures (cf. LØVTRUP 1965 a-c).

There are a number of interesting conclusions to be derived from the views presented here. BALDWIN (1937) stated: "Biologists have from time to time been impressed by the fact that the members of the animal kingdom fall into a relatively small number of types, in spite of a considerable degree of variation within each type ..." (l.c. p. 104). If the basic morphogenetic events, which of course must influence the pattern of all subsequent development, are determined mainly by the activity of these four cell types, then it seems obvious that the possible number of variations must be quite low. On the other hand, variations within each group of animals presumably is a result of differential protein synthesis, and here the possibilities are almost unlimited.

Another consequence is that phylogenetic evolution must largely be a result of changes in the cell distribution pattern during early embryogenesis. The first animal cell must have been a solitary cell, an amoeba or an amoebocyte. This archaic cell is, to this very day, represented by each egg cell. The solitary amoeba represents, from an evolutionary point of view a blind alley; only when this cell had acquired the possibility to transform to other cell types were new roads open. The first new cell type which arose apparently was the epitheliocyte, probably in the flagellate form. The reversible transformation amoebocyte  $\rightleftharpoons$  flagellate can be observed in certain protozoa (cf.

WILLMER 1960). The flagellate is also a solitary cell, but the epitheliocyte exists in another form which is adhesive, often also ciliated or flagellated. With this cell type it is possible to build up multicellular structures, one of the simplest form being a spherical hollow body. If the cell transformation be reversible, cells may enter the cavity in the form of amoebocytes.

This form is also, unless new devices are invoked, a blind alley. This is maybe best illustrated by the stereoblastula, a structure which may arise if, in the sea urchin embryo, the formation of mechanocytes be suppressed by animalizing agents. Under these circumstances all amoebocytes may transform into ciliated epitheliocytes; no further development is consequently possible. It is not possible here to trace the various morphogenetic mechanisms which have allowed to avoid the formation of stereoblastulae in the various invertebrates, but we may dwell a moment at the sea urchin embryo. Here are two conspicuous traits of utmost morphogenetic importance. The first one is the hyaline membrane, a structure which is typical for amoebocytes (cf. WOHLFARTH-BOTTERMAN 1960); strong adhesion obtains between the cell surface and this extracellular membrane. It seems that this force may be overcome during cell division when the cells exceed a certain size, presumably spatial factors are mainly involved in this mechanism. The separation between the cells and this supracellular structure is of great morphogenetic significance, allowing the formation of a blastula, in its absence a number of solitary amoebocytes would arise from the subsequent cell divisions. The only possibility for formation of a multicellular structure under these conditions would be the transformation of the amoebocytes to epitheliocytes, but this would lead to a solid aggregate of cells, or at best to a stereoblastula.

The other trait is the apical formation of a new cell type, the mechanocytes, which through their pseudopodal activity can accomplish the invagination of the endoderm (GUSTAFSON 1961). At the time this happens most other cells seem to have become immobilized by the acquisition of epitheliocyte properties.

This kind of invagination is possible only in quite small eggs, for obvious spatial reasons. In larger eggs primary invagination results from the activity of mechanocytes, but the following event, the epibolic movements, results from the apposition of two layers of amoebocytes (cf. LØVTRUP 1965b). A prerequisite for this type of gastrulation is obviously that the transformation amoebocyte→epitheliocyte is delayed relative to the formation of mechanocytes. Another requirement is that the primary invagination, in contrast to that in the sea urchin egg, occurs outside the polar region, implying that it is bilaterally symmetrical. The factors determining the site of invagination in the amphibian embryo have been discussed in a recent paper (LØVTRUP 1965a).

The epibolic movements, involving intimate contact between cells of different types, since some of the invaginating cells are sf-cells, is the prerequisite for superficial cells transforming into the mechano-epitheliocyte

type (cf-cells), and the formation of this cell type in the embryonic surface is again a condition for the formation of a nervous system. The presence of mechanocytes able to produce elastic membranes is necessary for notochord formation, and thus for longitudinal stretching. The anchoring of the notochord to the neural plate by mechanocytes (the neural keel cells) allows for the enlargement of the brain, cf. *Amphioxus*, in which this attachment does not occur. I believe these few examples suffice to illustrate the kind of changes in cell transformation etc. which has made evolution possible (a more detailed discussion is to be found in LØVTRUP 1965 b).

