I. STUDIES ON ALKALINE PHOSPHATASE ACTIVITY IN DEVELOPING SEA URCHIN EMBRYOS II. STUDIES ON THE ACTIVATION OF PROTEIN BIOSYNTHESIS IN SEA URCHIN EGGS AT FERTILIZATION

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ABSTRACT

I. Alkaline phosphatase activity in the developing sea urchin Lytechinus pictus has been investigated with respect to intensity at various stages, ionic requirements and intracellular localization. The activity per embryo remains the same in the unfertilized egg, fertilized egg and cleavage stages. At a time just prior to gastrulation (about 10 hours after fertilization) the activity per embryo begins to rise and increases 300 times over the activity in the cleavage stages during the next 60 hours.

The optimum ionic strength for enzymatic activity shows a wide peak at 0.6 to 1.0. Calcium and magnesium show an additional optimum at a concentration in the range of 0.02 to 0.07 molar. EDTA at concentrations of 0.0001 molar and higher shows a definite inhibition of activity.

The intracellular localization of alkaline phosphatase in homogenates of 72-hour embryos has been studied employing the differential centrifugation method. The major portion of the total activity in these homogenates was found in mitochondrial and microsomal fractions with less than 5% in the nuclear fraction and less than 2% in the final supernatant. The activity could be released from all fractions by treatment with sodium deoxycholate.

II. The activation of protein biosynthesis at fertilization in eggs of the sea urchins Lytechinus pictus and Strongylocentrotus purpuratus has been studied in both intact eggs and cell-free homogenates. It is shown that homogenates from both unfertilized and fertilized eggs are dependent on potassium and magnesium ions for

optimum amino acid incorporation activity and in the case of the latter the concentration range is quite narrow. Though the optimum magnesium concentrations appear to differ slightly in homogenates of unfertilized and fertilized eggs, in no case was it observed that unfertilized egg homogenates were stimulated to incorporate at a level comparable to that of the fertilized eggs.

An activation of amino acid incorporation into protein has also been shown to occur in parthenogenetically activated non-nucleate sea urchin egg fragments or homogenates thereof. This activation resembles that in the fertilized whole egg or fragment both in amount and pattern of activation. Furthermore, it is shown that polyribosomes form in these non-nucleate fragments upon artificial activation. These findings are discussed along with possible mechanisms for activation of the system at fertilization.

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I. STUDIES ON ALKALINE PHOSPHATASE ACTIVITY IN DEVELOPING SEA URCHIN EMBRYOS

INTRODUCTION

Changes in alkaline phosphatase activity per embryo during development have been reported for many types of animals (cf. 1 for review). From the studies of Zorzoli (2); Mazia, Blumenthal and Benson (3); Gustafson and Hasselberg (4); Mulnard (1); Løvtrop (5); Manganti and Mancusco-Palazzo (6) it appeared that in embryos of both vertebrates and invertebrates the alkaline phosphatase activity was low and remained the same throughout cleavage. Increases in activity per embryo then began at certain later embryonic stages. This increase was reported to begin at the onset of gastrulation in sea urchin embryos (3, 4) and eventually to reach a value approximately ten times that of the cleavage stages (see Appendix, p. 149). This, then, appeared to be a suitable system in which a closer specification of the amounts and times of activity changes could possibly serve as a basis for future examination of questions of how the enzyme(s) might be regulated during development and whether or not alkaline phosphatase might be involved in specific developmental processes.

Histochemical studies of alkaline phosphatase in developing sea urchin eggs indicated that the enzyme was distributed uniformly throughout the unfertilized and fertilized eggs, and cleavage stage embryos (7). An increase in activity was first noticed in the vegetal pole and migrating mesenchyme cells of the blastula stage, and as development progressed very strong reactions developed in both the gut and skeleton-forming areas (8). On the other hand, these reactions were greatly reduced in embryos cultured in the presence of beryllium (9). It was also noted that corresponding to

this lack of enzymatic activity there were gross abnormalities associated with the two enzyme rich areas, and it was suggested that at least in the case of skeletal development, the enzyme plays an important role in the differentiative processes (9). Since alkaline phosphatase activities from a wide variety of sources are known to commonly require ion cofactors for maximal activity (10, 11) it seemed that the variations in skeletal development observed in embryos cultured in calcium deficient or magnesium enriched sea water (12, 13) might be due to specific effects of these ions on the activity of the enzyme itself. In these studies the in vivo effects of the two ions were for the most part antagonistic, thus one might expect to see the in vitro activity of the enzyme also affected differently by changes in the concentrations of the two ions if indeed the primary effect was at the enzymatic level. It was with this in mind that the study of ion effects on sea urchin embryo alkaline phosphatase activity was begun.

It was shown by histochemical techniques that there was both nuclear and cytoplasmic alkaline phosphatase activity in cells of developing sea urchin embryos (7, 14). A similar study of the system by the differential centrifugation method (15) is reported herein. In these studies the above observations have been confirmed and extended, though not without considerable disagreement on the amounts of activity in the two fractions. This study was begun primarily for the purpose of locating the fraction with which the activity is associated, and secondarily it was hoped that this information might give insight into the function of the enzyme in development.

MATERIALS AND METHODS

Collection, fertilization and culturing of eggs

Eggs and sperm were collected from Lytechinus pictus adults either by injecting 0.55 molar KCl into the body cavity (16) or by cutting out the gonads and placing them in sea water to shed. The eggs were then washed with sea water until the wash fluid became clear. For fertilization the "dry" semen was diluted 1000-fold with 0.001 M EDTA (ethylenediaminetetraacetic acid) in sea water (17), and increasing amounts of this suspension were added to the eggs at 2 to 4 minute intervals until at least 95% fertilization was observed. In this way cultures containing large numbers of eggs in a small volume of sea water were obtained in which polyspermy was less than 1%.

The fertilized eggs were washed once to remove excess spermatozoa and cultured in filtered sea water at 21.5°C in shallow glass trays which were kept in a moist chamber. Development proceeded normally, and water loss due to evaporation was limited to less than 3% over a period of three days. For retarding bacterial growth penicillin G potassium (Squibb) was added to the cultures after fertilization to a final concentration of 100 units per ml and again 48 hours later to a concentration of 50 additional units per ml (cf. 18).

Where specified, the feeding-stage embryos were fed with cultures of Nitzchia closterium which are maintained at this laboratory. The amount supplied was adjusted not to be in excess of that eaten daily

by the embryos. No attempt was made to obtain a maximum growth rate, but care was exercised to insure that only cultures in which the embryos were active and otherwise normal appearing were used for assay.

Preparation and assay of fertilized eggs at different developmental stages

To insure uniform sampling, five 10 ml samples were taken with wide-mouth pipettes from each culture at various times after fertilization. The five samples from a single culture were combined. Each resulting 50 ml aliquot was then cooled in an ice bath to stop development and also to inactivate the swimming embryos. The embryos were washed once by settling in a graduated centrifuge tube and frozen (-15°C) after adjusting the final volume to one ml with sea water at one-half strength. They were stored frozen and used within four days after collection.

For counting the embryos, formaldehyde (to 4%) was added to separate 50 ml aliquots of the culture. These suspensions were later diluted to 500 ml, and counts were made on 8 to 10 one ml samples (wide-mouth pipette). Embryos with gross abnormalities were separately enumerated, and no culture containing more than 1% of these was used.

After thawing, the one ml samples received one ml of 2% sodium deoxycholate (DOC) at pH 10. They were homogenized by repeated passage (about 50 times) in and out of a glass syringe fitted with a 22 gauge needle and powered by a pipetting machine. Examination of the homogenates by light microscope showed complete cellular disruption. In addition to causing homogenization of the eggs or embryos, this treatment also resulted in a nearly complete release of enzymatic activity from particulate

fractions with very little change in activity (note Tables III and V). The activity was assayed either in the total homogenate or in the supernatant obtained after centrifugation at 12,000 times gravity (X g) for 20 minutes to remove large particulate matter. The temperature was kept at or below 4°C throughout the above procedure.

The change in optical density at 400 mμ due to hydrolysis of p-nitrophenyl phosphate[1] (19) was continuously measured at pH 10.15 and 30°C in 1 cm light path microcuvettes with a recording spectrophotometer (Cary Model 11MS). Thus, the rate of hydrolysis could be directly determined from the slope of the OD_{400} change with time. Since the reaction rates were no longer linear beyond the conversion of the first 10% of the substrate, comparison of the enzyme activities from different samples never involved the hydrolysis of more than this. Keeping this in mind it could then be shown that the activity is proportional to the amount of homogenate added over a wide range (Fig. 1). It was also shown that, by using an eight-fold difference in the amount of homogenate assayed while maintaining equal buffer and substrate concentrations as well as equivalent ionic strengths, the activities from a single culture could be maintained within the range previously shown to be representative of the amount of enzymatic activity present (Fig. 2). Either of two buffer systems, carbonate-veronal-HCl (20) or carbonate-bicarbonate (21), were found satisfactory for the incubation mixture which contained 0.1 molar buffer, 0.0002 molar $MgCl_2$ and 0.0002 molar substrate. The incubation mixture was adjusted in each case so that the concentrations of sea water salts were at one-sixth their

[1] Sigma Chemical Co.

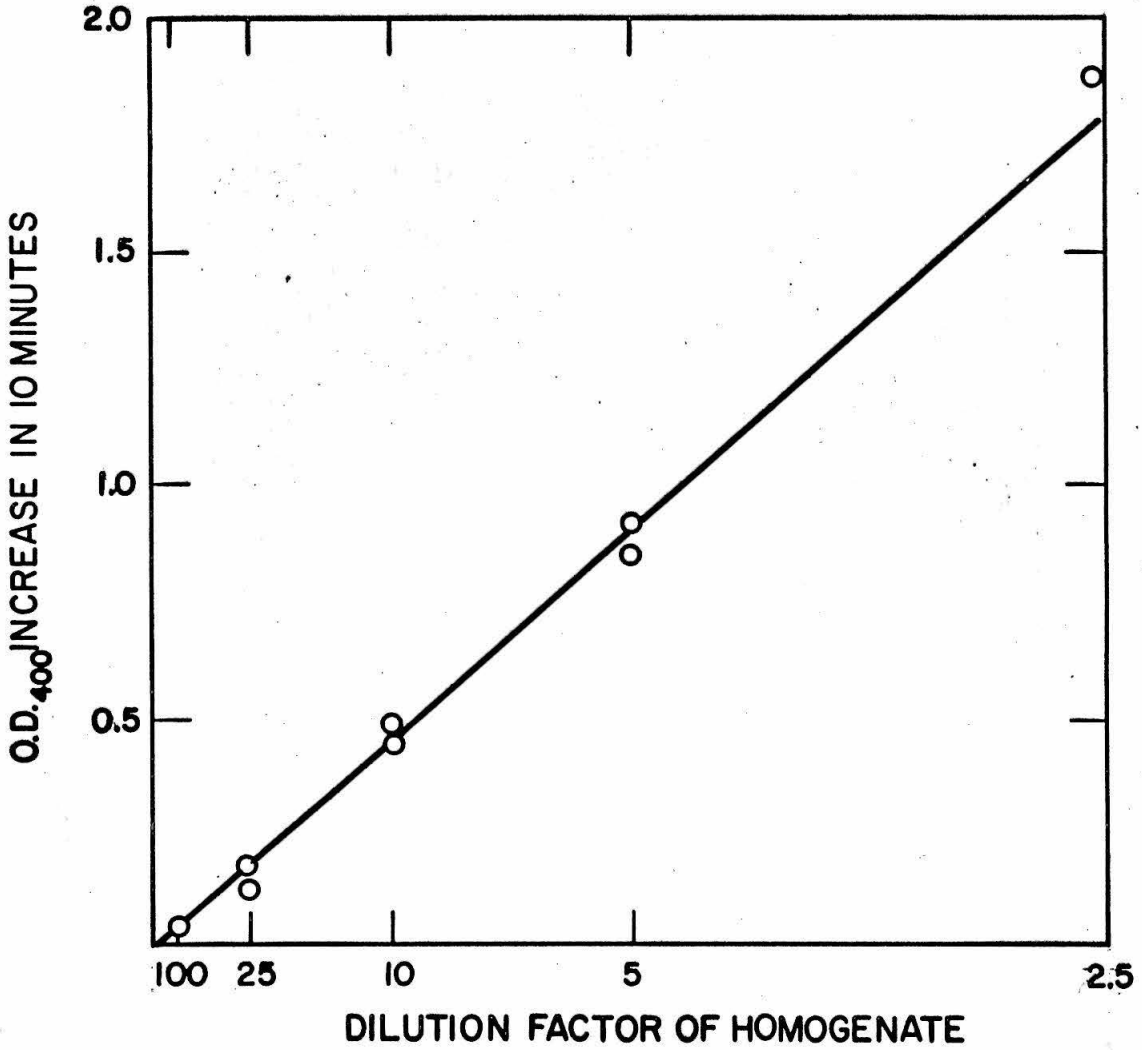

Fig. 1.-Spectrophotometric assay of alkaline phosphatase activity showing the linear relationship between the rate of product formation and the concentration of the homogenate which contains the enzyme activity.

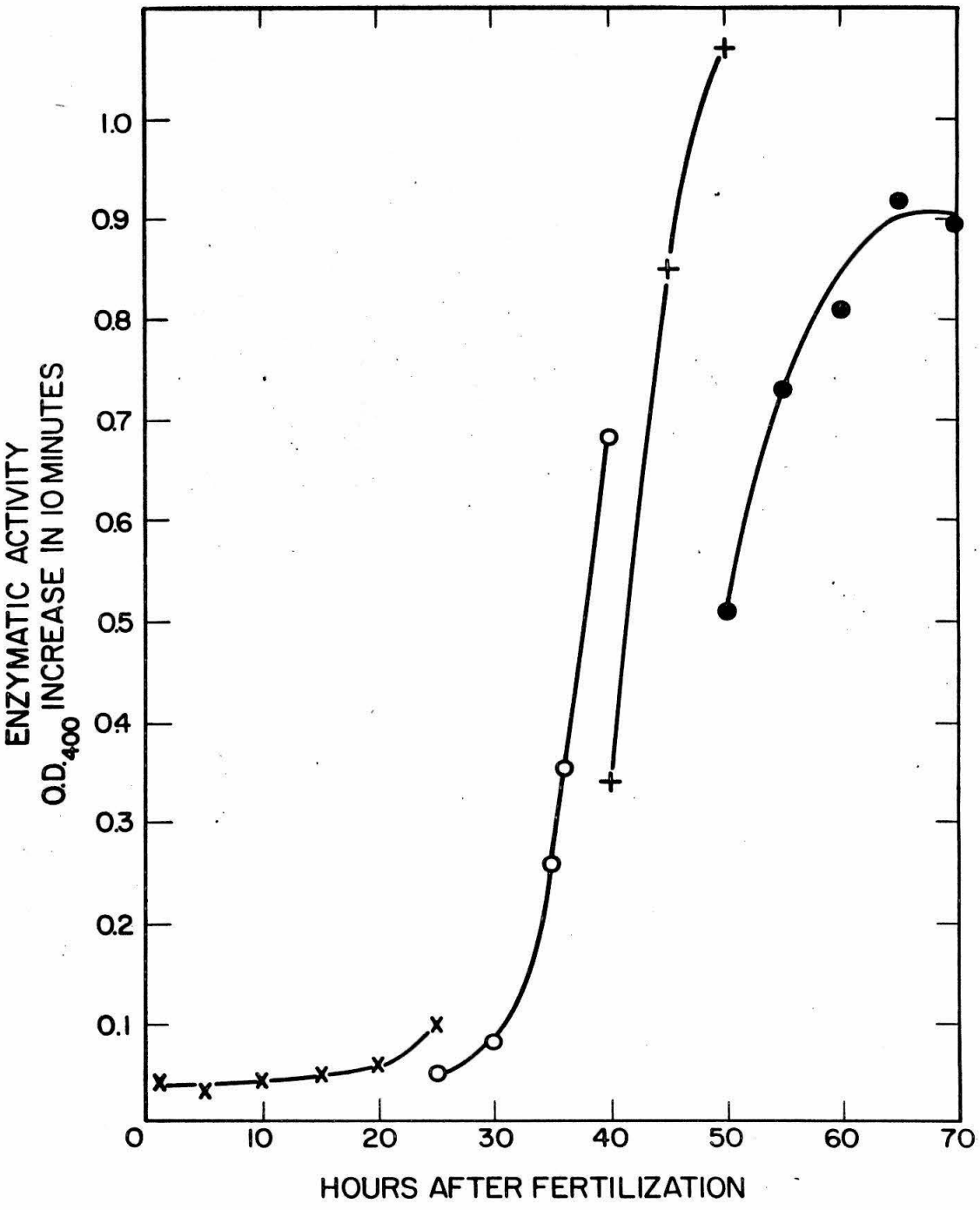

Fig. 2.-Dilutions needed in order to assay homogenates of eggs and embryos from single cultures within a safe range of activities. All samples contained equal buffer and substrate concentrations as well as equivalent ionic strengths. Symbols represent the amount in ml of homogenate in the 0.50 ml incubation mixture (crosses - 0.40; open circles - 0.20; pluses - 0.10; closed circles - 0.05).

normal value. Each sample tested had its own control which was placed in the reference beam of the spectrophotometer and contained the reaction mixture minus the substrate. Non-enzymatic hydrolysis of the substrate at this pH during the reaction time was found to be insignificant as was also the case for the activity in boiled homogenates of unfertilized eggs or larvae (Table I).

Preparation and assay for study of ion effects

Entire cultures of 72-hour plutei were used as a source of alkaline phosphatase activity. The embryos were collected by cooling the cultures with carbon dioxide gas falling from pieces of "dry" ice suspended in wire baskets above them. Under these conditions the embryos settled to the bottom of the culture vessel very quickly, allowing for rapid concentration and washing. The embryos were homogenized in a Potter-Elvehjem tissue grinder with Teflon pestle. The debris from a 15-minute settling period was discarded, and the remainder pelleted by a centrifugation at 105,000 X g for 120 minutes. This pellet was washed twice in distilled water or 0.55 molar KCl. Adjustment of the ionic strength to the test values was done in a 0.05 molar veronal-HCl buffer-substrate solution (pH 8.5) by addition of calculated amounts of chloride salts. The value of ionic strength as given in Figs. 5 and 6 refers to the added chloride salts and does not include the contribution of the buffer and substrate.

TABLE I.-The effect of 100°C treatment on alkaline phosphatase activity* in homogenates of unfertilized eggs and embryos of L. pictus.

Homogenate	Treatment	Activity
Unfertilized eggs	---	0.05
Unfertilized eggs	100°C for 10 minutes	0.00
Embryos	---	0.91
Embryos	100°C for 10 minutes	0.00

*Expressed as optical density change at 400 mμ during 10 minute incubation. Each sample of homogenate contained about 15,000 eggs or embryos.

Preparation and assay of homogenate fractions

For preparation of the homogenate fractions approximately 5×10^5 embryos were collected as above and washed twice by settling in a solution containing 0.55 molar KCl and 0.005 molar $MgCl_2$. They were finally suspended in a solution containing 0.24 molar KCl, 0.24 molar sucrose, 0.005 molar $MgCl_2$ and 0.05 molar veronal-HCl buffer at pH 8.5. The embryos were ground up in a Potter-Elvehjem homogenizer only until no intact cells were observed. The various fractions were separated in 0.328 x 1-15/16 inch centrifuge tubes using an International refrigerated centrifuge with high speed attachment and a Spinco Model L ultracentrifuge. The centrifugal forces listed were calculated with reference to r_{ave}. The various fractions were assayed at pH 10.15 in 0.1 molar carbonate-veronal-HCl buffer and 0.55 molar KCl.

RESULTS

Alkaline phosphatase activity during development

Results of a set of determinations of alkaline phosphatase activities in homogenates of L. pictus embryos at various stages of development are plotted in Fig. 3. The general pattern of the changes resembles that found by Mazia, Blumenthal and Benson (3) for A. punctulata and by Gustafson and Hasselberg (4) for P. miliaris.

The values of enzyme activity have been expressed in Fig. 3 as moles of p-nitrophenol formed per embryo per minute. The relationship of values expressed as activity per embryo to activity per mg of protein present has not been determined. There is only a relatively small decrease in total protein in sea urchin embryos up to the pluteus stage; namely about 16.5% decrease in S. purpuratus (22) and about 10% decrease in P. lividus (23). If it can be assumed that this is common to the development of sea urchins, one might expect that during development the changes in activity based on protein content will be similar to changes in activity per embryo. Furthermore, based upon approximate total cell numbers given by Mazia (24), the change in activity per cell would be one-third to one-half the magnitude of the change in activity per embryo during the period from 10 to 40 hours after fertilization (see Appendix, p. 149). However, since the enzyme appears to be localized in newly formed areas which represent only a fraction of the total number of cells, this type of comparison is not likely to be valid. Thus it remains a possibility that the activity per cell does not change in the gut and skeleton-forming cells and that the increase in enzyme activity is a result of the proliferation of these cells. Prior to

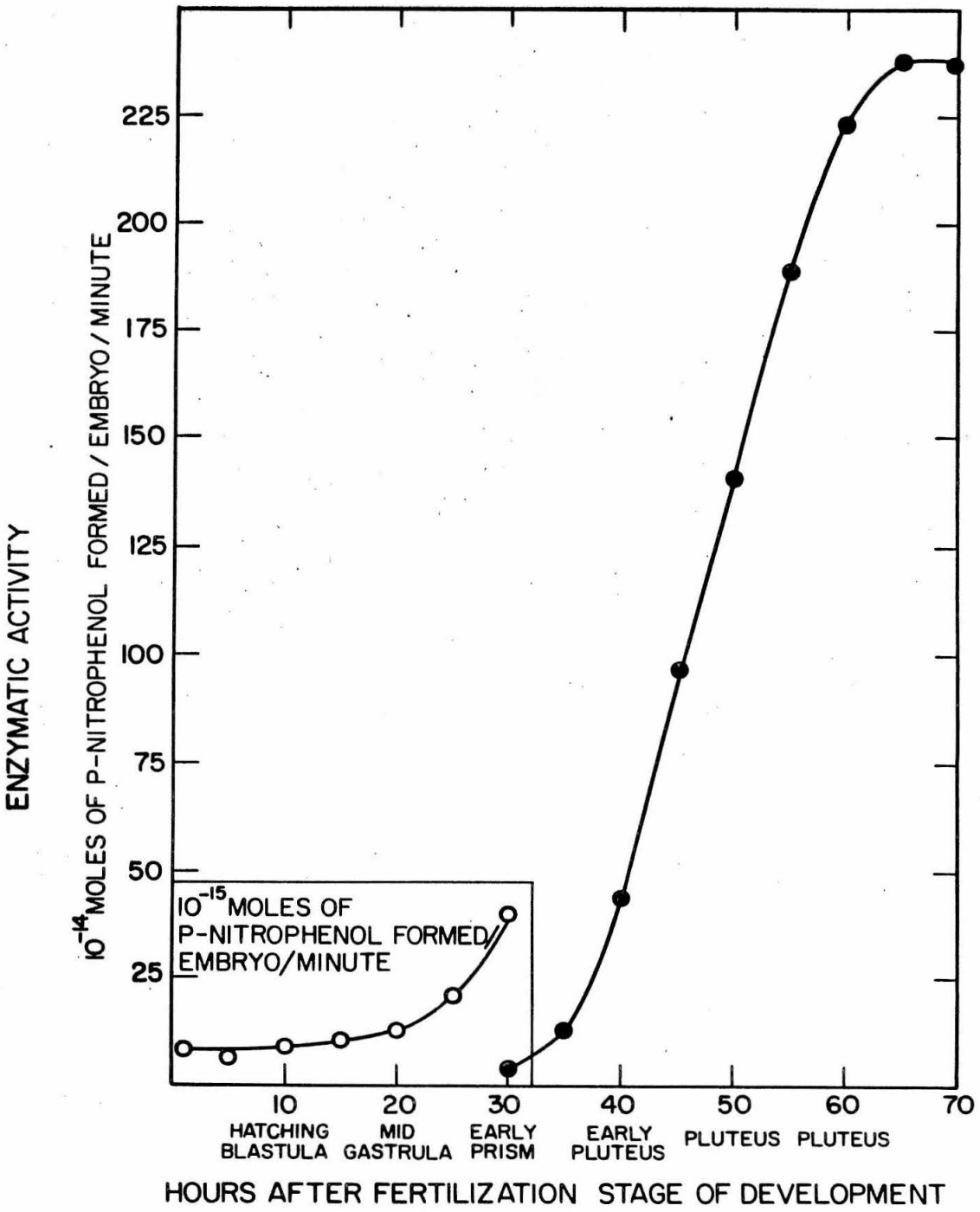

Fig. 3.-Alkaline phosphatase activity in homogenates of L. pictus eggs at different stages of development at 21.5°C. All points are from a single culture, and each point represents five separate samplings of the culture. After combining the five samplings, each resulting sample was assayed at 30°C in 0.1 molar carbonate - veronal - HCl buffer (pH 10.15), 0.002 molar $MgCl_2$ and 0.0002 molar p-nitrophenyl phosphate. In addition, sea water salts were present at one-sixth the concentration in regular sea water.

the increase in activity per embryo which begins at about 10 hours after fertilization, the activity per cell decreases approximately one thousand-fold, though the activity per unit volume does not change.

Samples containing equal aliquots of unfertilized, aged unfertilized, and newly fertilized eggs (Table II) indicate that alkaline phosphatase activity is not affected by fertilization. The unfertilized eggs were given the same prefertilization treatment as the other two classes but were put on ice at the time that fertilization took place. The aged unfertilized eggs received the same treatment as the fertilized eggs, and both were placed on ice after the fertilized eggs had developed for one and one-half hours. All sperm were removed from the fertilized eggs by washing several times. The slight difference in activity between unfertilized eggs and fertilized eggs cannot be attributed to fertilization since the aged unfertilized eggs also showed a slightly increased activity.

A detailed study showed that alkaline phosphatase activities remained constant during cleavage stages and began to increase shortly after hatching (Fig. 4). The exact time that this increase began after fertilization in the three cultures which were grown under reasonably identical conditions appeared to vary nearly four hours. The cause of this variation was not studied though the simplest explanation is that some difference in culture conditions was responsible. The unfertilized eggs did not show any increase in activity after as long as 18 hours under culture conditions.

Cultures of embryos which were fed could be maintained several days longer under crowded conditions than those which were not. The activities of homogenates from feeding larvae did not level off after 65 hours, as

TABLE II.-Alkaline phosphatase activity* in homogenates of equal aliquots of unfertilized, aged unfertilized and fertilized eggs of L. pictus

Sample number	Activity
Unfertilized eggs No. 1	0.04
" " No. 2	0.0375
" " No. 3	0.04
Aged unfertilized eggs	0.045
Fertilized eggs No. 1	0.0475
" " No. 2	0.045
" " No. 3	0.0475

*Expressed as optical density change during 10 minute incubation. Each sample contained about 15,000 eggs.

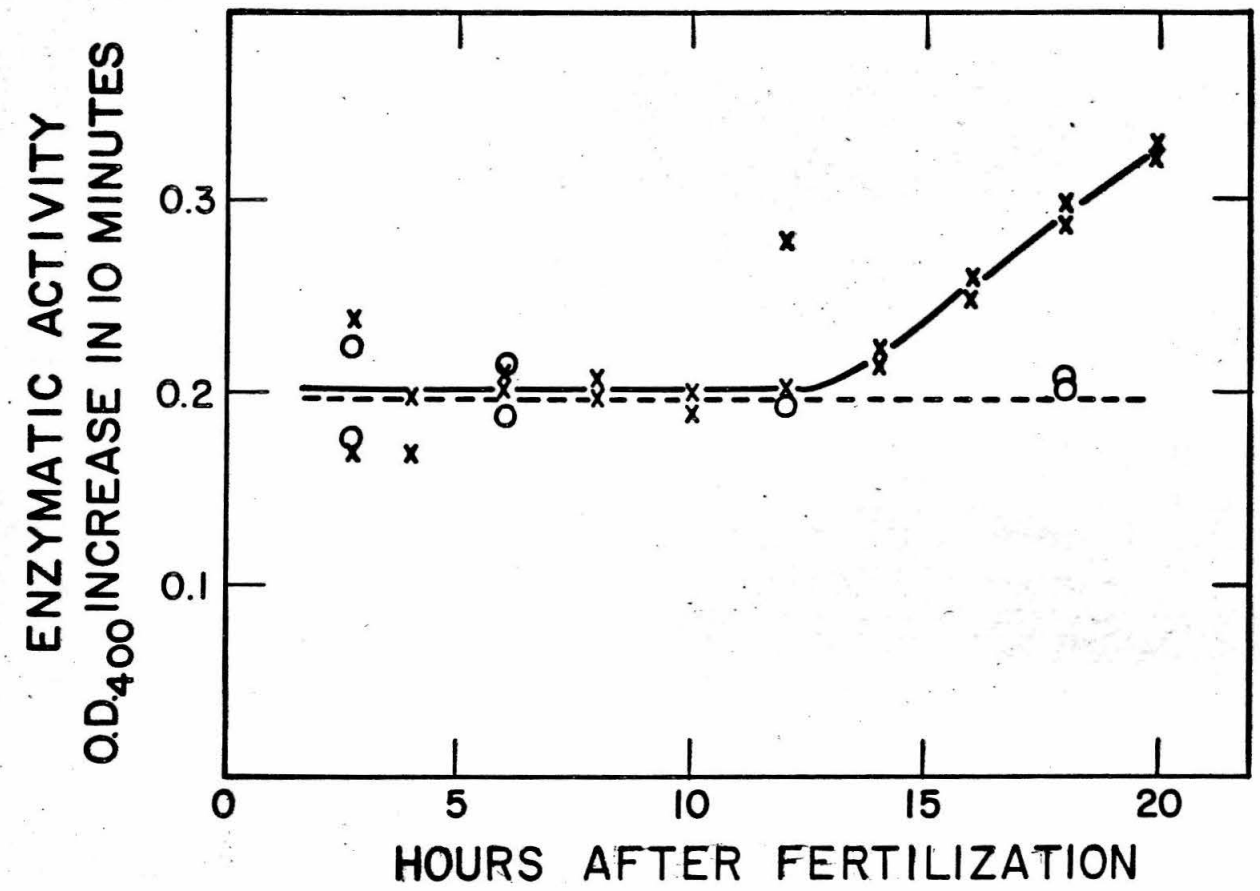

Fig. 4.-Alkaline phosphatase activity in homogenates of L. pictus eggs during early development at 21.5°C. All points are from a single culture, and each point represents five separate samplings of the culture. Each point was obtained from a sampling of about 65,000 eggs or embryos. **Symbols:** Circles - unfertilized eggs; crosses - fertilized eggs.

was the case with non-fed cultures, but continued to increase. The activity at the 80-hour stage was 50% higher than the activity in the 60-hour embryos.

Ionic requirements for optimum alkaline phosphatase activity

The ionic strength optimum for alkaline phosphatase activity was studied in solutions containing several developmentally significant cations. In order to view the relative importance of ionic strength and of specific ions, one set of tests was run by adding only the salts to the buffer-substrate solution. This set gave a range of total ionic strengths of approximately 0.05 to 1.25 (Fig. 5). In the other set the buffer-substrate contained 0.6 molar KCl in addition to the added salts and represented an approximate total ionic strength range of only 0.65 to 1.85 (Fig. 6). From these it can be seen that optimum activity is obtained at a total ionic strength in the range of 0.6 to 1.0 and that it is relatively insensitive to specific ion effects. On the other hand, the two divalent ions, magnesium and calcium, showed a more specific maximal activation in the region of 0.02 to 0.07 molar under both experimental conditions.

EDTA inhibits alkaline phosphatase activity at concentrations of 0.0001 molar and higher. For purposes of comparison with the effects of other salts at similar ionic strengths the EDTA was treated as a trivalent ion. This could be done because the pH of the buffer which was used fell midway between the pK_3 and pK_4 of EDTA at a point where a titration curve with KOH made a sharp inflection (25). Addition of

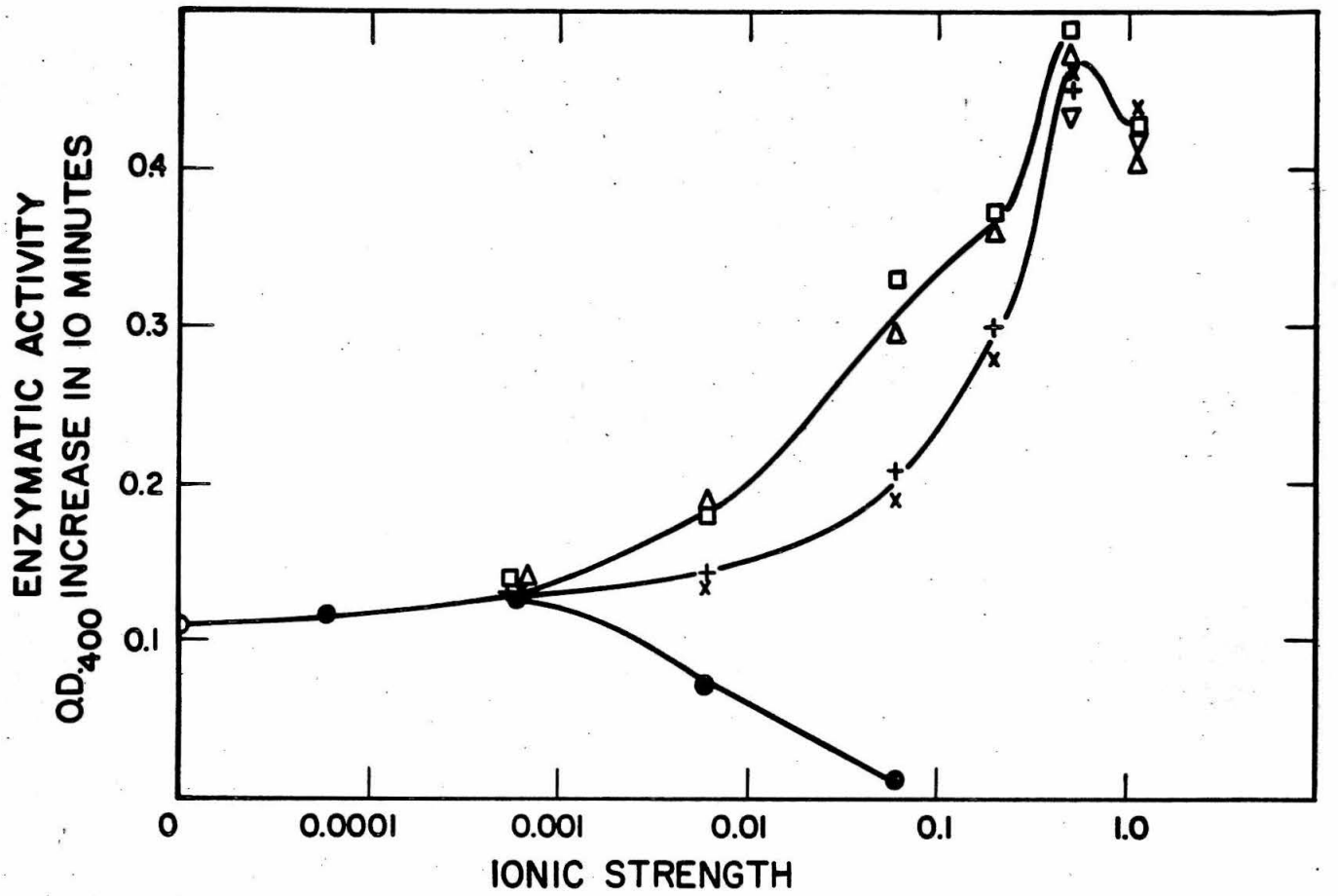

Fig. 5.- The effect of varying the concentration of different ions on the activity of alkaline phosphatase from embryos of L. pictus. All samples were assayed at 30°C in 0.05 molar veronal - HCl buffer (pH 8.5) at a substrate concentration of 0.0002 molar. The ionic strengths refer to the salts which were added and do not include the contributions by the buffer or substrate. Symbols: Squares - Mg; triangles - Ca; crosses - K; pluses - Li; inverted triangles - Na; closed circles - NaEDTA; open circles - buffer and substrate only.

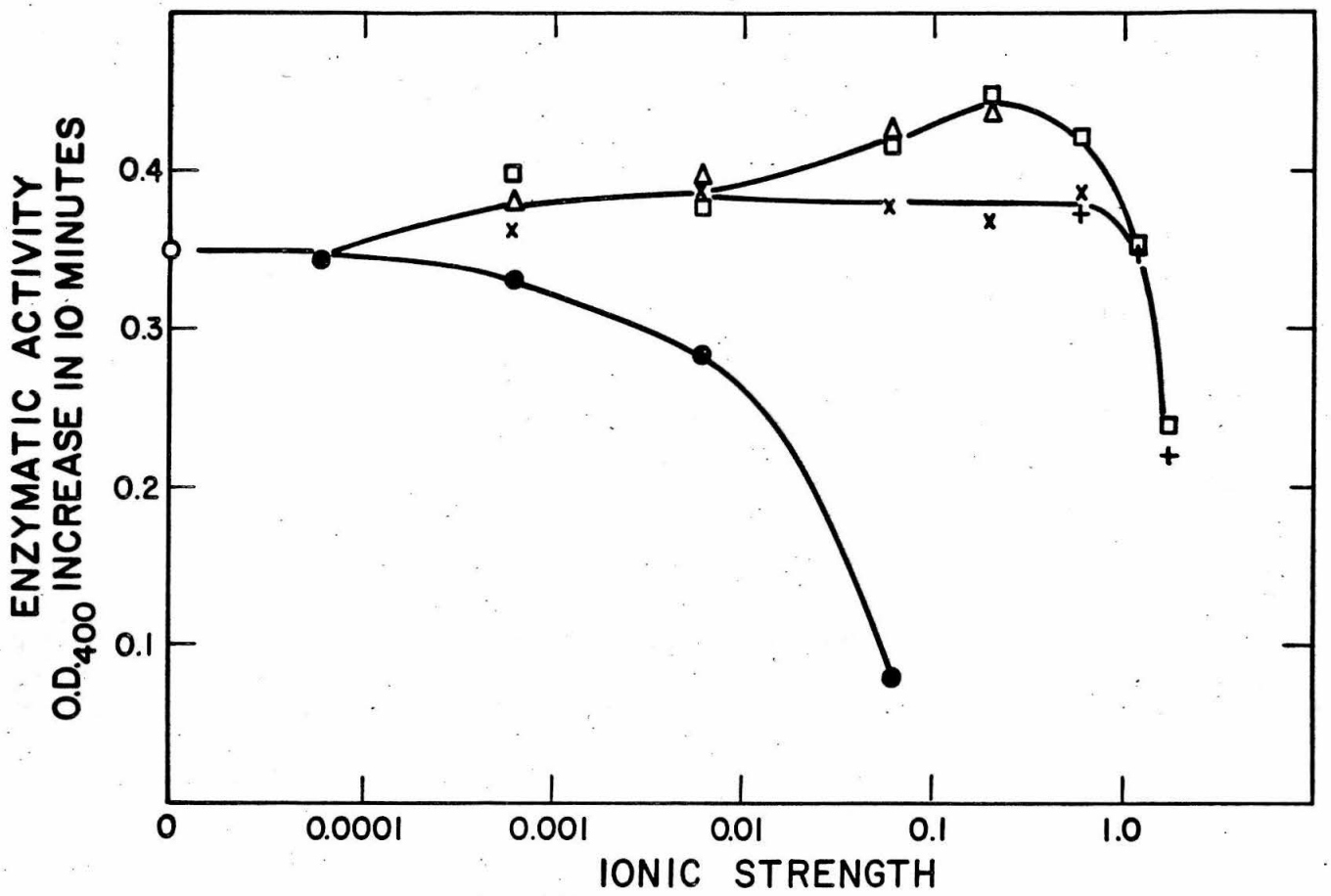

Fig. 6-The effect of varying the concentrations of different ions on the activity of alkaline phosphatase from embryos of L. pictus. All samples were assayed at 30°C in 0.05 molar veronal - HCl buffer (pH 8.5), 0.6 molar KCl and 0.0002 molar substrate. The ionic strengths refer to the salts which were added and do not include the contributions by the buffer, substrate or 0.6 molar KCl. Symbols: Squares - Mg; triangles - Ca; crosses - K; pluses - Li; closed circles - NaEDTA; open circles - buffer, substrate and 0.6 molar KCl.

EDTA depressed the pH of the reaction mixture through chelation of the divalent ions and corresponding liberation of protons. However, at the concentration where inhibition of activities was first noticed the pH depression was insignificant.

In vitro identification of alkaline phosphatase activity in cell fractions

Alkaline phosphatase activity of the 48-hour embryo was spread throughout most of the fractions when subcellular particles were isolated by differential centrifugation (Table III). Each fraction was washed twice with suspending medium and divided so that it could be assayed before and after DOC treatment. A third portion of the fraction was frozen at -60°C immediately after collection and used as an activity control for the other two samples which necessarily remained at 0°C for at least eight hours. The washings from each fraction were not combined with the supernatants but assayed separately. In this experiment, as in the one that follows, the activity of each fraction is expressed as a percentage of the total activity of the initial homogenate.

Light microscope examination showed that the first fraction contained mostly skeletal fragments. Fraction II (note Table III for sedimenting forces) contained nuclei and a few whole cells. The number of whole cells is probably indicated by the increase in activity in this fraction after DOC treatment. The third fraction contained a few nuclei and some smaller particles. Fractions IV and V fall into the range of centrifugal forces which have been used to sediment sea urchin mitochondria (26, 27).

TABLE III.-Activity* of fractions of L. pictus homogenate prepared by the differential centrifugation method

Fraction number	Centrifugal forces required to sediment pellet from supernatant of previous fraction (all except final fraction are pellets)	Activity of untreated fraction	Activity of DOC treated fraction	Activity of frozen fraction**	Activity of washings***
I	Allowed to settle 10 minutes	0.4	1.35		0.7
II	300 X g for 5 minutes	4.5	8.1	5.9	6.5
III	600 X g for 10 minutes	1.8	2.5	2.3	9.2
IV	3,500 X g for 15 minutes	10.7	9.9	8.4	8.0
V	12,000 X g for 10 minutes	6.5	6.0	6.1	10.6
VI	14,000 X g for 20 minutes	4.8	6.0	5.3	4.9
VII	105,000 X g for 120 minutes	15.3	14.5	14.9	0.5
VIII	105,000 X g for 120 minutes supernatant	1.6	2.3	1.8	
	Total recovery	86.0	91.1	85.1	

*Expressed as percentage activity of original homogenate. All samples were assayed at 30°C in 0.1 molar carbonate - veronal - HCl buffer (pH 10.15), 0.0002 molar substrate and 0.55 molar KCl.