## 2. *Biochemistry and phylogenesis*

It seems possible to distinguish two phases in the history of the young branch of science, chemical embryology, specially with respect to the question about energy supplying mechanisms. At a certain stage it was observed that the processes obtaining in eggs and early embryos in various ways differed from those found in adult tissues, thus the difficulties associated with the demonstration of phosphorylation in embryos at a certain time led to the belief that lack of phosphorylation was typical for embryonic metabolism (cf. NEEDHAM 1942).

Later observations showed this view to be erroneous, and subsequently the contention was spreading that no significant differences exist between embryonic and adult energy metabolism. The various attempts to demonstrate glycolysis and cytochrome *c* in the sea urchin egg must be regarded as expressions of this opinion (cf. ROTHSCHILD 1956).

I have tried to show in the present paper that this standpoint may be wrong. Our present scanty knowledge of comparative biochemistry shows that on the chemical level there is a recapitulation of phylogenesis during ontogenetic development, and if we can extrapolate this principle back to the egg cell then we must expect this to represent a very early stage in animal evolution, viz., a solitary amoeboid cell. The closest relatives to the egg cell must thus be found among present day protozoa.

It is therefore interesting that the counterpart to a number of peculiar biochemical traits in the sea urchin egg concerning carbohydrate, and possibly also nucleic acid metabolism, as well as the content of phosphagen and various enzymes, has been observed in protozoa.

As during the development the original amoebocytes are transformed into other cell types these primitive features disappear and are replaced by others known from the tissues of metazoa. Thus the primitive, rather inefficient mitochondria disintegrate, and the extramitochondrial cytochrome oxidase as well as the enzymes associated with the special oxidative glucose localization disappear. Instead new types of mitochondria are produced, together with enzymes specific for the new cell types arising. There is no question that this comparative biochemical study could be extended to other features than those discussed here, to mention only one case I would like to point to the

observations of BÄCKSTRÖM (1956, 1957) that ascorbic acid increases during development and that it is higher in vegetalized and normal embryos than in animalized ones. This observation may bear some relation to the observation that this compound is a specific growth requirement for Trypanosomas, since these as suggested above may be protozoa which possess the f-property, being cf-cells. Ascorbic acid may also promote the growth of other protozoa (amoebae and flagellates, etc.) but in this case it may be replaced by other reducing agents (LWOFF 1951). I shall not discuss the implications for biochemical evolution inherent in the metabolic peculiarities of the sea urchin egg except by mentioning that glycolysis in animal development apparently does not represent the most primitive metabolic pathway for glucose utilization.

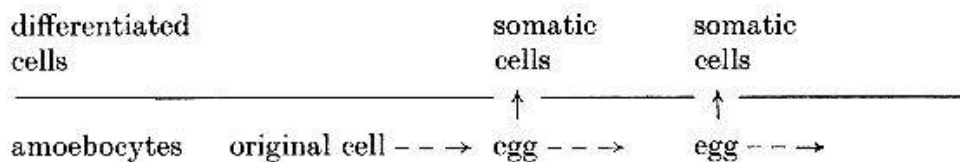
The thesis that an egg cell to all measures and extent is a very primitive cell may appear unlikely and incredible in view of the innumerable mutations which have occurred during animal evolution. Even if it can be stated with confidence that most of these mutations have been concerned with the synthetic capacities of differentiated cells at later stages of development, the possibility remains that certain mutations have been of direct influence upon the properties of the egg cell. About this there can be no doubt, the morphogenetic importance of such changes were discussed in the preceding chapter. Also on the chemical level changes might occur; I do not think it is entirely impossible to imagine that for instance cytochrome c might be present in other eggs than those of the sea urchin, claims to this have certainly been advanced (cf. ROTHSCHILD 1956).

However, there seems to be a limit to the extent of such changes, if this is transgressed ontogenetic development may no longer be possible. Thus with respect to the cell type it seems quite obvious that if the egg mutated into any of the other cell types, embryogenesis would be excluded, for no other cell type than the amoebocyte can form a blastula with further developmental possibilities.

Even concerning the chemical properties the range of permissible deviation may be narrow. If namely, as envisaged long ago by MONOD (1947) and SPIEGELMAN (1948), differentiation consists of the gradual establishment of unique enzyme patterns, resulting from interaction between nucleus and cytoplasm, then it seems to follow that a very specific sequence in the cytoplasmic changes be a prerequisite for normal chemical differentiation. Any mutation tending to interfere with this particular sequence would automatically lead to developmental arrest.

All these considerations suggest that there is terribly little margin for variations in the properties of the egg cell, and that all the changes in the genome which have been responsible for phylogenetic evolution have been expressed in cells at higher levels of differentiation.