**Frozen at -60°C immediately after collection and stored at -20°C until its assay.

***The washings from each fraction were assayed separately. The wash solution contained 0.24 molar KCl, 0.24 molar sucrose, 0.005 molar $MgCl_2$ and 0.05 molar veronal - HCl buffer (pH 8.5).

Fraction IV was quite large and may have been the major mitochondrial fraction. The main function of Fraction VI was to rid the supernatant of any remaining mitochondria-sized particles, and its actual composition is unknown. Fraction VII can be considered as the major microsomal pellet (27, 28), and Fraction VIII contains the supernatant materials. It is indicated from these data that the major portion of activity is concentrated in the mitochondrial and microsomal fractions with very little in the nuclear and cell debris fractions and virtually none in the supernatant fraction. Assay of the quickly frozen samples confirmed the two initial values for each fraction.

The above conclusions are also supported by the following experiment during which a different fractionation scheme was used (Table IV). A homogenate made from 72-hour plutei was freed of skeletal fragments by settling and centrifuged at 14,000 X g for 20 minutes to pellet the nuclei and mitochondria. This pellet was then resuspended and washed eight separate times with the suspending medium. The activity was checked in the initial supernatant, the washing fluids and the final pellet. The pellet was then fractionated in the following manner. The nuclear fraction prepared by centrifugation at 600 X g for 10 minutes was washed twice, and the washings combined with the 600 X g supernatant. This was then centrifuged at 12,000 X g for 10 minutes to bring down the mitochondria. This fraction was washed once, and the wash was combined with the 12,000 X g supernatant. Assay of these fractions after DOC treatment showed most of the activity to be associated with the mitochondria as opposed to the nuclei. Two separate experiments of this type were run, and each gave

TABLE IV.-Activity* of three major fractions of L. pictus homogenate prepared by the differential centrifugation method

Fractionation scheme			Activity
14,000 X g for 20 minutes	supernatant		19.5
"	"	1st wash	9.6
"	"	2nd "	7.3
"	"	3rd "	3.4
"	"	4th "	3.0
"	"	5th "	2.5
"	"	6th "	2.0
"	"	7th "	1.6
"	"	8th "	1.5
"	pellet		46
600 X g for 10 minutes	pellet		3.3
12,000 X g for 10 minutes	pellet		34.6
12,000 X g for 10 minutes	supernatant		8.2
Total recovery			96.5

*Expressed as percentage activity of original homogenate. All samples were assayed at 30°C in 0.1 molar carbonate - veronal - HCl buffer (pH 10.15), 0.0002 molar substrate and 0.55 molar KCl. The wash solution contained 0.24 molar KCl, 0.24 molar sucrose, 0.005 molar $MgCl_2$ and 0.05 molar veronal - HCl buffer (pH 8.5).

exactly the same pattern of activity distribution with very similar quantitative results.

Alkaline phosphatase activity was found in both the mitochondrial and microsomal fractions of two earlier stages in development, early blastula (6 hour) and gastrula (24 hour). However, in contrast to the later stages, almost all of the activity was in the microsomal fraction instead of being approximately distributed equally between the mitochondrial and microsomal fractions.

A nearly complete solubilization of microsomal alkaline phosphatase results from a 10-minute agitation of the fraction in a 1% DOC solution (Table V). On the other hand, the untreated sample retains most of the bound enzyme throughout the four washings. The activity associated with the mitochondria also responds to DOC treatment in the same manner.

TABLE V.-The effect of deoxycholate treatment on L. pictus microsomal fraction alkaline phosphatase

Fractionation scheme			Activity	*Activity after DOC treatment
105,000 X g for 60 minutes	supernatant		0.07	0.77
"	"	1st wash	0.02	0.15
"	"	2nd "	0.03	0.02
"	"	3rd "	0.01	0.02
"	"	4th "	0.01	0.01
"	pellet		0.82	0.02

*Expressed as optical density change during 10 minutes incubation. Wash solution and conditions of assay the same as that reported in Table IV.

DISCUSSION

The control of alkaline phosphatase activity during development

The observation that there is a change in alkaline phosphatase activity at fertilization (29) was not confirmed, and the present measurements show that it first begins to increase shortly after hatching of the blastula. Since the leveling of activities of the embryos at 65 hours after fertilization could be delayed by adding food to the cultures, this leveling is not likely to be an indication of the sort of functional maturation of an organ or tissue which has been suggested by Rogers (30,31) for alkaline phosphatase activity plateaus in developing avian nervous systems. In the cultures to which food had been added an activity plateau was reached as the embryos began to die off. These results also indicate that for feeding stages (after 40 hours) the data presented here may have very little value in reference to activities of embryos in natural environments except perhaps to suggest strongly that their activity continues to increase for some time beyond 80 hours after fertilization.

From experiments with echinoid hybrids in which paternal influence upon the activity during development was observed, Flickinger (32) concluded that there is evidence of a nuclear control of alkaline phosphatase activity. It is, of course, generally assumed that all cellular enzymes are direct or indirect products of nuclear activity, and there does not seem to be any reason to doubt this for the enzyme or enzymes concerned in the alkaline phosphatase activity. In the developing embryo, however,

the pertinent questions relate to the time and place at which specific genes become active. Conceivably the enzyme for alkaline phosphatase activity could be present in the unfertilized egg or synthesized soon after fertilization in masked form and become unmasked (rather than newly synthesized) during development. In addition, from the studies which will be presented later, it can be postulated that cytoplasmic elements instead of the nucleus might be directly responsible for the onset and continued synthesis of the enzyme.

The increase in alkaline phosphatase activity which is begun during the late blastula stage is the only point in the early development of the sea urchin embryo at which a change in activity so obviously implies that a controlling mechanism has been switched from off to on. Though the nature of this control mechanism has not yet been extensively studied, a considerable amount of evidence can be presented which suggests that the genetic material contained within the nucleus begins actively directing development at this time. Prior to this stage the phenomena which go to make up cleavage appear to originate at a cytoplasmic level (cf. p. 50). The rate of cleavage is always maternal even when non-nucleate fragments are fertilized with heterologous spermatozoa (33, 34, 35). On the other hand, development which follows gastrulation has been observed to show both maternal and paternal characteristics, including a compromise in the rate of development (34, 36). It is also notable that approximately 80% of the known lethal hybrid combinations of amphibians pass through the cleavage and blastula stages before arresting (cf. 37). Sea urchin embryos, reared in sea water containing 5-iododeoxyuridine and 5-bromodeoxyuridine, incorporated these agents into their DNA with no apparent effects upon development

through the blastula stage (38, 39). Further development was abnormal and death soon resulted for almost all of the embryos. The few survivors were in every case abnormal. In addition, as reviewed elsewhere, artificially activated non-nucleate fragments (p. 50) and actinomycin D treated embryos (p. 46) never develop beyond the blastula stage.

The above is not to imply that the nuclear genetic material is necessarily inactive during the cleavage stages. There is a high positive correlation which exists between respiratory rate and DNA content in fertilized sea urchin eggs (35, 40). Moreover, nuclear activity may be indicated by the RNA synthesis which is going on during the cleavage stages (p. 44 for review; p. 133 for discussion).

The view that the increase in alkaline phosphatase activity represents a turning-on of the appropriate genes at the gastrula stage is consistent with the customary findings that gastrulation represents the earliest stage at which nuclear influences on embryonic development are observed. Whether or not this increase represents synthesis of enzyme at that time or a genetically directed activation of a pre-existing inactive form is not known. Borrowing from other systems, at least three possibilities along this latter line can be put forth. The first suggests that if active sea urchin alkaline phosphatase is composed of dimers as is the case for E. coli alkaline phosphatase (41, 42, 43), the activity would ultimately depend upon the ability of the two monomers to combine. Thus they might be present in the egg and restricted from combining. A second possibility is that the active enzyme might require an activator molecule which is not supplied until the observed time of increase. The phenomenon, though not known to apply to alkaline phosphatase, has been shown to occur in numerous other

enzyme systems (44), and its importance in the maintenance of normal cell functions is not difficult to surmise. Finally, it has been shown that alkaline phosphatase activity increases two to three times in the 14-to 21-day mouse embryo duodenum as a result of the administration of puromycin and actinomycin D (45). These findings strongly suggest that the enzyme itself is already present in an inactive form; probably inhibited by a labile protein. These possibilities cannot, however, be seriously considered until the question of synthesis or activation at the time of increase in activity has been resolved.

The synthesis of specific proteins at the gastrula stage is strongly indicated by studies using the Ouchterlony method of immunological analysis (46, 47). In these studies the appearance of new antigens in normal embryos and hybrids was first detected at this stage. Furthermore, paternal antigens could not be detected until this time. Autoradiogram studies (48, 49, 50) also give support to the idea of a synthesis of enzyme which begins at the late blastula stage. Mesenchyme blastulae and other later stages which had been allowed to incorporate radioactive protein and RNA precursors showed that both protein and RNA synthesis were increasing rapidly in the regions where histochemical studies had indicated that the alkaline phosphatase activity was localized. Since, however, more recent studies were in direct contradiction to the above observation with respect to amino acid incorporation (51), final judgment on even this matter must await further developments.

The alkaline phosphatase of the developing chick embryo has been found to be "adaptive" in the sense that it responded to the addition of substrate by a two-to four-fold increase in activity (52). Though this is an attractive possibility, attempts to induce activity in cleavage stage sea urchin

embryos with exogenous substrates have met with no success (32).

Ionic effects on alkaline phosphatase activity

In these studies magnesium and calcium ions show negligible differences of effect on *in vitro* alkaline phosphatase activity, whereas a considerable difference was noted in their effects on morphological development in sea urchin embryos (12, 13). There remains, however, a serious limitation to this type of comparison; namely, that one does not know the effective concentrations of the responsible ions *in vivo*. In the absence of such information, it cannot be said that these two ions do not specifically affect the *in vivo* activity in bringing about the observed developmental effects. However, the similarity of the magnesium and calcium effects on the *in vitro* activity appears to favor this conclusion. Other studies indicate that exogenous ion changes can affect protein synthesis as well as development (53). This suggests the possibility that the synthesis of either alkaline phosphatase or the organic matrix of the skeleton might be inhibited under the conditions which alter morphological development in these later stages.

Several observations may be made concerning the response of alkaline phosphatase to the different ions and ionic strengths. The loss of alkaline phosphatase activity from sea urchin embryo preparations after the addition of EDTA indicates that a cation or cations chelated by this agent is required to activate the molecule. The identity of the ion(s) remains unknown since calcium and magnesium,

two prime suspects (11) have relatively little effect on the activity, especially at higher salt concentrations. The data presented here suggest two possibilities; either that there was not a significant amount of the cofactor washed from the enzyme by the methods used or that an ion different from these two was the activator. Perhaps a situation exists similar to that found for purified alkaline phosphatase prepared from swine kidney (54). In this case zinc appeared to be intimately associated with the enzyme molecule. In addition, a magnesium ion optimum existed although at a much higher concentration (5×10^{-3}M) than the estimated concentration of enzyme (10^{-8}M). This led to the proposition that the magnesium salt of the phosphate ester is the preferred substrate for the enzyme while zinc is the actual cofactor. Although the requirements shown here for sea urchin alkaline phosphatase could conform to the above activation pattern, there is no direct evidence in its support as yet.

There are a number of possible ways in which changes in ionic strength may affect alkaline phosphatase activity. As summarized by Webb (55) an increase in enzyme activity with an increase in ionic strength can, in general, be attributed (a) to an increased efficiency in the formation of the enzyme-substrate complex, (b) to an increased rate of release of the product by the enzyme, (c) to direct influences on the structure of the enzyme molecule, or (d) to an effect of ionic strength upon the substrate itself especially when the pH of the reaction system is close to a dissociation constant (acidic or basic) of the substrate. These experiments on sea urchin alkaline phosphatase provide another example of the susceptibility of an enzymatic activity to changes in ionic strength but do not contribute to the analysis of the phenomenon.

A final observation which concludes this part of the discussion is that chloride ions have been shown to activate some enzymes (56). Since no discrimination was made with regard to this possibility, it remains as a possible activator.

Alkaline phosphatase activity in different cell fractions

The two different procedures by which subcellular fractions were prepared accomplished different purposes. The first procedure, as outlined in Table III, minimized contamination of the later fractions by particles damaged during the washings and at the same time still clearly showed the lower limit of activity in all of the fractions. On the other hand, the second scheme (Table IV) gave a quantitative result while minimizing contamination of the faster sedimenting particles with the microsomal fraction. By these two methods one feels reasonably certain that both qualitative and quantitative results have been obtained.

The activity which could be attributed to nuclei was never over 5% of the total activity. It does not seem likely that this small amount would account for the observations that the histochemical alkaline phosphatase reactions always developed first in the nuclei of the larval cells and also that the increase in activities seen during the larval stages appeared to take place almost entirely in the nucleus (7, 14). The first question which might be asked is if this difference represents a leaching of the native enzyme from the nuclei during the isolation procedures. However, keeping in mind that there was almost no activity in the non-particulate fraction (Table III) this possibility seems very

unlikely. In view of this apparent inconsistency it can be suggested that the histochemical methods used for this type of localization were subject to diffusion artifacts (cf. 57). A clear example of this has recently been shown. Whole-tissue histochemical studies on the nervous system of the developing chick embryo have consistently indicated that most of the activity of the cells was associated with the nuclei (58, 59). However, fractionation of homogenates of these tissues by differential centrifugation has shown most of the activity to be located in the cytoplasmic fractions (60). Histochemical studies on these fractions have gone further to indicate that the amount of residual activity associated with these isolated nuclei was directly proportional to the amount of cytoplasm remaining with the nuclei. In agreement with this latter finding, it has been suggested by Duve (61) that especially when the enzyme is also localized in the microsomal fraction, one might expect to find small strands of endoplasmic reticulum still attached to the nucleus after homogenization. Another possibility is that the outer membrane of the nuclear envelope has enzymes associated with it. This association could be similar to that between enzymes and the endoplasmic reticulum since the latter appears to be structurally identical with the outer nuclear membrane (62-65). There is also a possibility that the activity shown here for the nuclear fraction results from mitochondrial contamination. However, this does not seem likely since the combined wash fluids from this fraction do not show a great deal more activity than the fraction itself (Table IV).

In these experiments the mitochondrial fractions contained approximately 45% of the total activity. Since the fractions which were isolated

correspond roughly to the mitochondrial and lysosomal fractions of rat liver homogenates (66) it suggests that perhaps sea urchin embryo alkaline phosphatase may itself be contained within these particles. This is, on the other hand, not likely in view of its lack of need for hypotonic or detergent activation which seems to be characteristic of lysosomal and some of the mitochondrial enzymes (67, 68). The evidence for the existence of special particles for each individual lysosomal enzyme was very inconclusive, though it was highly suggestive in the case of uricase (69, 70). Alkaline phosphatase may have a particle of its own as suggested above or it just as plausibly may be found on the outside of particles in these fractions as has been suggested for some other mitochondrial enzymes (71). The possibility also remains that the activity in these fractions comes from contamination by broken nuclei. This, however, does not appear to be a reasonable hypothesis since the nuclear fraction itself contains such a small amount of the activity.

Approximately 50% of the total activity of the homogenate was found in the microsomal fraction. Nearly all of the enzyme could be solubilized by use of a detergent with no apparent gain or loss of activity. The detergent used, sodium deoxycholate, is known to disrupt mitochondria (72) and to free ribosomes from the endoplasmic reticulum (73) presumably by dissolution of the lipid portions of the membranes. These observations suggest that sea urchin alkaline phosphatase is probably structurally associated with the lipid portions of membranes in both the mitochondrial and microsomal fractions.

The mechanism by which the enzyme becomes distributed in these locations remains an open question. The recent investigation into the

phenoloxidase system of Calliphora erythrocephalas (74) suggests a possible model. Inactive proenzyme circulated in the hemolymph and became activated only when brought in contact with a factor localized in the cuticle. If activated in the mitochondria-free supernatant of a cuticle preparation the active unit remained in the supernatant even after additional centrifugation. If, on the other hand, the activation took place in the total homogenate the active unit then became associated with the mitochondrial fraction. It was also found that the specificity of the enzyme varied depending upon whether or not it was particulately associated. These findings suggest that there can be specificity in the association between an enzyme and a subcellular fraction and that the particle containing the enzyme does not necessarily represent the site of synthesis of the enzyme. They suggest further that in a system where there is no non-particulately associated activity the enzyme might be transported from the site of synthesis to the particle in an inactive form and become active only on associating with the particle. Finally, the above findings showing that enzymes may become associated with particles in homogenates also signal that cell fractionation studies may possibly contain artifacts of preparation. Though this type of artifact cannot be ruled out, there is as yet no reason to suspect it in the data presented here.

As visualized by Moog (75) the location or change of location of an enzyme within a cell may be of ultimate importance as a factor governing the function and perhaps developmental role that it will have. The experiments reported here are inherently restricted in their functional application largely because entire embryos had to be used. However, it is interesting to view the possibilities that the alkaline phosphatase

activities which were found in the two intracellular fractions, i.e. mitochondrial and microsomal, were from different enzymes and that the alkaline phosphatase-containing particles in these two fractions came respectively from cells of either of the two alkaline phosphatase-rich organs. In this regard electrophoretic studies have demonstrated two separate bands containing alkaline phosphatase activity in extracts of later stage embryos (76). Furthermore, on the basis of differences in pH and temperature optima for reaction rates, it has been suggested that two different alkaline phosphatases exist separately in adult sea urchin organs (77) which correspond roughly to the alkaline phosphatase-rich embryonic organs.

The functions of alkaline phosphatase within the cell are most likely related to substrate hydrolysis rather than synthesis or transfer reactions (61). In this capacity, it has been proposed that alkaline phosphatase has a role of providing inorganic phosphate to the cell (78). Furthermore, in view of the ability of the enzyme to hydrolyze monophosphate esters of a wide variety of compounds, including primary and secondary aliphatic alcohols, sugar alcohols, cyclic alcohols, phenols and mononucleotides at the 2', 3' or 5' position (cf. 10), it may also be useful in degrading these compounds to where they can be more widely used in synthetic processes. The enzyme in sea urchins is associated with organs which commonly contain alkaline phosphatase in other invertebrates and in vertebrates (cf. 79). Judging from the organs in which it is found, the enzyme appears to function somehow in calcification of the skeleton and resorption of food from the intestine. Though very little is known about the actual role of the enzyme in these processes, it is

interesting to note that inhibition of alkaline phosphatase activity coincided with abnormal development of activity-rich organs or structures during embryogenesis (9, 80, 81).

SUMMARY AND CONCLUSIONS

Alkaline phosphatase activity in the sea urchin L. pictus remains constant during the cleavage stages and begins to increase at the beginning of gastrulation; eventually increasing at least 300 times. On the basis of several lines of evidence it seems likely that this increase is due to a synthesis of new enzyme.

The enzyme activity showed an ionic strength requirement which was optimum somewhere between 0.6 to 1.0. Calcium and magnesium showed a more specific additional optimum concentration at 0.02 to 0.07 molar. Since these two ions affected the in vitro activity in a similar manner it does not seem likely that defects in skeleton development caused by these two ions in abnormal amounts in sea water were due to ion effects at the enzyme level.

Finally, even though activity was distributed throughout all of the homogenate fractions which were collected it seems very unlikely on the basis of the small amount of activity consistently found in the nuclear fraction, that the enzyme becomes a nuclear enzyme in later development as was reported in histochemical studies.

II. STUDIES ON THE ACTIVATION OF PROTEIN BIOSYNTHESIS IN SEA URCHIN EGGS AT FERTILIZATION

INTRODUCTION

Protein synthesis in sea urchin eggs

It appears that in sea urchin eggs (as in many others) there is no increase in total protein (22, 23) or total nitrogen per embryo (82) during development. Nevertheless it has been shown that numerous enzymes increase in activity during development (83) and also that new proteins appear (46, 47). This implies that protein synthesis is taking place in these eggs and that it does so at the expense of materials already present within them.

It is well known that many biochemical activities alter upon fertilization; generally in the direction of increased activities (83). The penetration of labelled protein precursors into the intact sea urchin egg was first noted by Hultin (84) to increase at this time. Paralleling this increased uptake of N^{15}-NH_4Cl was also an increased labelling of the proteins of the egg. The interpretation of these experiments was very difficult because the change in amount of incorporation into proteins upon fertilization correlated very closely with the observed change in permeability. In similar experiments with N^{15} labelled glycine and DL-alanine the phenomenon of increased incorporation into the protein fraction after fertilization was confirmed (85) though the possibility of a permeability change was not investigated.

A striking increase in the labelling of proteins coincident to the formation of the blastula was found in embryos reared in heavy water (86). Based upon several lines of reasoning which are now immaterial, the authors interpreted this labelling of the proteins to represent increased amino

acid incorporation. Since, however, there was very little difference in the amounts of deuterium incorporated into protein by the unfertilized and fertilized eggs after as long as three and one-half hours of development, nothing could be concluded with respect to the immediate effect of fertilization. Even if there had been some indication of an increased activity the interpretation would have had to remain ambiguous largely due to reports that the sea urchin egg became more permeable to water (and presumably D_2O) as well as other substances immediately after fertilization (87-91).

An increase in incorporation of labelled amino acid into protein was unequivocally shown to occur upon fertilization of sea urchin eggs (27) only after a method was developed for preloading the ovarian eggs in vivo so that effects caused by permeability changes in the egg would be eliminated (89). This method consisted mainly of injecting the labelled precursor, S^{35}-methionine, into the body cavity of the adult female sea urchin and allowing approximately four hours for its penetration into the eggs. Eggs treated in this manner contained the label primarily in the acid soluble fraction. Fertilization or artificial activation by butyric acid treatment resulted in an increase in the labelling of the acid insoluble fraction (93). Fractionation by centrifugation of homogenates from eggs at different times after fertilization showed that the label was incorporated into the microsomal and supernatant fractions almost exclusively for the first four or five hours (26, 94). Thereafter a considerable amount of the label became associated with the mitochondria.

Experimental investigations of protein synthesis in sea urchin eggs

Evidence that the ribosomal fraction is activated upon fertilization

The *in vitro* demonstration of the difference in incorporation rates of unfertilized and fertilized eggs (28, 95) opened new avenues of study to the now well-confirmed phenomenon. One of the first techniques to be employed with the cell-free system was the isolation by centrifugation of the microsomal fractions from unfertilized and fertilized egg homogenates. These fractions were then recombined with the supernatant fractions of either the unfertilized or fertilized egg homogenates and allowed to incorporate. Comparison of the activities of the various possible combinations indicated that either supernatant fraction could support active amino acid incorporation while only the combinations with fertilized egg microsomes showed the increased activity. These results immediately suggested that some change in the ribosomes was responsible for the increase in incorporation rate following fertilization. In this series of experiments an attempt to bring about the *in vitro* activation of the ribosomes was made by using several different agents with more or less specific chemical or biological activities. The agents which were tried at various concentrations were trypsin, ficin, ribonuclease, glutathione, spermadine, heparin, sialic acid, periodate, $CaCl_2$, EDTA, hypertonic KCl, butyric acid and potassium butyrate buffer at pH 5.2. Though no data were presented, it was stated that a small amount of activation was observed with several agents; namely, trypsin, ficin, EDTA, heparin, butyric acid and butyrate buffer. Of these, the two least specific agents, butyric acid and butyrate buffer, gave the highest activation though never exceeding 200 to 300%.

In view of the amount of activation which was observed upon fertilization (ca. 1400%), these amounts which were effected in vitro have not as yet had a great deal of importance attached to them.

Evidence that ribosomes are as competent before fertilization as after

The discovery that the synthetic polynucleotide polyuridylic acid (poly U) stimulated a bacterial cell-free amino acid incorporating system to incorporate phenylalanine exclusively into acid-precipitable material (96) suggested that it could act in the capacity of a messenger RNA (mRNA). The isolation of the product synthesized in this poly U stimulated system appeared to be identical with poly-L-phenylalanine and thus substantiated the role of poly U as a mRNA.

The effect of adding poly U to homogenates of unfertilized and fertilized eggs was soon investigated in three different laboratories (97-101). In all three cases the results were very clear that the synthetic mRNA could stimulate unfertilized egg homogenates to incorporate a much greater amount of phenylalanine into acid-precipitable material than the controls. In comparing the amounts of poly U stimulation in unfertilized and fertilized eggs it was first reported by Nemer (97) that there was more activity in unfertilized homogenates. Tyler (99, 100), on the other hand, reported that the activities of unfertilized and fertilized homogenates were very similar. Later Nemer and Bard (98) also reported this similarity. The results from these poly U stimulated homogenates gave a very strong case for equal numbers of available ribosomes in the unfertilized and fertilized eggs. They further suggested that mRNA is lacking in the unfertilized egg.

This led to the proposal that the observed increase in amino acid incorporation is due to a production of new mRNA at the time of fertilization (97, 98).

Evidence that messenger RNA synthesis is begun at fertilization

The synthesis of RNA during early development of the fertilized sea urchin egg had not been widely studied with radioactive tracers prior to the interest drawn to the system by the homogenate fractionation and poly U experiments mentioned above. The investigations previous to this time had shown that there was very little over all net change in total RNA content during development (102-106). This then meant that if there was synthesis of new RNA taking place in the egg it must have relied upon endogenous precursors ultimately supplied primarily from the breakdown of existing RNA.

The question of the presence or absence of a synthesis of new RNA in either the unfertilized or newly fertilized sea urchin egg is still a matter of differing opinions. In general, it can be said that the evidence of Brachet, Decroly, Ficq and Quertier (107), Ficq, Aiello and Scarano (108) and Wilt (109) indicated that there was no synthesis in the unfertilized egg while that of Markman (50), Nemer (97, 110) and Gross, Spindel and Cousineau (111) indicated that there was; though the latter authors wished to regard the incorporation which they had reported as marginal. The fertilized egg showed an increase in incorporation of precursors into RNA according to all of the above reports except that of Brachet et. al. (107) and Ficq et. al. (108) who found none.

The above evidence largely favored the hypothesis that the egg upon fertilization began to synthesize, at an increased rate, new RNA which could then be the messenger necessary for the activation of the protein synthesizing system. When eggs were allowed to incorporate radioactive precursor and phenol extracted, the labelled RNA which was analyzed by sucrose gradient centrifugation appeared to be quite heterogeneous (109, 110, 112). The label was spread throughout much of the gradient with the major peak of counts associated with the 4s fraction. Other peaks which were found could not be readily identified, indicating that the new synthesis was not likely to be ribosomal RNA. It does indeed seem likely that at least part of the new RNA was messenger. In keeping with this idea, similar experiments with actinomycin D treated fertilized eggs (112) showed the complete absence of the labelled heterogeneous non-4s fraction. In demonstrating the production of RNA soon after fertilization, these findings gave very strong support to the likelihood of the above mentioned proposal that the increased amino acid incorporation is due to the production of new mRNA.

Evidence that the increase in protein synthesis at fertilization does not require production of messenger RNA

The evidence in this thesis does not favor the hypothesis that mRNA production is responsible for the increase in protein synthesis and in fact rules it out in as much as it pertains to the production of new mRNA by the nucleus. This conclusion was given strong support by studies on the effects of actinomycin D on sea urchin eggs.

The antibiotic actinomycin D has become an important tool in the study of development primarily because of its ability to inhibit DNA-dependent RNA synthesis (113, 114). This inhibition has been graphically confirmed recently in microscopic and autoradiographic studies of its effects on in vitro uridine incorporation by the well known polytene chromosomes of insects (115, 116) and the lampbrush chromosomes of amphibian oocytes (117). It is interesting to note that this actinomycin D induced inhibition of uridine incorporation was also accompanied by structural changes in the chromosomes, namely loss of both the "puffs" and the loops.

In sea urchin eggs the effect of the antibiotic was to depress uridine incorporation by the fertilized egg without affecting the rate of amino acid incorporation into proteins (107, 118, 119). Furthermore, the eggs continued to cleave and incorporate thymidine into DNA though at a slower rate than the controls. These actinomycin D treated eggs did not develop beyond the blastula stage (120), and they did not show the increase in amino acid incorporation usually seen subsequent to this stage of development. The morphological development of these treated eggs very greatly resembled the development of parthenogenetic anucleate eggs and egg fragments (118). Perhaps more important from this work was the evidence that the increase in amino acid incorporation upon fertilization was not due to a synthesis of new mRNA.

From the initial results the above conclusion could not be completely substantiated because it was found that even after prolonged treatment the eggs always retained some ability to incorporate C^{14}-uridine into ribonuclease sensitive material. As this actinomycin D insensitive incorporation

was, in some cases, as high as 50% of the total it was thought to be labelling of soluble RNA. This was later found to be true when phenol extracted sea urchin egg RNA was examined after centrifugation in a sucrose gradient (112). No RNA other than the 4s fraction contained labelled uridine in the preparations from actinomycin D treated eggs. However, the gradients from untreated eggs did show labelling of larger species of RNA in addition to the 4s fraction.

Evidence for the formation of active protein synthesizing units at fertilization

The demonstration that active protein synthesis in the rabbit reticulocyte took place primarily on ribosomal aggregates (121) suggested that the ability of cells to synthesize proteins may depend largely upon whether or not they contain the aggregates. Indications that the aggregating factor was indeed mRNA were already in the literature (122, 123). This was soon confirmed with similar poly U experiments, and in addition the incorporation ability of isolated ribosomal aggregates (polysomes) placed in cell-free amino acid incorporating systems was also demonstrated (124, 125).

In view of the above mentioned experiments with homogenate fractions (28) and poly U in sea urchin eggs (97, 99, 101) it seemed likely that the increase in amino acid incorporation observed upon fertilization might be accompanied by a formation of polysomes. A search for their presence quickly showed that the above expectation was correct and that as development progressed there was a continued increase in their numbers (126, 127, 128). Though small amounts of RNase resistant material from unfertilized

eggs sedimented into the polysome region of the gradient, the results consistently indicated that the mRNA and ribosomes were restricted in their ability to get together in the unfertilized egg and that their union after fertilization was probably the final step in the activation of the amino acid incorporating system.

Anucleate cells as indicators of nucleus-independent cellular functions

Developmental capacities of anucleate cells

As mentioned earlier, there was a considerable amount of evidence which favored the hypothesis that the activation of amino acid incorporation into protein of sea urchin eggs at fertilization is brought about by a synthesis of mRNA. Since this synthesis of RNA would presumably occur in the nucleus (129-131), it seemed that the above hypothesis could be tested by comparing amino acid incorporation by parthenogenetically activated non-nucleate egg fragments with incorporation by fertilized eggs. This type of comparison was not unreasonable to make since several different types of cells, including the sea urchin egg, were known to be capable of mimicking normal development for considerable lengths of time in the absence of nuclei.

One of the first indications of development that was observed in anucleate sea urchin eggs that had been parthenogenetically activated was cleavage. This as well as aster formation was first reported by Wilson in 1901 (132) for magnesium chloride treated non-nucleate egg fragments prepared by vigorous shaking. These results could not be viewed as conclusive mainly because of the method of fragmentation.

Though it was widely used at that time, a careful study of the fate of nuclei passing through this treatment showed that often they were fragmented along with the egg and some apparently disintegrated (133). In both cases they were shown to have been dispersed within the egg fragments. Thus a certain amount of nuclear contamination within the non-nucleate fragments was assured, and the clarity of the results suffered accordingly. Wilson's results were for the most part confirmed later in experiments with parthenogenetically activated (butyric acid treated) anucleate eggs which had been enucleated by micro-dissection (134). However, only about 11% of these activated eggs were able to show signs of cleavage which could at best be described as incomplete or asymmetric.

In studies with enucleated amphibian eggs fertilized with x-ray treated or otherwise genetically incapacitated sperm it was often found that the resulting blastulae contained clones of cells which were free of nuclei and presumably of chromatin as well (135, 136). Examination showed that these clones were caused by unequal chromosome distributions during cleavage; eventually resulting in the complete loss of chromatin material from some cells with very little, if any, loss in their ability to continue to divide. These results clearly showed that cell division could take place in the absence of a nucleus, however, since parthenogenetically activated anucleate amphibian eggs did not show any inclination to cleave, these results were interpreted to indicate that nuclear material was needed in order to initiate cleavage (cf. 137).

The development of a simple, consistent centrifugation method for fragmenting sea urchin eggs (138) soon led to confirmation of the observations that parthenogenetic non-nucleate egg fragments raised fertilization and hyaline membranes as well as showed astral formation. Cleavage of these fragments was not observed in this series of experiments, and in keeping with the current thought, it was stated by Harvey (138) that one could not expect them to. It was later found that by the use of a different parthenogenetic agent, hypertonic sea water, the unexpected consistently occurred (139, 140). These induced cleavages were usually slower and more irregular than those of fertilized eggs, but the fragments continued to divide and often groups of more than a hundred cells were obtained having the overall general shape of blastulae though lacking blastocoels. It was observed that they hatched and may have had cilia; however, there was no suggestion of spindles, chromosomes or centrioles in any of these cells.

In addition to these observations on sea urchin egg fragments it has more recently been shown that anucleate amphibian eggs which had been stimulated to cleave by genetically inactivated sperm (toluidine blue treated) resulted in essentially anucleate blastulae complete with blastocoels (141, 142). The latter investigators were also able to obtain a few unequal, irregular cleavages in parthenogenetically activated anucleate eggs.

Blastomeres from fertilized sea urchin eggs which had been enucleated with a micropipette behaved essentially the same as their parthenogenetically activated counterparts (143). An interesting difference was that

in these cells astral formation and multiplication took place for several hours without an indication of cleavage; then all at once the cell divided into many smaller cells. The one thing that all of these anucleate cells or organisms that have been considered so far had in common was that they never showed any signs of gastrulation. This lack of ability to pass on into this stage of development which marks the onset to tissue and organogenesis is consistent with the idea that nuclear messages are actively directing development from this time on.

Another cell which did remarkably well in the anucleate condition was Acetabularia. This alga, because of its large size and richness of structure, could be very easily cut in two yielding a comparatively large nucleus-free segment of the cell (144). Contrary to what was found in the amphibians and echinoderms, with the possible exception of cilia formation, enucleated Acetabularia could differentiate structures, notably the "cap". This organelle showed a very distinct species specificity, leaving little doubt of its genetic origin. The length of survival of these anucleate cells was several months, suggesting that the cytoplasm has the ability to maintain itself independent of the nucleus.

The amoeba, which could be easily enucleated by merely pushing the nucleus from the cytoplasm with a probe soon recovered and showed periods of amoeboid movement interspersed with periods of quiescence (145-147). These cells rarely lived more than 20 days in the anucleate condition, much of the later part of it as non-moving spheroids covered with short,

blunt pseudopodia. This reaction appeared to be primarily due to their inability to capture food rather than the loss of the nucleus since normal amoebae deprived of food showed a very similar response. Some of the more notable differences in nucleate and anucleate amoebae was that the anucleate were no longer negatively phototactic and no longer possessed the ability to make the usual adjustments to changes in oxygen tension, temperature or cyanide treatment made by the normal cells.

Protein, RNA and DNA synthesis in cells without nuclei

As mentioned previously, cells lacking nuclei were in certain cases capable of limited development suggesting perhaps an inherent capacity for synthesis of cell constituents necessary for prolonged survival and synthesis of at least some of the components responsible for development. One might then expect to observe protein synthesis in anucleate cells which were capable of growth and morphogenesis. This has indeed been found to be true in Acetabularia, with net increases in total protein often reaching as high as two fold (144, 148-151). Though the increase in total protein paralleled that in the nucleate cells for only 2 to 3 weeks, amino acid incorporation took place in the anucleate cells for several months thereafter without a net increase or decrease in protein. Other studies showed that growth (increase in size and dry weight) and morphogenesis (cap formation) were always accompanied by protein synthesis in anucleate Acetabularia cells, while on the other hand, protein synthesis in the absence of development was quite common (152). Protein synthesis thus seems to be a basic feature of the ground cytoplasm whereas

morphogenesis, while being dependent upon the synthesis of protein, does not appear to be an inherent capability of these nucleus-free cells. Further studies indicated that morphogenesis appears to be dependent upon materials supplied by the nucleus. These so-called morphogenetic substances could be shown to be accumulated in the cytoplasm under certain conditions (144, 152).

Concerning the proteins that were being synthesized by the anucleate Acetabularia cells little is known except that they did continue making invertase (153) and if given H_2O_2 in the medium they reacted by showing increased amounts of catalase activity (154). The mechanism of this latter phenomenon is admittedly suggestive of an adaptive enzyme synthesis though it has not been shown as yet. In addition, net increases in activities of aldolase (151), fructosidase and phosphorylase (152) have been noted. On the other hand, the acid phosphatase which appeared to be associated with the microsomal fraction showed a decrease in total activity immediately after enucleation (153, 155). This suggested perhaps a nuclear role in the maintainance of acid phosphatase even though chloroplasts did have the ability to synthesize it in small quantities (156).

In addition to the well known case of mammalian reticulocytes (157), several other cell types; Tetrahymena, Paramecium, Acanthamoeba, Amoeba proteus and amphibian tissue culture cells (158-160) when enucleated continued to display a very active protein synthesis. Anucleate amoebae maintained proteinase and amylase activities which were comparable to the controls for up to two weeks, and curiously enough the latter enzyme

consistently showed activities which were twice as high as the controls on the second and third day after enucleation (161, 162). On the other hand, dipeptidase activity rapidly decreased after enucleation and finally came to lie at a level approximately one-half that of the nucleate controls. As was also the case in Acetabularia, acid phosphatase immediately decreased in amoebae after enucleation (163).

The RNA content of anucleate Acetabularia cells usually showed a slight decrease over a period of several weeks (164, 165) though it had been observed on occasion to show a small net increase initially (151). This net increase could apparently be brought about by allowing the cytoplasm to accumulate the "morphogenetic substances" in the dark prior to enucleation (166). In the absence of a net increase in RNA the anucleate cells showed a relatively constant rate of incorporation of adenine, orotic acid or CO_2 into RNA primarily of the chloroplast fraction (167, 168). The ratio observed when comparing incorporation by the nucleate and anucleate cells remained very similar for as long as two months. Though morphogenetic development of anucleate Acetabularia cells was relatively insensitive to solutions of actinomycin D and 5-fluorodeoxyuridine or 5-fluorouridine, the development of the nucleate cells was grossly inhibited (169, 170). Ribonuclease treatment inhibited cap formation and protein synthesis in both fragments; however only in the nucleate cells was it a reversible condition (171, 172).

Anucleate Acetabularia cells, in addition to being able to convert thymidine into uridine and cytosine which were ultimately incorporated into RNA, incorporated the nucleoside itself into the nucleic acid fraction

(156, 173). This material was RNase resistant, was removed by DNase and primarily localized in the chloroplast fraction. Use of a fluorometric method (174, 175) for determining the presence of DNA has given values significantly above the DNase treated controls to suggest that it was actually DNA that was present in very small quantities. Its role in the prolonged survival of these cells is not known, though it would appear to be minor since, as mentioned above, RNase treatment of these fragments was irreversible.

The RNA content of anucleate amoebae decreased rapidly while the nucleate cells maintained a constant amount (176, 177). The incorporation of labelled adenine into the RNA by anucleate amoeba cells has been reported by Plaut and Rustad (178), though it was not confirmed by Prescott (179) who found ample incorporation by both the whole cells and nucleate fragments. The question has not yet been resolved though a possible explanation is that ingested food particles, bacteria, etc. somehow contribute to this incorporation (159).

It has also been reported that the cytoplasm of amoebae contained particles which were the sites of tritiated thymidine incorporation into acid-insoluble material (180, 181). Both the label and the affinity for acridine orange were removed from the particles by DNase treatment; indicating that the material was DNA. Enucleation of amoebae enhanced the thymidine incorporation, and there was marked increase in the number of DNA containing particles. This strongly suggested that the cytoplasmic DNA was capable of replication and that the particles themselves might also be relatively autonomous.

It has been reported recently that parthenogenetically activated anucleate as well as normally developing amphibian eggs showed approximately a two-fold net increase in cytoplasmic DNA 30 hours after activation (182). However, before concluding that these eggs were capable of synthesizing DNA in the cytoplasm, it must be cautioned that in this case the use of the term DNA is not clear. Since the microbiological assays for thymine or thymidine and thymidylate were performed upon total homogenates of the eggs, it seems likely that the designation of the term must not only include polynucleotides but also smaller molecules such as di- and trinucleotides as well as other enzymatically degradable thymidine containing compounds such as those found in sea urchin eggs (183). There is other evidence that during oogenesis the amphibian oocyte does indeed incorporate thymidine into a class of small molecular weight compounds (184). Though there cannot yet be a direct connection between the two observations, it appears likely that this increase in cytoplasmic DNA only represented an accumulation of small molecular weight thymidine containing compounds.

The DNA content of sea urchin eggs

When considering the use of non-nucleate egg fragments as a test system for indicating nucleus-independent cellular functions, it is implied that cellular functions which are not directly controlled by the genetic material are being tested for at the same time. While this may be the case, it can be questioned on the basis of the evidence that cytoplasmic DNA exists in some anucleate cells and in chloroplasts

(185) and mitochondria (186, 187). It would seem that if all of the DNA present in sea urchin eggs could be accounted for as nuclear DNA there would be no reason to consider cytoplasmic DNA along with a study of protein synthesis by anucleate fragments. However, it would appear that there is more than the haploid amount of DNA in the unfertilized sea urchin egg and that this DNA is for the most part extra-nuclear.