The relation between the original mother cell of animal evolution, egg cells, and differentiated somatic cells may be illustrated as follows:



It follows that the egg cells in each generation must derive from the blastomeres before cell transformation or any other differentiation process has begun. Studies on the origin of germ cells support this contention (cf. BOUNOURE 1939; MINTZ 1961; BLACKER 1961). The present view on this question may probably best be summarized by quoting the last author: "the primordial germ-cells ..., or endodermal cells closely associated with them, are directly ancestral to the definitive gametes" (l.c. p. 28).

### *3. Biochemistry, preformism, and epigenesis*

In closing the present discussion I would like to deal briefly with a question recurrent in papers dealing with chemical differentiation. It is suggested (recently by WRIGHT 1964) that enzyme determinations be of limited value because enzyme activity may depend as much on removal of inhibitors as upon synthesis of new enzyme protein. This point is of course correct, but it must be stressed that disappearance of an inhibitor is just as much a sign of differentiation as would be synthesis of any specific enzyme. However, the danger of the argument is the hidden preformistic point of view, i.e., that all the enzymes are there awaiting only to be activated, for instance by inhibitor elimination.

Obviously nobody would carry this argument to that extreme today, but the possibility is still discussed now and then in the literature: "The well-known question of 'preformation' or 'epigenesis' arises in trying to solve the problem of the origin of the enzymes. It is still to be proved whether the egg contains all the necessary enzymes or whether some of them are formed only at subsequent stages. Our knowledge of enzymatic properties is not sufficient to provide a precise answer, and is complicated by the confusion between the enzymatic molecule as considered as a chemical entity and enzymatic activity as displayed by the molecule itself in vivo [and in vitro]. An enzyme, in fact, may be present, but inactive ... a satisfactory solution to the problem will only be made possible by an objective examination of the data available and further research" (URBANI 1962, p. 98-99).

Although inclined to accept the epigenetic view URBANI discusses the preformistic one and decides that future research must decide the question. I am afraid that if we lean on this approach it will be as with the question of Creation, for each step our understanding advances the scope for participation of God diminishes, but there will always be plenty of possibilities for interference beyond the limits of our knowledge.

If the introduction of more and more refined techniques still led to negative results, it would be possible to reduce the maximum limits for the amounts of enzyme present, but it would be impossible to exclude that one

or two molecules of any enzyme was present, and thus the question remained unsettled. There is, in my opinion, a shortcut to the solution of the problem, i.e., the logical approach. I presume that, in contrast to opinions held in earlier times, nobody maintains today that the embryo is preformed in the egg, ready to develop by what may be called a growth process. In other words, none of the morphological entities, liver, brain ... etc. are present, only the genetical information required for the establishment of these structures, if and when the developmental processes proceed according to a certain, causally determined spatio-temporal pattern. However, if the various organs are absent it would seem an obvious inference that no organ-specific proteins can occur, since the synthesis of these compounds must depend upon the activity of the respective differentiated cells.

A particularly complex situation arises if it is contended that organ-specific proteins are present and that they, in order to exert their (determinative?) function must become distributed in the embryo in accordance with the organ and tissue differentiation. This mechanism would seem to imply that the substances be distributed according to a very intricate pattern already in the unfertilized egg. It is very difficult to see how such a requirement be reconcilable with various results obtained in experimental embryology, e.g. by the rotation experiments of ANCEL and VINTEMBERGER (1948). Anyhow, enzyme molecules are not self-reproducing units; what is required for synthesis is not an enzyme prototype, but the code which is present in the nucleus of any cell.

The question of epigenesis-preformism may also be approached from the phylogenetic point of view. According to this way of thinking the unfertilized egg, in spite of its highly complex organization in certain respects, must represent the archaic cell type, on the basis of whose properties all later development rests. It follows that this cell can contain only such substances as are typical for this stage of development, any enzyme or other protein which is characteristic for cell types derived from the original one by differentiation cannot be present, even though, of course, the template for their formation is present in the genome.

*Acknowledgement.*—The appearance of the present work owes much to Dr. TRYGGVE GUSTAFSON, my friend. Not only has his own contributions to many different aspects of the research on sea urchin embryology captured my fascination many years ago, but it has been strengthened during many stimulating and instructive discussions.

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### *Rectification*

concernant l'article U. ROSA et al., *Bull. Acad. suisse sci. méd.* 21, No. 3/4, p. 185-196 (1965): Les expériences qui ont fait l'objet de l'article ont été développées en partie sous le contrat EURATOM 053-63-10 RISI.