The amount of DNA contained in the sea urchin egg has been a topic of exceptional concern over the years as judged by the number of reports in the literature. Values for the egg ranged from nearly 1000 times the haploid value to none. On the other hand, the DNA content of spermatozoa showed a surprising lack of variation: From six different species of sea urchins the amount in picograms (10^{-12} grams) per sperm varied from 0.67 to 1.0 (188-193). While the different methods used for the determinations on sperm all gave similar values they appeared to be largely responsible for the large variations in the amount of DNA found in the egg rather than gross variations among batches of eggs. Methods which relied upon the different labilities of the nucleic acids toward acid or alkali for the separation and direct assay of DNA from the bulk of the egg RNA and proteins consistently gave high values. The value of 1000 times the haploid amount was obtained by phosphorus assay (102) and a value of over 400 times by a direct spectrophotometric assay (194, 195). Even when these extracts were subjected to the Dische colorimetric method for DNA determination (196), values of over 200 picograms per egg are obtained (188). Microbiological assays suitable for thymine or its nucleoside and nucleotide gave values closer to 20 picograms (190, 191) and thus probably were

the most reliable estimate of the DNA content per egg. The claim that the sea urchin egg contained no DNA was made by Marshak and Marshak (192) and was not based upon their inability to find DNA by an isotope dilution method in extracts of eggs but rather upon their notion that the amount of contamination by polar nuclei and ovarian cells was sufficient to account for all of the DNA present. Their reason for assigning DNA to polar nuclei and somatic cells but not to egg nuclei was largely because they found the egg nucleus to be Feulgen negative while the other nuclei were Feulgen positive. While the lack of affinity of the egg nucleus for the Feulgen reagents has been confirmed (197), it has by no means been unanimous (198-200). More recently the result of the latter investigators was confirmed with nuclei isolated from unfertilized eggs (193). In addition to being Feulgen positive the nuclei were shown by both the Dische and the microbiological assay methods to contain the haploid amount of DNA (0.89 ± 0.25 picograms). Though little is known concerning the nature of the so-called cytoplasmic DNA this will be discussed later along with its possible role during cleavage.

Isotope experiments have clearly indicated that the synthesis of DNA for the first cleavage began soon after fertilization and lasted for only 10 to 20 minutes (38, 201, 202). This pattern of early interphase synthesis was repeated during subsequent divisions. The ease with which the DNA was labelled even in the very first cleavage indicated that the endogenous pool of precursors was probably quite small. However, the time that net synthesis of DNA began as judged by an increase in amount per embryo depended almost completely upon the amount of cytoplasmic DNA found by the method of detection being used.

Aims of the present study

It now appears that one of the most important problems to face those interested in fertilization and early development is that of working out the exact steps, beginning with sperm penetration, which lead to the activation of the protein synthesizing system of the egg. It was with this problem in mind that a study of factors influencing protein synthesis was begun. From investigations carried out by others it remained a possibility that certain changes occurring in the ionic constitution within the egg at fertilization might be directly responsible for the inherent difference in the incorporation rates of unfertilized and fertilized eggs. Some of these possibilities have been investigated by cell-free amino acid incorporation techniques. These studies are admittedly incomplete in the sense that very few of the possible influencing factors have been investigated. Of the known ionic changes which occur upon fertilization, the two most likely to affect protein synthesis are those involving magnesium and potassium since they are known to be directly involved with maintenance of an active amino acid incorporating system in other organisms (203-205). In view of the unconfirmed evidence that there was a loss of magnesium from the sea urchin egg upon fertilization (206) it can be proposed that a decrease in magnesium activates the amino acid incorporating system of the unfertilized egg.

Likewise, potassium within the egg became twenty times more exchangeable with the potassium of the sea water upon fertilization. This perhaps indicates a release or decompartmentalization of the ion (91, 207) possibly effecting an activation of the system since potassium

is also known to be important for protein synthesis in sea urchin eggs (28). These studies will be reported in following sections.

Another approach to the problems of activation of the protein synthesizing system which has been followed was the investigation of the response of non-nucleate sea urchin egg fragments to parthenogenetic activation. Non-nucleate sea urchin egg fragments have been known for some time to incorporate labelled glycine into protein at a rate comparable to nucleate fragments and unfertilized whole eggs (208). It has only recently been shown that non-nucleate fragments when parthenogenetically activated respond by an increase in protein synthesis which is comparable in rate and amount to both parthenogenetically activated or fertilized nucleate fragments or whole eggs (99, 100, 209-211). These results show that anucleate cells can maintain an active protein synthesis and in addition indicate that they have an extra-nuclear mechanism for stimulating or perhaps even activating cytoplasmically localized protein synthesizing units as evidenced by the formation of ribosomal aggregates. These findings will also be presented in detail in following sections.

MATERIALS AND METHODS

Preparation of homogenates

The homogenates used for preliminary cell-free amino acid incorporation experiments were prepared from eggs of the sea urchins Lytechinus pictus and Strongylocentrotus purpuratus by the method outlined by Hultin (28). However, as investigations into the optimum conditions for incorporation were begun soon thereafter the method was altered many times. In all cases where comparisons were made between unfertilized and fertilized or parthenogenetically activated eggs or fragments equal aliquots were used. Other than this, the first revision to be made in the procedure was the substitution of a KCl enriched calcium and magnesium-free artificial sea water for the wash solutions in order that the magnesium optimum could be determined. Later as a result of the studies of development in solutions of different ionic composition, 0.55 molar KCl was substituted as the wash solution. Thus the eggs could easily be washed free of essentially all other ions including those within the perivitellin space without any apparent harm to them. In this way all of the constituents of the homogenization fluid other than KCl could be added in accurate amounts without need for consideration of egg volumes, interstitial and perivitellin effects. Thus for all experiments except where variations have been tested and are noted in the results, the composition of the homogenization buffer added to the eggs suspended in 0.55 molar KCl was such as to bring the final concentration in the total homogenate of added magnesium acetate to 0.0033 molar, the tris (tris (hydroxymethyl) aminomethane)-HCl buffer pH 7.8 to 0.033 molar and sucrose

to 0.066 molar. The homogenization buffers were also designed so that the final homogenate volumes were always constant within a given experiment; usually three or four times the volume of the pelleted unfertilized eggs. Since equal aliquots of fertilized and unfertilized eggs were always compared there was a considerable difference in the volumes of the pelleted samples. The increased volume of the fertilized eggs was primarily due to the fertilization membranes which are known to be permeable to KCl (212). Thus, the volume difference between the unfertilized and fertilized egg samples, which had been washed in 0.55 molar KCl, could be assumed to be made up with the KCl contained in the perivitellin space and could be adjusted for by merely drawing off the KCl of the final wash down to the level of the pelleted fertilized eggs in both fertilized and unfertilized egg samples.

In experiments other than those where the amount has been varied the usual concentration of KCl was 0.18 molar. The concentrations given for KCl refer to the concentration of the salt in the added homogenization fluids only. Since several different methods for measuring potassium had indicated that the concentration of potassium within the egg was close to 0.20 molar (28, 91, 213, 214), the actual concentration of potassium in these homogenates was essentially 0.18 molar except in cases where the concentrations have been varied.

A typical procedure follows. Using stock solutions of 0.5 molar tris-HCl buffer pH 7.8, 1.26 molar sucrose and 0.25 molar Mg acetate; a homogenization buffer concentrate was made up to 125 ml with

deionized distilled water after 26.4 ml of the tris-buffer, 19.6 ml of the sucrose and 4.0 ml of the Mg acetate stock solutions had been added. Equal aliquots of unfertilized and fertilized eggs were given two 20-fold washes with ice cold 0.55 molar KCl and tightly pelleted in graduated centrifuge tubes. The KCl was drawn down to the level of the packed fertilized eggs in both cases. Judging from the volume of the pelleted unfertilized eggs, there was usually three-fourths of a volume of 0.55 molar KCl contained in the perivitellin spaces of the fertilized eggs. A volume of homogenization buffer concentrate equivalent to one and one-fourth volumes of the pelleted unfertilized eggs was added to each of these samples, and they were transferred to Potter-Elvehjem type homogenizers with Teflon pestles. Homogenization was considered complete when microscopic examination revealed no intact eggs or nuclei.

For washing ribosomal pellets and diluting homogenates another solution was developed which closely approximated the actual optimum concentrations of magnesium and potassium in the homogenates. Based upon an intracellular magnesium concentration of 0.011 molar as determined by Rothschild and Barnes (214) for eggs of Paracentrotus lividus and found by Timourian (215) to be approximately the same for eggs of S. purpuratus, the actual optimum magnesium concentration was calculated from the optimum added concentration (Fig. 15) to be 0.005 molar. Taking into consideration the optimum potassium concentration (Fig. 16) the solution was composed of the following: 0.03 molar tris-HCl buffer pH 7.8, 0.005 molar Mg acetate, 0.06 molar sucrose, and 0.18 molar KCl.

Amino acid incorporation

The incubation system which was used was very similar to the one reported by Hultin (28). Incubation mixtures contained 225 µl of homogenate and 25 µl of a reaction mixture containing per ml: 100 µmoles of 2-phosphoenolpyruvic acid, sodium salt (PEP)[1]; 10 µmoles of adenosine triphosphate, dipotassium salt (ATP)[2]; 0.2 mg of PEP kinase[3]; 2.5 microcuries of C^{14}-L-valine, uniformly labelled (4.8 curies per mole[4] or 200 curies per mole[5]); 0.5 µmoles of each of the other 19 amino acids (gly, ala, ser, asp, asn, glu, gln, ilu, cys, his, tyr, pro, thr, met, arg, lys, phe, leu). In some experiments the PEP kinase and the C^{12}-amino acids were not included with apparently no effect upon the incorporation rate. In other experiments C^{14}-L-phenylalanine, uniformly labelled (9.8 curies per mole[4] or 360 curies per mole[5]), was used instead of valine.

As soon as possible after preparation the homogenates were added to disposable plastic test tubes[6] already containing the reaction mixture in an ice bath. The samples were then incubated at 25°C for various lengths of time, usually one hour. The reaction was stopped by quenching with excess C^{12}-valine and by cooling in an ice bath. The time zero controls (t_o) were prepared the same way as the other samples, i.e. 225 µl homogenate and 25 µl reaction mixture, the only difference being that the excess C^{12}-amino acid was added

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[1]Calbiochem

[2]Sigma Chemical Co.

[3]Boehringer Chemical Co.

[4]Nuclear Chicago Corp.

[5]New England Nuclear Corp.

[6]17 x 100 mm (Falcon Plastics)

immediately. While the other samples were incubating, the t_o controls were kept in an ice bath. After the incubation had been stopped, the samples were ready to be prepared for counting.

Incubation of intact eggs or fragments in C^{14}-amino acid was usually done in 0.5 to 1.0 ml volumes of sea water in disposable plastic test tubes at 20°C. The amino acid added was usually in the range of 0.06 to 0.20 microcuries per ml and the egg concentrations near 10^4 per ml. The above ranges of values are given as general indications of the various experimental conditions and are not to be interpreted to indicate variation within an experiment or series of experiments. In time sequence or pulse experiments the eggs or fragments were cultured in larger vessels, and aliquots were removed at the proper times to the tubes for incubating. As was also the procedure for the homogenates, the incubation of intact eggs was stopped by adding a great excess of the C^{12}-amino acid and by placing the samples in an ice bath. The procedure for preparing the whole eggs or homogenates for radioactive assay will be described next.

Determinations of radioactivity

During the initial experiments protein was prepared for counting by a procedure similar to that of Siekevitz (216). The protein was precipitated at 0°C from individual one ml samples containing excess C^{12}-amino acid by adding 20% trichloroacetic acid (TCA) to a final concentration of 10% and was sedimented by centrifugation. The precipitate was dissolved in 1.0 normal NaOH to which two drops of

0.5 molar C^{12}-amino acid had been added and then reprecipitated with 20% TCA to a final concentration of 10%. The precipitate was heated at 85-90°C for 10 minutes before it was centrifuged, and the above procedure of dissolution, reprecipitation and heating was repeated once. The precipitate was given one final 10% TCA wash which also included a 10 minute heating. Next, lipid extraction was undertaken with two 1:1 100% ethanol-ether washes; two 1:1 100% ethanol-chloroform washes and two final 100% ethanol washes. The precipitate was then dissolved in a known volume of 1.0 normal NaOH from which an aliquot was taken for protein determinations. The remainder was pipetted onto 4 x 8 cm #1 Whatman filter paper strips (217) which were placed around the inside vertical walls of standard 20 ml low K^{40} scintillation vials and were dried under a current of air. Twenty ml of a scintillation fluid containing ethanol (218) was added, and the samples were counted on a Tri-Carb Spectrometer Model 314-EX (Packard Instrument Co.) at approximately 48% efficiency.

Later the method of Mans and Novelli (219) by which the native proteins were first dried and purified then counted on filter paper was adapted to use with the sea urchin material. The procedure for removing non-protein counts had to be altered slightly due to the weakening and subsequent tearing of the paper strips after prolonged hot TCA treatment. It was found that in place of the 30 minute hot TCA treatment used by Mans and Novelli, two 3 minute 90°C TCA washes were sufficient to remove all of the hot TCA soluble counts (Table VI) with very little damage to the papers. In the same experiments, it

TABLE VI.-Length of hot trichloroacetic acid treatment necessary to remove hot acid soluble counts*

	Minutes of treatment with 5% TCA at 90°C					
	0	3	6	9	12	15
Counts per minute of duplicate samples (average of five 10 minute counts)	418 421	357 357	324 331	327 331	326 327	329 332

*A fertilized egg homogenate was prepared and allowed to incorporate as described in the text. Aliquot samples were pipetted onto paper strips and thoroughly dried. All samples received one ice cold 10% TCA and one ice cold 5% TCA rinse prior to the hot treatment. Thereafter all of them received two 95% ethanol, three 100% ethanol and two ether rinses prior to counting. T_6 samples contained 41 and 43 counts per minute respectively.

was also found that there was a great deal of consistency among the identical samples once the hot acid soluble material had been removed. It was also found that identical samples purified on the paper gave more reproduceable results than aliquots purified in a similar manner in test tubes which were carefully centrifuged after each wash. The test tube samples also showed consistently lower values even though the t_o samples were very similar. C^{14} labelled amino acid mixed with different amounts of egg protein, dried on papers and counted at the optimum high voltage setting of 4.20 showed a negligible change in the counting efficiencies over the range of protein concentrations normally used (Fig. 7). In addition, it would also appear that the presence of proteins and other constituents of the unextracted egg had very little influence on the counting efficiency through filter paper (Table VII). The efficiency also remained constant over a great range of radioactivities (Fig. 8). For these and all subsequent experiments, a scintillation fluid was used which contained in grams per liter of reagent grade toluene, the following: 2.89 PPO (2,5-diphenyloxazole) and 0.34 dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene) (Packard Instrument Co.). With the change to this fluid it seemed likely that the papers, once counted, could be removed from the vials without having contaminated the scintillation fluid since it was highly non-polar and the solubilities of the products being counted were extremely low. This was found to be true, and the re-use of both vials and scintillation fluid was routinely practiced in this laboratory. After removal of the papers, the vials containing only scintillation fluid were counted, and any with

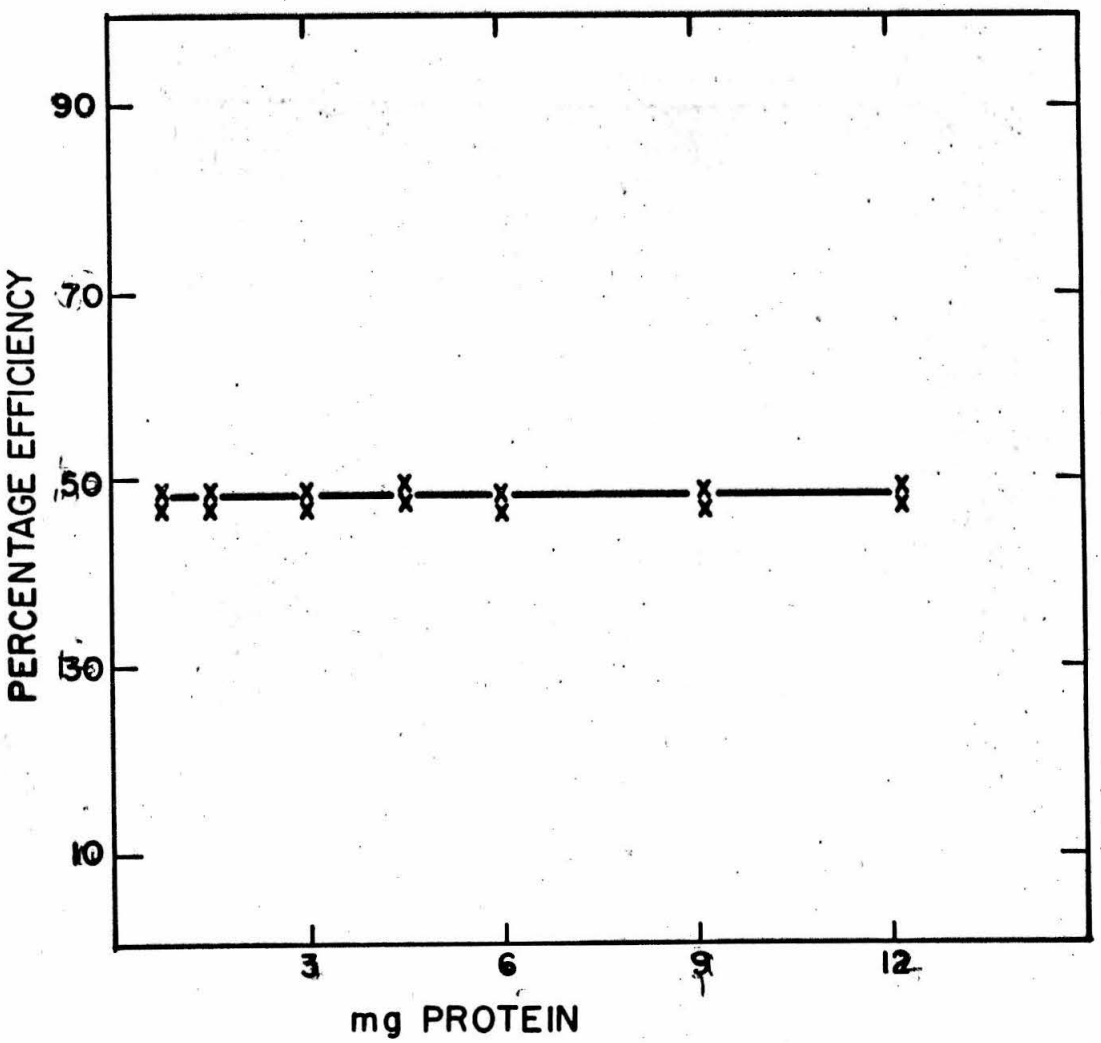

Fig. 7.-The effect of differing amounts of protein on the efficiency of scintillation counting of C^{14}-valine on filter paper strips. Samples containing the above amounts of protein were removed from a homogenate of unfertilized eggs and placed in individual test tubes. Equal aliquots of C^{14}-valine were added and mixed-in thoroughly. Each sample and two subsequent tube rinses were then pipetted onto 4x8 cm #1 Whatman filter paper strips and dried. The papers were counted at a high voltage setting of 4.20. Based upon the manufacturers specifications, the amount of C^{14}-valine added to each sample was calculated to contain 5.33×10^4 disintegrations per minute.

TABLE VII.-The effect of adding unextracted sea urchin eggs to paper strips containing C^{14}-uridine*

	Counts per minute
C^{14}-uridine alone	5188, 5327
C^{14}-uridine plus unextracted sea urchin eggs	5051, 5033

*Equal aliquots of C^{14}-uridine were pipetted onto the paper strips prior to the addition of the eggs to some of the papers. Approximately 30,000 eggs were contained in each sample, and all of the papers were thoroughly dried before counting.

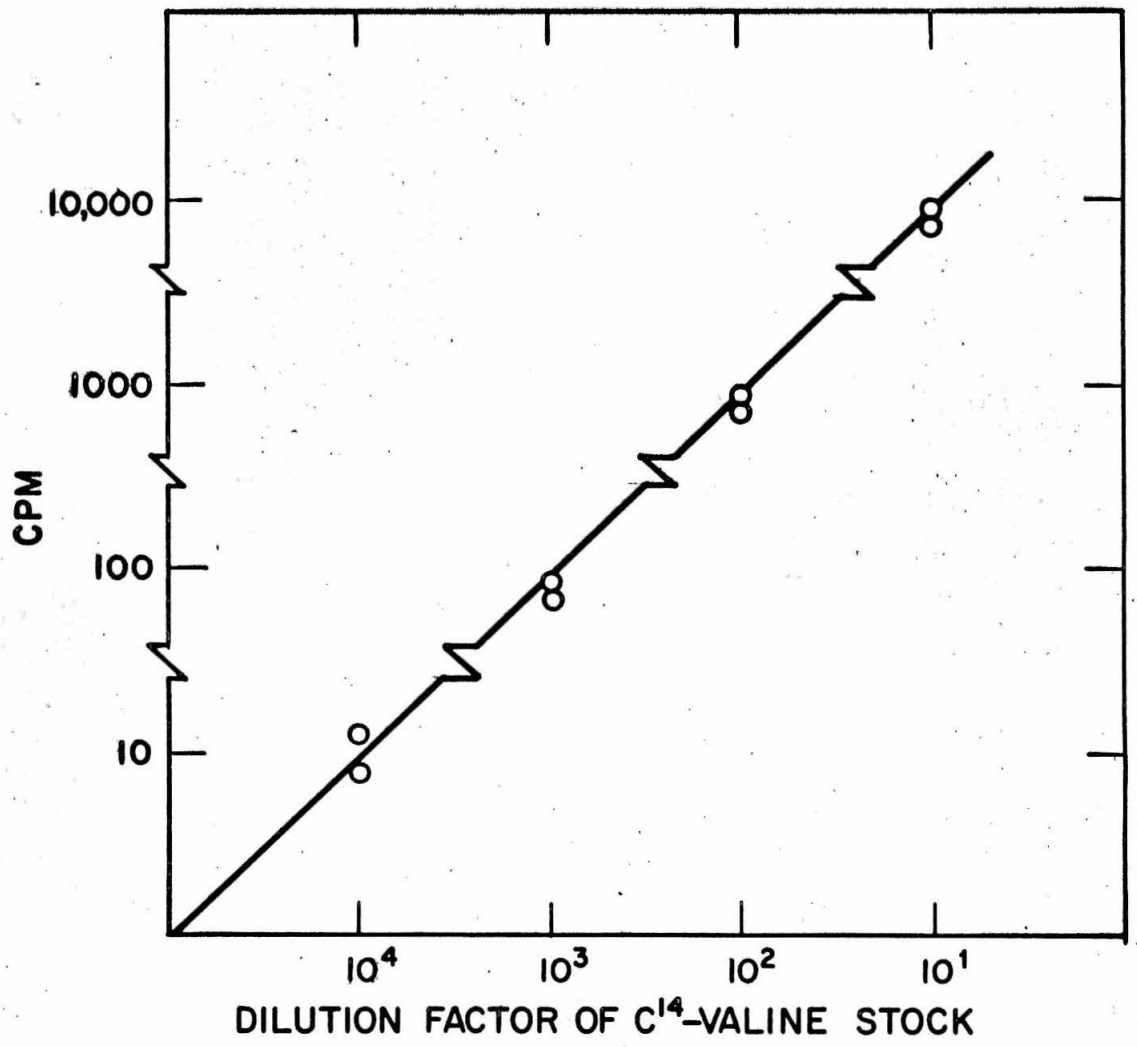

Fig. 8.-Scintillation counting of C^{14}-valine applied to filter paper strips containing extracted L. pictus egg proteins showing the linear relationship between the amount of radioactive material present and number of counts. One-tenth ml samples from dilutions of a stock C^{14}-valine solution containing approximately one microcurie per ml were plated on paper strips. To this was added 4.2 mg of extracted L. pictus protein in a solution of 0.5 normal NaOH. Samples were thoroughly dried before counting.

counts over background were discarded. Usually about 1% of them did contain some counts, and these were rarely ever over 10 counts per minute. All vials were thoroughly washed, rinsed and dried before reuse. Scintillation fluid older than two months was discarded.

The extraction procedure used for many of these experiments was that described in Table VI which used two 3 minute 90°C TCA washes. This procedure was later shortened (53) when it was found that the number of washes could be reduced without affecting the t_o values. Thus the procedure which has been in use most recently is the following. The sample, followed with 100 µl of tube rinse fluid, was pipetted onto a filter paper strip and air dried. The strips were then immersed in 100 ml of ice cold 10% TCA and given two subsequent 100 ml washes with 5% TCA at 90°C. Three 95% ethyl alcohol washes, one 100% ethyl alcohol wash and two washes with reagent grade acetone or ether completed the lipid and pigment extraction and the dehydration. Usually no more than 30 filter paper strips were washed at one time in a 1500 to 2000 ml beaker.

This method of preparation of proteins for counting has also been used for suspensions of intact eggs. The eggs were pipetted onto the paper strips and thoroughly air dried before running them through the extractions. These samples also showed very good internal consistency when equal aliquots were extracted and counted (Table VIII).

The proteins, after purification and counting on the paper, could be quantitatively extracted from the paper with alkali and determined

TABLE VIII.-Preparation of aliquot samples of whole fertilized L. pictus eggs which have been allowed to incorporate C^{14}-valine.*

	Sample number			
	1	2	3	4
counts per minute	20157	20524	20623	20819

*The eggs were fertilized and allowed to develop 30 minutes. At this time two microcuries of C^{14}-valine was added to 30 ml of the culture, and the eggs were allowed to incubate for another 30 minutes. Samples were pipetted with a wide-mouth pipette onto paper strips and allowed to dry. As outlined in the text, the samples were extracted with cold TCA, hot TCA and lipid solvents prior to being counted.

by the Ellman method (220). For this purpose the scintillation fluid was poured off, and the papers were given three 100% ethanol washes while remaining in the vials. After drying, 6 ml of 1.0 normal NaOH were added to each vial, and the papers were minced with a small glass rod. The vials were then centrifuged, and the supernatants were used for protein determinations. The values which were obtained from blank papers were subtracted in order to cancel any effect by the paper itself. This procedure again showed that there was a great deal of internal consistency within the paper strip method (Table IX) and that the amounts of protein recovered from the papers though slightly higher were very similar to the samples extracted in test tubes.

The small differences in counts and total protein noted between the two methods were probably due to small losses attributable to the washing of precipitates by centrifugation. The consistency among filter paper samples suggests that if equal aliquots of protein are used throughout an experiment and net protein synthesis is negligible, then one need assay no more than two samples for total protein in order to place the entire experiment on a per mg protein basis (in practice the t_o controls).

Protein determinations

The protein determinations were done by the Ellman (220) modification of the biuret reaction which relied upon the 263 mμ absorption of Cu-peptide bond complexes in strong alkali. Since the maximum absorbance for this complex was very similar to many other highly absorbing materials which were found in the egg, this method would appear to be unsuitable.

TABLE IX.-Quantitative recovery of aliquot samples of sea urchin egg protein from paper strips.*

	Sample number					
	1	2	3	4	5	6
mg protein recovered	8.5	8.4	8.4	8.9	8.5	8.8

*Equal aliquots (0.20 ml) of an egg homogenate were pipetted onto paper strips and dried. Samples were carried through the usual procedure for preparing protein for counting. They were then placed in individual scintillation vials, and six ml of 1.0 normal NaOH were added. After mincing papers with a glass rod all vials were centrifuged, and the supernatants were used for the determinations.

However, by virtue of the previously mentioned preparatory procedures most of the possible contaminants were removed prior to the assay.

For our purposes a standard curve was prepared from unfertilized L pictus egg proteins which had been purified by the procedure reported previously (p. 65). The proteins were given their final drying in a vacuum desiccator over P_2O_5 and kept there until used. They were then quickly transferred to weighing flasks containing a known weight of 0.1 normal NaOH and dissolved. The final weight then gave the concentration in mg of protein per ml of solution, assuming a density of one gram per ml for the solvent. The reagent mixture was a solution of 0.2 molar $CuSO_4$ in 2.0 normal NaOH, and the blank was 2.0 normal NaOH alone. The protein solution was added to the reagent or blank at a ratio of 1:10 or a 11 fold dilution and allowed to stand at room temperature for approximately 30 minutes. Once the complex had formed it was stable for several hours at room temperature and several days under refrigeration.

When the values for the control samples were subtracted from the reagent containing samples, an absorption coefficient of 5.83 O.D. units per mg per ml per cm of light path for the complex of copper and sea urchin egg proteins was obtained. The value reported by Ellman (220) for bovine serum albumin was 5.3. The plot of several dilution series (Fig. 9) from which the above value for egg proteins was obtained represents the actual amount of protein present. From this a factor (1.894) was derived which gave the concentration of protein in mg per ml in the original solution when multiplied by the O.D. of the reagent containing sample minus the blank. Since blank values for these proteins

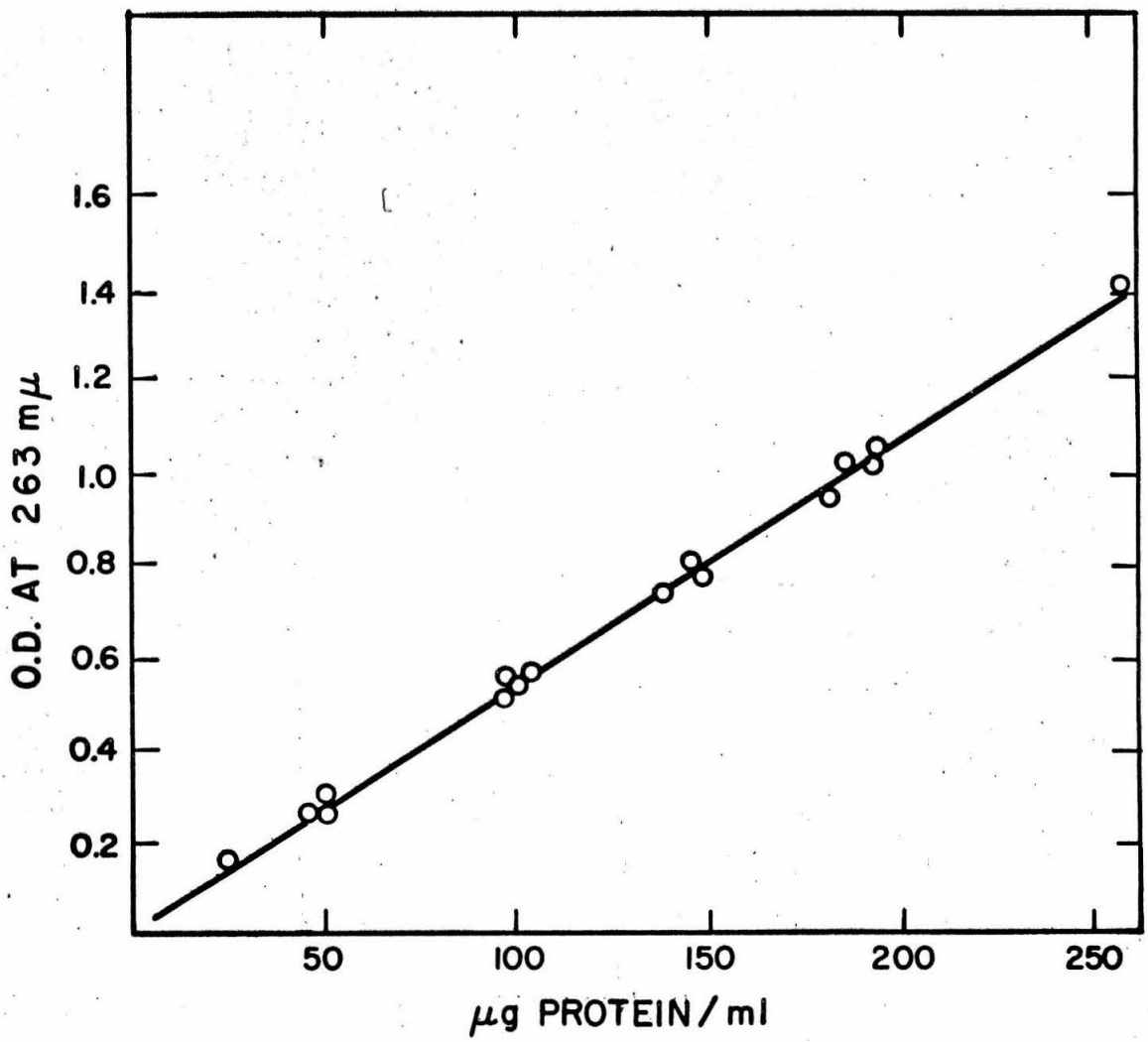

Fig. 9.-Standard curve for the determination of L. pictus egg protein by the method of Ellman (220). The curve is the composite of several different dilution series, and the individual points represent the value of the reagent containing sample minus that of the blank. The reagent solution contained 0.2 molar $CuSO_4$ in 2.0 normal NaOH, and the blank solution was 2.0 normal NaOH alone. The protein solution was added to the reagent or blank solutions at a ratio of 1:10 respectively, and the mixture was allowed to stand at room temperature for 30 minutes prior to measuring the O.D. at 263 mμ. Thoroughly extracted proteins from unfertilized eggs were used; note text for procedure of extraction.

as well as the values for the reagent samples were quite consistent, a plot was made of the reagent containing samples only (Fig. 10). From this a factor of 1.56 was derived to relate the O.D. to the mg protein per ml in the original solution. This value has been used most often and has not been used on anything but sea urchin proteins which had been thoroughly extracted.

Preparation of egg fragments

The procedure used for separating eggs into nucleate and non-nucleate fragments was based upon a centrifugation method first devised by F. R. Lillie (221) for fragmenting Chaetopterus eggs. It was first used for splitting sea urchin eggs by E. B. Harvey (138). The principle involved was that when eggs were subjected to a centrifugal force they elongated forming a density gradient within the egg and eventually splitting into fragments of different densities. The nucleus, probably due to properties of the nuclear plasm, always came to lie in the clear area just beneath the so-called oil cap at the least dense end of the stratified egg. By using solutions of the proper densities, in this case mixtures of a sucrose solution which was isotonic with sea water (1.1 molar in distilled water) (17), the nucleate and non-nucleate fragments could be separated.

The eggs were first de-jellied either by slight acidification (16) or swirling in a small volume of sea water then washed and given a five minute low gravity centrifugation (ca. 1000 X g) on top of a 5.5:4.5 mixture of 1.1 molar sucrose and sea water in the case of Lytechinus eggs and a 6.5:3.5 mixture in the case of Strongylocentrotus eggs. Only the eggs remaining on top of these mixtures were used. Since the

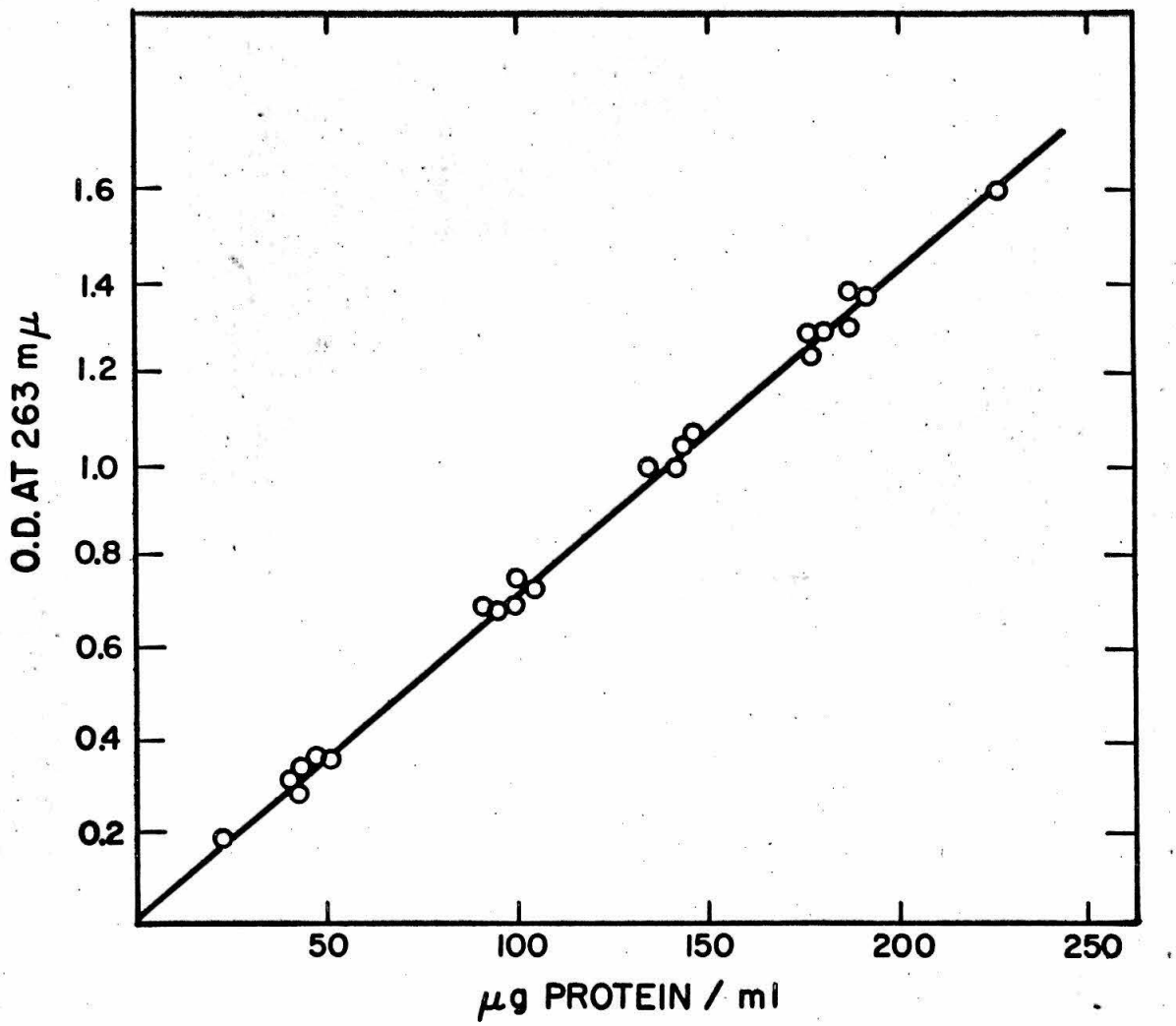

Fig. 10.-Standard curve for the determination of extracted L. pictus proteins. Details similar to those of Fig. 9 with the exception that this curve is based upon the values of the reagent containing samples only.

density of eggs would vary from different females as well as during different parts of the season, the importance of this preliminary centrifugation was that the eggs used were known to be less dense than the solution they had been centrifuged over and could be counted on rising through a slightly more dense solution.

The eggs were packed, and enough sea water was added to them so that 3 additional volumes of 1.1 molar sucrose would bring the final volume to at least 2.0 ml. This 3:1 mixture of 1.1 molar sucrose and egg suspension was the lowest layer placed in the 1 x 3 inch tubes designed for use with the SW 25.1 rotor of the Spinco model L ultracentrifuge. The 8 ml middle layer contained in the case of L. pictus eggs a 3:2 mixture of 1.1 molar sucrose and sea water while that for S. purpuratus was a 6.7:3.3 mixture. The top layer consisted of 17 ml of sea water. The three layers were introduced into the tubes in reverse order, thus the top layer was placed in the tube first. Next, the middle layer was added from a wide-mouth serological pipette which passed through the first layer to the bottom of the tube, and finally the egg containing bottom layer was added in a similar manner. A propipette attached to the serological pipette was used for adding the middle layer, and a serological syringe attached to the pipette was used for the addition of the bottom layer so that all of the egg suspension could be added. Addition of the layers in this order gave a minimum of mixing and could be done quite rapidly. As soon thereafter as possible centrifugation was begun at 10,000 rpm (10,000 X g r_{ave}) for 15 minutes. After centrifugation the non-nucleate fragments were

banded at the interphase of the bottom and middle layers while the nucleate fragments were located near the top of the middle layer. Since any remaining whole eggs were less dense than the middle layer by virtue of the preliminary centrifugation step, they were also found layered near the nucleate fragments.

The fractions could either be collected by pipetting off the layers from the top or by puncturing a hole in the bottom of the tube large enough for the fragment suspension to pass through and collecting them by drops. The latter method was preferred especially in cases where the fragment bands were separated by small distances. After collection the fragments were washed free of sucrose and resuspended in sea water. All of the above steps beginning with the shedding of the eggs were carried out in ice cold solutions primarily because the percentage of fertilization of the eggs and fragments remained much higher than those at room temperature if they were kept cold for the several hours necessary for preparation.

Purity of nucleus-free preparations

The determination of the purity of non-nucleate fragments of L. pictus eggs simply amounted to observing the number of nucleate fragments present in the preparation since the nuclei were readily microscopically visible (Figs. 11 and 12). Many of the preparations were completely free of nucleate fragments, and none of the experiments that are reported had more than 0.2% contamination. S. purpuratus eggs in addition to being considerably more dense were also quite opaque. In this case the first indication of contamination was the obvious

Fig. 11.-Nucleate fragments prepared from unfertilized L. pictus eggs. Light spots near centers of fragments are nuclei. Fragments are magnified 150 times.

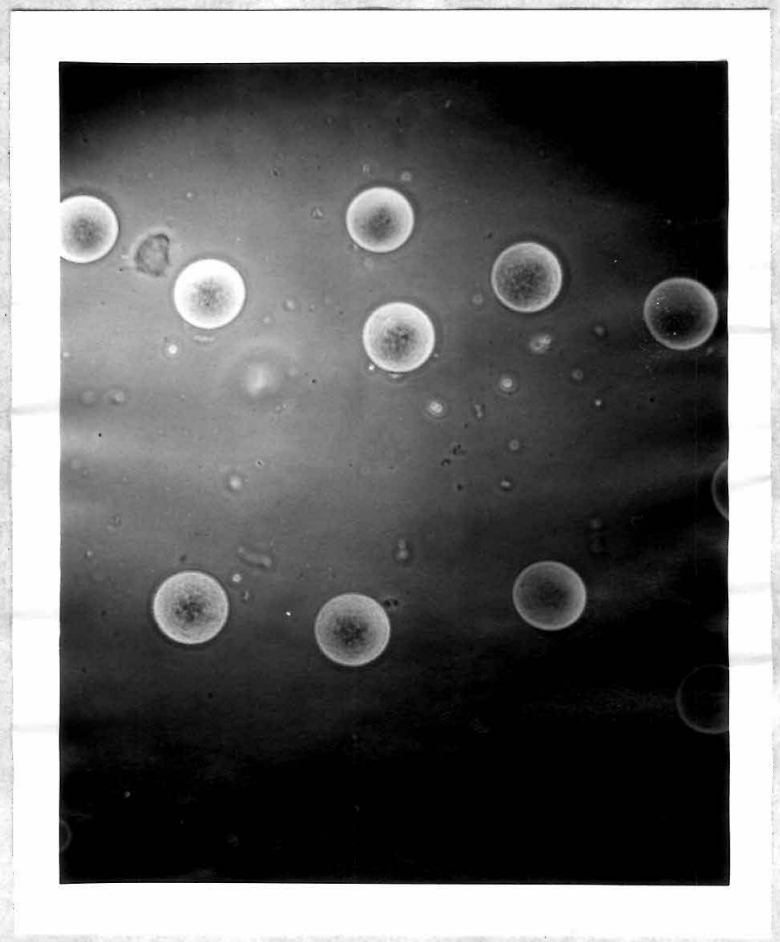

Fig. 12.-Non-nucleate fragments prepared from unfertilized L. pictus eggs. Magnification the same as in Fig. 11.

presence of whole eggs in the non-nucleate fraction. Secondly, nuclei could be distinguished if preparations of flattened eggs or fragments were viewed in dim light in a microscope which was slightly out of focus. Attempts to stain the nuclei of these eggs selectively have so far given far less success. The above method using flattened eggs has consistently confirmed the original observation of Harvey (138) that the nucleus lies close to the oil cap in the clear area. Thus, the oil cap itself which was easily microscopically visible could be used as a nuclear indicator. The preparations of non-nucleate fragments from S. purpuratus eggs which were used for the polysome experiments were approximately 99% pure.

Procedure for parthenogenetic activation

There are many agents which are effective parthenogenetic activators (cf. 222); however, in order that a valid comparison, timewise, could be made between parthenogenetic non-nucleate fragments and fertilized fragments or whole eggs a method was needed which activated the eggs quickly. For this reason the butyric acid method of Loeb (223) has been used almost exclusively in these experiments. Moreover, it had already been shown that butyric acid activated eggs incorporated amino acids into proteins at a rate comparable to the fertilized eggs (26, 28).

After centrifugation the fragments were washed free of sucrose with ice cold sea water and allowed to warm to 20°C. A 0.0074 molar solution of butyric acid in sea water was then added to the egg or fragment suspensions to bring them to the desired butyric acid

concentration. During the course of these experiments the final butyric acid concentrations in sea water necessary for activation remained within a range of 0.004 to 0.006 molar. These values will be reported individually in the results. Likewise the lengths of time that the eggs or fragments were left in the activating agent were also limited, ranging from 30 to 120 seconds. The simplest method for stopping the treatment was to dilute the suspension greatly at the proper time. However, if the eggs or fragments were needed in a small volume of sea water soon after activation, effective dilution was achieved by pelleting the eggs just prior to the end of the treatment and resuspending them with sea water at the proper time. One additional wash by centrifugation was given to remove any remaining traces of butyric acid. The use of sea water enriched with $NaHCO_3$ instead of normal sea water for the rinse solution (28) had not given any indications of being advantageous so it was not considered to be a necessary step in the procedure. Criteria for determining the amount of activation achieved by these treatments will be considered in a section of the Results along with the visible effects produced.

Detection of polysomes

The eggs or fragments were mechanically stripped of their fertilization membranes by a method developed in this laboratory. A 30 ml suspension of fragments was squirted against the side of a beaker (100 to 250 ml) three times beginning at 40 seconds after fertilization or artificial activation. This usually resulted in a greater than 90% removal of fertilization membranes with only 10-20% cytolysis. They were then given two 0.55 molar KCl washes to prevent clumping

and given at least three sea water washes to remove the KCl. Just before pulsing they were allowed to settle to the bottom of the culture vessel so that as much as possible of the sea water could be drawn off before they were transferred to a graduated centrifuge tube. After adjustment of the fluid volume to a range of 2.5 to 4.0 ml, a 5 minute pulse of 1.0 microcurie per ml of C^{14}-valine (200 curies per mole) or phenylalanine (360 curies per mole) was given in a flat bottomed 20 ml vial. The eggs were cooled in ice and given two cold 0.55 molar KCl washes. An amount of KCl equal to the egg volume was left on top of the eggs, and a volume of ice cold solution equivalent to six times the volume of the pelleted eggs was added. This solution was composed of the following: 0.05 molar tris-succinate buffer (pH 7.6), 0.025 molar KCl, 0.004 molar $MgCl_2$ and 0.005 molar mercaptoethanol (126). The eggs were gently mixed with the solutions and allowed to sit in ice for 10 minutes in order to swell and begin cytolysing. Only 25 seconds of gentle shaking were needed to get nearly complete cytolysis. The homogenate was then centrifuged at 10,000 rpm for 5 minutes in the SW 39 rotor of the Spinco model L ultracentrafuge, and the supernatant was divided into two 0.5 ml aliquots. To one aliquot, 0.05 ml of a 0.01 mg per ml solution of bovine pancreatic ribonuclease (RNase)[1] in the above buffer was added, and to the other buffer alone was added. The RNase containing samples were allowed to sit at $4^{\circ}C$ for 45 to 60 minutes while the other samples remained at $0^{\circ}C$. At the end of this

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[1]Sigma Chemical Co.

time, 0.05 ml of a 5% sodium deoxycholate solution was added to each sample which was then layered on a 4.5 ml concentration gradient of sucrose dissolved in the homogenizing buffer solution. Beginning at the bottom, the different layers of the gradient were as follows: 0.5 ml of 60% sucrose, 1.0 ml of 30%, 1.0 ml of 25%, 1.0 ml of 20% and 1.0 ml of 15%. The gradients were made up just before they were needed and thus had not had time to diffuse into a continuous gradient. After centrifugation at 27,500 rpm for 105 minutes in the SW 39 rotor, 0.5 ml fractions were collected from a hole punctured in the bottom of the gradient containing tube. From these, 0.225 ml were pipetted onto filter paper strips for radioactive assay in the manner described earlier, and the remaining part of the fraction was checked for optical density at 260 mμ in a Beckman model DU spectrophotometer.

RESULTS

Cell-free amino acid incorporation into protein

In agreement with Hultin (28), homogenates prepared from fertilized Lytechinus pictus eggs incorporate amino acid into hot acid precipitable material to a much greater extent than homogenates of unfertilized eggs (Table X). The time course of *in vitro* amino acid incorporation (Fig. 13) reveals that the rate is maximal for somewhat less than 10 minutes. The fertilized and the unfertilized egg homogenates show a similar pattern, and once the incorporation has stopped the amount incorporated remains constant for at least two hours. These results indicate either that protein synthesis has stopped and there is no proteolysis or that proteolysis has increased to a rate which is comparable to synthesis. These possibilities will be discussed later. It is also shown that the amount of labelled amino acid removed by hot TCA treatments (possibly the sRNA-amino acid complex) is fairly constant during the course of the incubation and that the amount in the unfertilized and fertilized egg homogenates is very similar. Nearly a four-fold stimulation of amino acid incorporation is obtained by the addition of energy-rich compounds to the fertilized egg homogenate while essentially no effect is brought about in the unfertilized egg homogenates (Fig. 14). The time courses for the samples with and without the energy-rich compounds are very similar though there may be a slightly faster leveling off in the latter.

TABLE X.-Incorporation of C^{14}-valine into protein by homogenates of unfertilized and fertilized L. pictus eggs.*

	Counts per minute per mg protein
Unfertilized eggs	5, 5
Fertilized eggs	81, 83

*The eggs were homogenized in the presence of 0.033 molar tris - HCl buffer (pH 7.8), 0.0033 molar Mg acetate 0.066 molar sucrose and 0.18 molar KCl. The homogenates were incubated at 27°C for 60 minutes in the presence of 10 µmoles per ml of PEP, 1 µmole per ml of ATP and 0.25 microcuries per ml of C^{14}-valine (4.8 curies per mole).

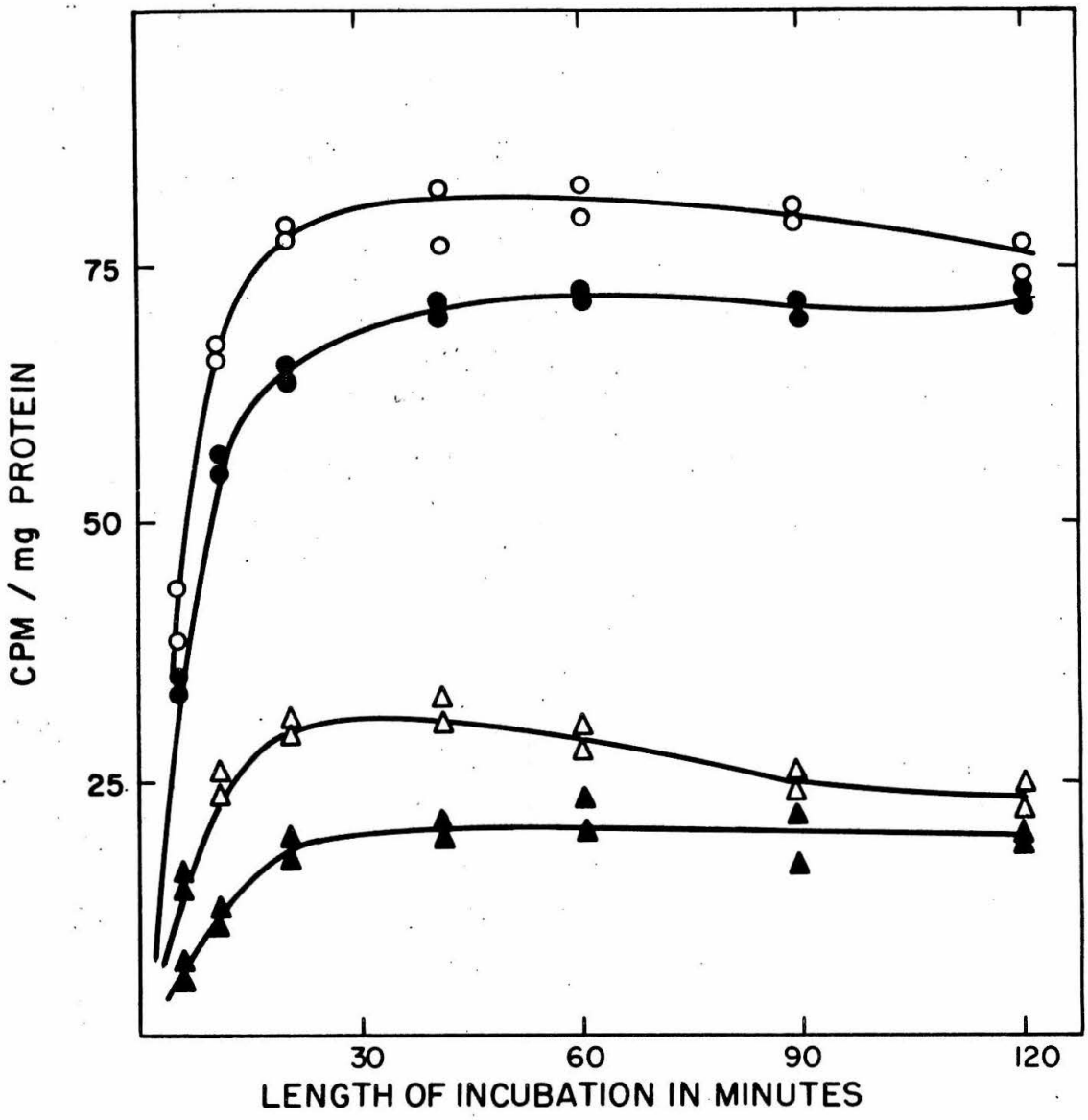

Fig. 13.-Time course of amino acid incorporation into protein by homogenates of L. pictus eggs at 25°C. The homogenates were prepared as described in Table X. At the various times after the beginning of incubation, aliquots were removed and placed in test tubes. These were placed in ice and excess C^{12}-valine was added. After being pipetted on paper strips and dried, all samples were thoroughly extracted with ice cold 5% TCA and lipid solvents. In addition, various aliquots were given six minute 90°C treatments with 5% TCA, Symbols: Open circles - fertilized egg homogenate without 90°C treatment; closed circles - fertilized egg homogenate with six minute 90°C treatment; open triangles - unfertilized homogenate without 90°C treatment; closed triangles - unfertilized egg homogenate with six minute 90°C treatment.

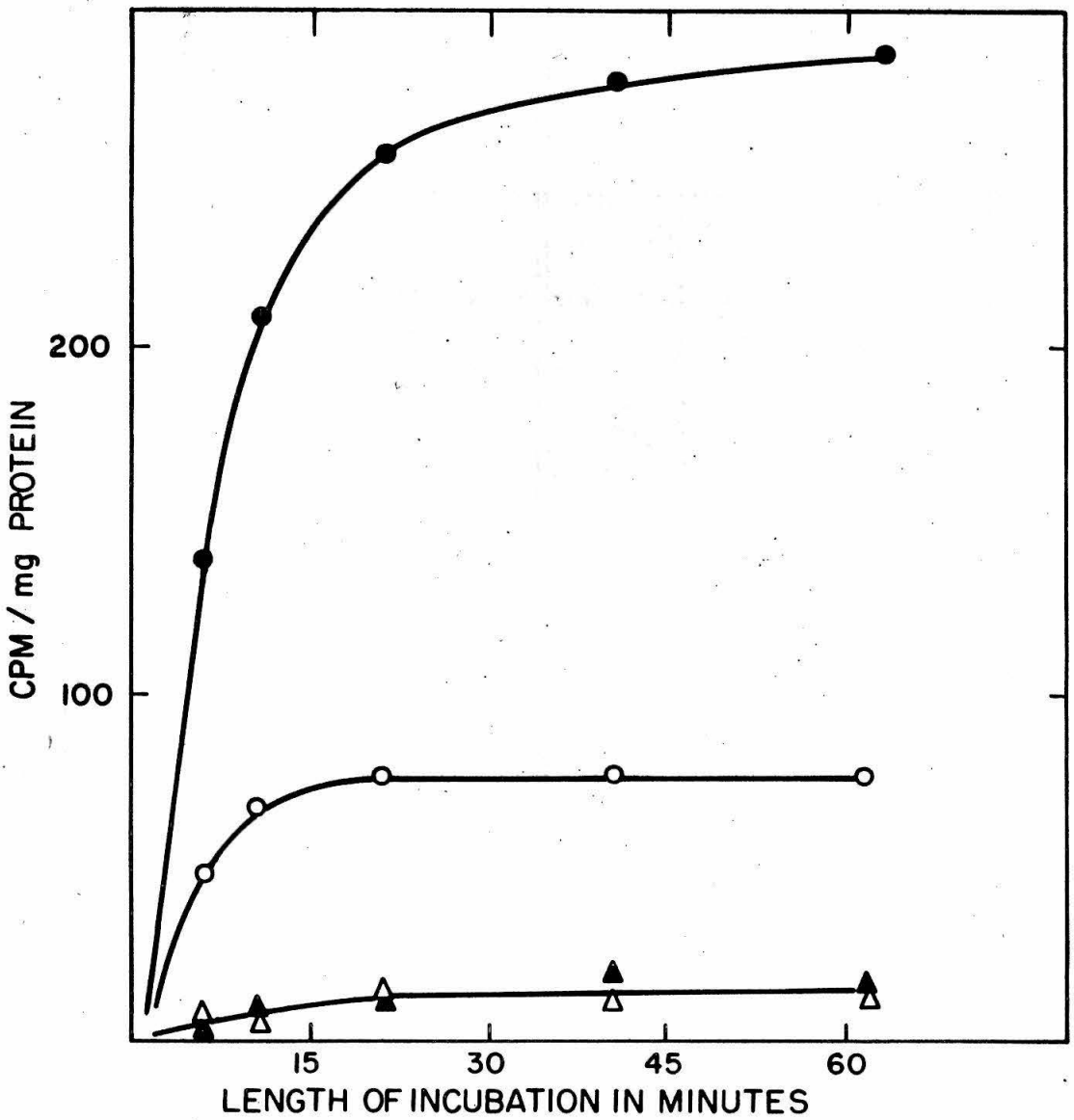

Fig. 14.-The effect of PEP and ATP on cell-free amino acid incorporation by homogenates of S. purpuratus eggs at 25°C. Homogenates were prepared as described in Table X. All samples contained 0.25 microcuries per ml of C^{14}-valine (4.8 curies per mole). Symbols: Closed circles - fertilized egg homogenate with added PEP and ATP (10 micromoles and 1 micromole per ml respectively); open circles - fertilized egg homogenate; closed triangles - unfertilized egg homogenate with PEP and ATP; open triangles - unfertilized homogenate.

The effect of changing from the use of C^{14}-valine with a specific activity of 4.8 curies/mole to valine with a specific activity of 200 curies/mole might be expected to increase the amount of label incorporated by a factor of more than 40. However, if the increase is less than this, it might be suspected either that there is a sizable endogenous pool or that there is a small pool which is rate limiting to amino acid incorporation at the higher specific activity and non-rate limiting at the lower. The change to the high specific activity accounted for only an increase of 50% in the amount of label incorporated (Table XI). Since other measurements favor the idea that sea urchin eggs contain a sizable pool (22, 23) it seems likely that the above findings can best be interpreted as indicating a large pool. Furthermore, it had been shown that the pool varied a great deal from stage to stage (22, 23), thus calculations of either the actual amount of protein being synthesized or the amount of amino acid being incorporated are of dubious validity unless pool measurements are made for each separate experiment.

The magnesium ion optimum for the incorporation of C^{14}-valine was determined in homogenates of fertilized and unfertilized L. pictus and S. purpuratus eggs (Fig. 15). The optima for homogenates of fertilized eggs of the two species are very similar; the final added concentrations falling very close to 0.003 molar. The curves for the unfertilized homogenates of the two species suggest that the optima for these may be closer to 0.002 molar. The range of activities at sub-optimum concentrations of magnesium indicates that there is a considerable amount of dependence upon magnesium ions by the amino acid incorporating system.

TABLE XI.-The effect of varying the specific activity of C^{14}-valine upon incorporation in homogenates of fertilized L. pictus eggs

Specific activity*	Counts per minute
201 curies per mole	1730, 1578
4.8 curies per mole	1117, 1102

*All samples contained 0.06 microcuries of C^{14}-valine, and the specific activities refer only to this added quantity. The specific activities are based upon the manufacturers specifications. All samples were from the same homogenate.

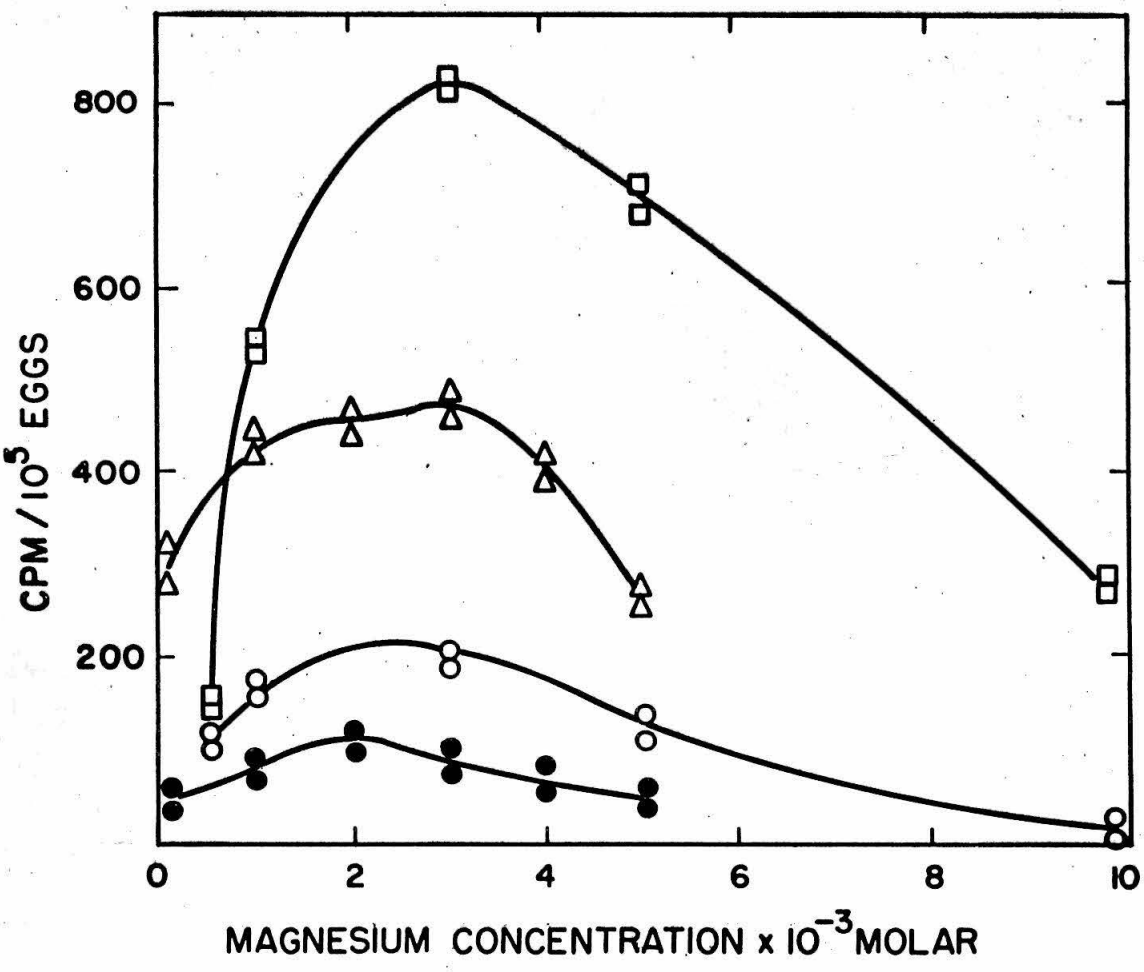

Fig. 15.-The effect of varying the concentration of added magnesium acetate on the incorporation of C^{14}-valine into proteins by homogenates of L. pictus and S. purpuratus eggs. Except for varying the concentrations of magnesium, the homogenates were prepared and allowed to incorporate as described in Table X. Symbols: Squares - fertilized S. purpuratus eggs; triangles - fertilized L. pictus eggs; open circles - unfertilized S. purpuratus eggs; closed circles - unfertilized L. pictus eggs (redrawn from 53).

The potassium ion optimum for amino acid incorporation into proteins of fertilized egg homogenates is at 0.18 molar with negligible side effects over a broad range (Fig. 16). Concentrations higher than 0.24 molar are definitely inhibitory though even at 0.30 molar there is only a 50% decrease in activity. Unfertilized egg homogenates did not show a clear optimum; however, since the activities were quite low during these studies it is doubtful that an optimum could have been recognized.

Amino acid incorporation into proteins of non-nucleate fragments of sea urchin eggs

Intact fragments

Non-nucleate fragments of unfertilized L. pictus eggs show an increased amount of amino acid incorporation into proteins after treatment with butyric acid (Table XII). The ratios of activity of the treated samples over the untreated samples (T/U) are on the whole generally smaller than the values usually obtained upon fertilization of whole eggs. This suggests that there is some basic difference in the abilities of parthenogenetically activated fragments and fertilized eggs to synthesize proteins. However, a comparison of the activities of non-nucleate fragments which have been artificially activated or fertilized shows a great deal of similarity (Table XIII) and indicates that the butyric acid treated samples can incorporate amino acid at a rate comparable to those that are fertilized. Furthermore, it was apparent from additional unreported data in experiment 1 of Table XII that the nucleate fragments of L. pictus are less active than the non-nucleate fragments when the activities are compared on a milligram protein basis.

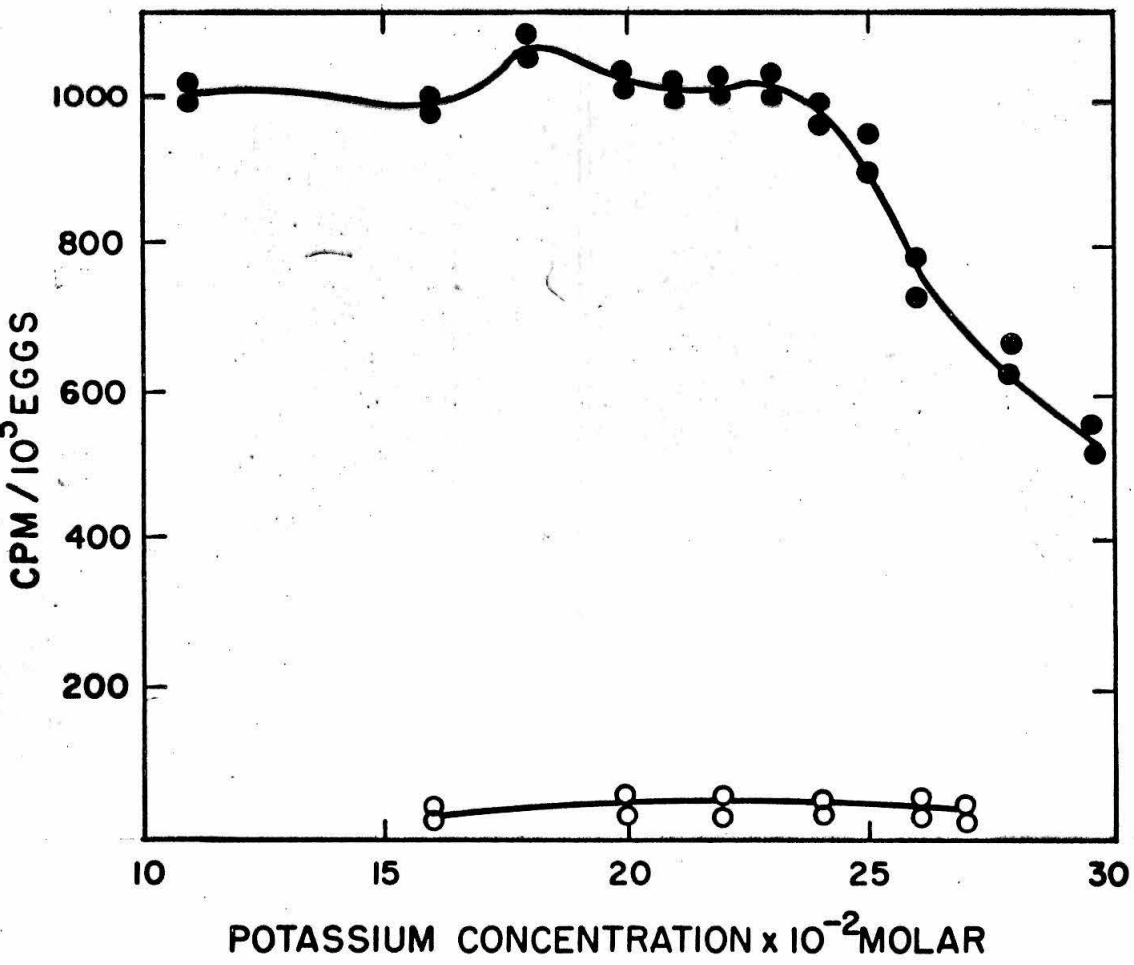

Fig. 16.-The effect of varying the concentration of potassium chloride on the incorporation of C^{14}-valine into proteins by homogenates of S. purpuratus eggs. Except for varying the concentrations of potassium, the homogenates were prepared and allowed to incorporate as described in Table X. Symbols: closed circles - fertilized eggs; open circles - unfertilized eggs (redrawn from 53).

TABLE XII.-The effect of butyric acid treatment on incorporation of C^{14}-valine by non-nucleate fragments of L. pictus eggs

Experiment number*	Counts per minute Untreated (U)	Treated (T)	Ratio T/U
1	25650, 25230	72750, 72300	2.8
2	1040, 920	3340, 2663	3.1
3	5861, 5584	8877, 8669	1.5
4	1475, 1281	3384, 3017	2.3

*Values for experiment one are per mg protein. For the other experiments the values are for aliquot samples. All samples were treated with 0.004 molar butyric acid in sea water for one minute. Incubation was in 0.1 microcurie of C^{14}-valine per ml at 20°C for 15 minutes to 2 hours.

TABLE XIII.-The comparison of the effect of butyric acid treatment and fertilization on the incorporation of C^{14}-valine by non-nucleate fragments of L. pictus eggs

Experiment number*	Counts per minute Untreated (U)	Treated (T)	Fertilized (F)	Ratios T/U	F/U
5	9540, 6700	20753, 17467	19348, 18451	2.3	2.3
6	247, 220	788, 769	655, 663	3.3	2.8
7	1005, 833	8885, 7526	8969, 8122	8.9	9.3
8	999, 879	4627, 4307	7645, 7404	4.8	8.0

*Same as above except that in experiment five C^{14}-phenylalanine was used instead of C^{14}-valine. The comparisons of butyric acid treated and fertilized samples within an experiment were always done between non-nucleate fragments that had been fertilized or parthenogenetically activated at similar times. The lengths of development prior to the incubation in C^{14}-amino acid were also the same.

Homogenates

Homogenates prepared from artificially activated fragments of L. pictus and S. purpuratus eggs show an increase in amount of amino acid incorporated into protein which is comparable to the intact fragments (Table XIV). Homogenate experiments also give evidence confirming the observation that artificially activated non-nucleate fragments of L. pictus eggs are more active per mg protein than the activated nucleate fragments (Table XV). Two other problems studied in this experiment have given additional preliminary information. The first, a variation of the length of butyric acid treatment, indicates very strongly that the protein synthesizing system is very sensitive to the amount of treatment. Whether or not the decreased amino acid incorporation is a direct result of the excess treatment or only the secondary result of a more generalized metabolic inhibition has not yet been determined.

The second part of the study was that of mixing of homogenates from artificially activated non-nucleate fragments with those of unactivated nucleate fragments just in case the nucleate fragments contained some necessary factor for protein synthesis that the non-nucleate did not. Obviously the results from the non-nucleate fragments by themselves excludes this possibility, though it is still interesting to note what little effect, if any, the nucleate fragment homogenate had upon homogenates of activated anucleate fragments.

TABLE XIV.-Incorporation of C^{14}-valine by homogenates of butyric acid treated non-nucleate fragments of L. pictus and S. purpuratus eggs

Experiment number*	Counts per minute Untreated (U)	Treated (T)	Ratio T/U
1	16.3, 14.3	68.4, 72.8	4.6
2	2.6, 1.4	6.6, 5.6	3.0
3	19, 17	98, 92	5.3

*Experiments one and two were performed on fragments of L. pictus eggs, and the values are per mg protein. Experiment three was done with S. purpuratus and the values are for a packed fragment volume of 0.075 ml. Using 0.005 molar butyric acid in sea water, the lengths of treatment were 30, 90 and 60 seconds respectively. The preparation of the homogenates and their incubation with C^{14}-amino acid was essentially the same as that described in Table X.

TABLE XV.-Incorporation of C^{14}-valine into protein of homogenates of butyric acid treated nucleate and non-nucleate fragments of *L. pictus* eggs

Sample number	Fragment treated	Length of treatment in seconds*	Counts per minute per mg protein
1	Non-nucleate	30	72.8
2	Nucleate	30	8.8
3	Non-nucleate	60	27.8
4	Nucleate	60	6.9
5	Non-nucleate	90	13.1
6	Nucleate	90	4.8
7	Non-nucleate	120	15.7
8	Nucleate	120	6.0
9	Non-nucleate	--	16.3
10	Non-nucleate	--	14.3
11	Nucleate	--	3.6
12	Nucleate	--	5.0
13	Non-nucleate plus nucleate	30 --	33.0
14	Non-nucleate plus nucleate	60 --	15.0
15	Non-nucleate plus nucleate	90 --	8.0
16	Non-nucleate plus nucleate	120 --	9.8
17	Non-nucleate plus nucleate	-- --	2.8
18	Non-nucleate plus nucleate	-- --	3.2

*Samples allowed to develop for one hour at 20°C prior to homogenization. Incubation of homogenates was for 45 minutes at 23°C. The butyric acid concentration in sea water was 0.005 molar. Homogenate preparation and incubation similar to that described earlier. Sample numbers 13 through 18 contained, in a 1:1 ratio, homogenates of untreated nucleate fragments and non-nucleate fragments, treated for various lengths of time with butyric acid.

Kinetics of activation of intact fragments

The kinetics of the activation of the amino acid incorporating system in artificially activated nucleate and non-nucleate fragments of S. purpuratus eggs are very similar for at least four hours (Fig. 17). Also a similar agreement can be seen between the fertilized nucleate and non-nucleate fragments. In comparing fragments which have been fertilized with the artificially activated fragments, it is again apparent that the rates of uptake are of the same order after they level off. However, the artificially activated fragments increase at a rate faster than the fertilized fragments. Though the kinetic curve for whole eggs does not clearly show this, it had been reported by Hultin (28) that artificially activated eggs of Paracentrotus lividus showed a more rapid increase in amino acid uptake than fertilized eggs. Both of the fertilized samples show dips at about 60 minutes after fertilization which are not found in the artificially activated samples. Since the artificially activated samples did not cleave and the fertilized samples did, the data is consistent with the idea suggested by Gross and Cousineau (119) that this decrease in incorporation rate is due to the cleavage which takes place at this time. A feature which is consistently observed in these studies is that even though the artificially activated samples show a sharper increase than the fertilized samples they begin to show a decrease in incorporation ability by four hours whereas the fertilized eggs and fragments continue to show a slightly increasing rate.

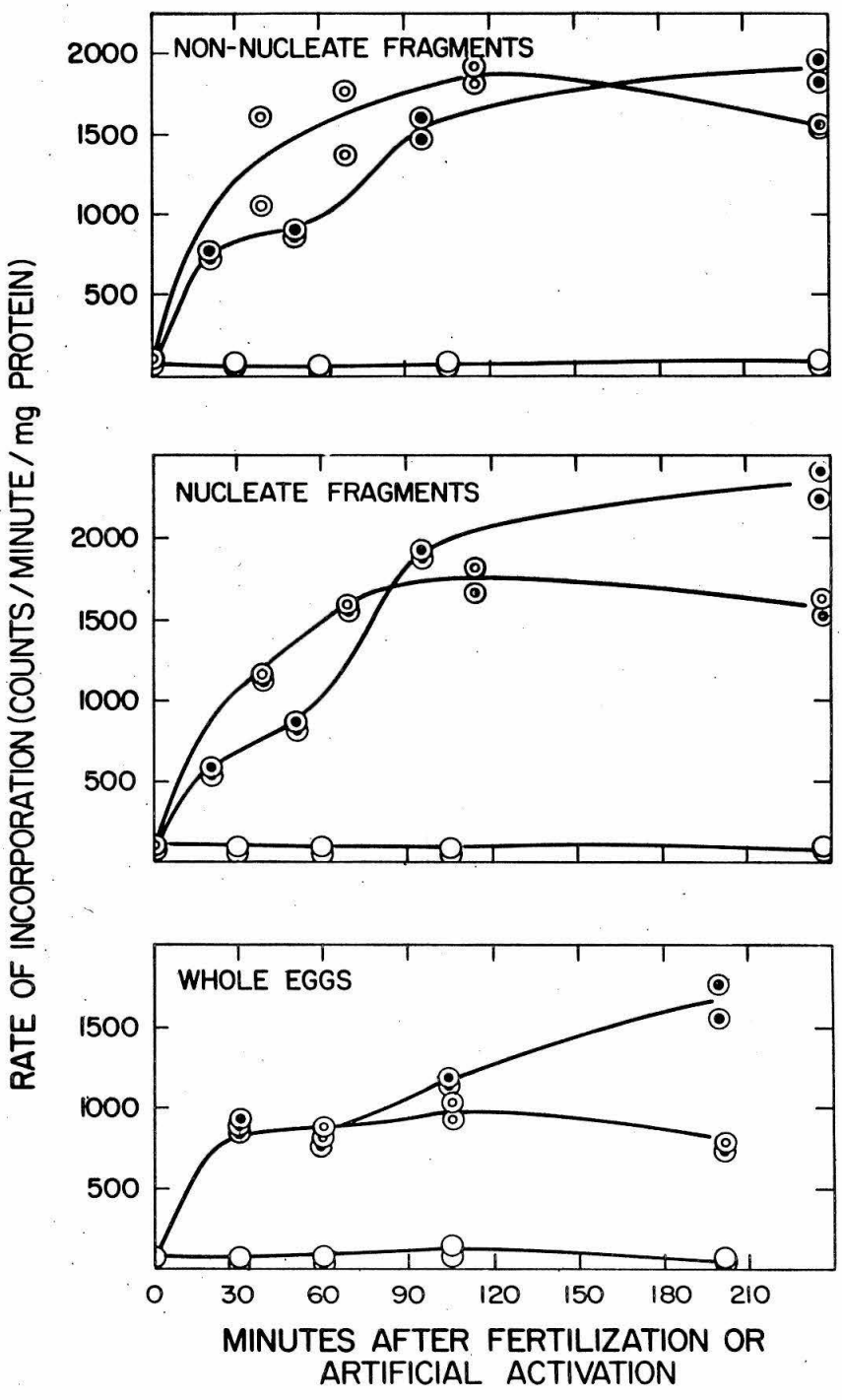

Fig. 17.-Comparison of the kinetics of activation of C^{14}-valine incorporation into proteins of unfertilized, fertilized and artificially activated non-nucleate and nucleate fragments and whole eggs of S. purpuratus. All samples were allowed to incubate for 10 minutes at 20°C in C^{14}-valine at a concentration of 0.1 microcuries per ml. Parthenogenetic activation was accomplished by a 90 second treatment with 0.005 molar butyric acid in sea water.

The ratio of activities of the treated and untreated (T/U) non-nucleate fragments of S. purpuratus eggs are usually over 20 and in these kinetic studies are almost 40. These T/U values compare very favorably with the values obtained upon fertilization of whole eggs. On the other hand, the incorporation by L. pictus fragments does not compare favorably with fertilized eggs since the fragments rarely ever exceed a T/U value of five and the whole eggs upon fertilization show a 20-40 value. In view of the evidence that fertilized Lytechinus fragments do not incorporate at a rate significantly different from their activated counterparts; that unfertilized whole eggs incorporate very little and that untreated fragments show a sizable incorporation, it would appear that there is some change brought about in the fragments during their preparation which causes an increase in incorporation. In order to pinpoint the portion of the procedure responsible for this increase in activity, certain steps have been checked independently. Probably the most logical place to start was with the solution itself since the high concentration of sucrose changed the ionic strength considerably. In experiments with whole eggs (Table XVI) it can be seen that the sucrose-sea water mixture has very little effect upon the incorporation rate unless it is accompanied by a period of time at 0°C. Furthermore, it can be seen that 40 minutes at 0°C in sea water alone is enough to cause a two-fold increase in activity. Other studies with both whole eggs (Table XVII) and whole egg homogenates (Table XVIII) also show the stimulatory effect upon unfertilized Lytechinus eggs caused by a 0°C treatment. These experiments also

TABLE XVI.-The effect of isotonic sucrose (1.1 molar) upon C^{14}-valine incorporation by unfertilized L. pictus eggs

Treatment for 40 minutes prior to incubation with C^{14}-valine at 20°C	Counts per minute
Sea water at 20°C	557, 530
Sucrose* at 20°C	636, 598
Sucrose at 0°C	1112, 862
Sucrose at 0°C for 10 minutes and at 20°C for 30 minutes	883, 835
Sea water at 0°C	1094, 1050

*Sucrose solution in all samples was composed of a 3:1 mixture of 1.1 molar sucrose and sea water respectively. The eggs were washed with sea water at the end of the treatment and allowed to incubate in the presence of 0.1 microcurie of C^{14}-valine per ml for two hours.

TABLE XVII.-The effect of 0°C treatment on the incorporation of C^{14}-valine by unfertilized L. pictus eggs

Treatment in sea water prior to incubation with C^{14}-valine* for one hour at 20°C	Counts per minute
20°C for one hour	194, 219
" for five hours	179, 234
" for twenty-four hours	456, 620
0°C for one hour	548, 415
" for five hours	347, 496
" for twenty-four hours	639, 531

*0.1 microcuries per ml of sea water

TABLE XVIII.-The effect of 0°C treatment on the incorporation of C^{14}-valine by unfertilized L. pictus egg homogenates

Treatment prior to homogenization*	Counts per minute
Homogenized immediately after shedding	71, 71
Sea water at 20°C for one and one-half hours	21, 28
Sea water at 0°C for one and one-half hours	193, 191

*The homogenates were prepared and allowed to incorporate as described in Table X.

indicate that the activity of unfertilized eggs decreases as they sit at room temperature. Thus, cold treatment can cause as much as a seven-fold increase in the amino acid incorporation ability of unfertilized eggs. It, therefore, seems likely that the low T/U values are indeed due to a stimulatory effect upon the eggs or egg fragments which is caused by low temperature storage.

Butyric acid activation

As mentioned earlier, both nucleate and non-nucleate fragments respond to the length of butyric acid treatments in a way that suggests an optimum treatment for activating protein synthesis. Further studies (Tables XIX and XX) show that an optimum does exist and that it is quite narrow with respect to both concentration of butyric acid and length of treatment.

The ultimate criterion for judging when total activation of fragments or whole eggs was caused by butyric acid treatment was the comparison of their activities with those of 100% fertilized samples. This was especially necessary for L. pictus eggs and fragments in these experiments since they did not raise recognizable fertilization membranes after artificial activation even though electron micrographs indicated that cortical vesicle breakdown had taken place (224). Six hours after activation they do, however, show a high percentage of blebbing (Fig. 18). S. purpuratus eggs and fragments, on the other hand, responded to butyric acid treatment by raising normal appearing fertilization membranes. The percentage membrane elevation appeared to be indicative of the amount of activation

TABLE XIX.-The effect of varying the concentration and time of butyric acid treatment on C^{14}-valine incorporation by L. pictus eggs

Concentration of butyric acid in sea water	Length of treatment in seconds	Counts per minute*
Untreated	--	111, 116
Fertilized	--	3474, 3151
0.004 molar	30	205, 207
0.004 molar	60	193, 161
0.005 molar	30	402, 315
0.005 molar	60	1737, 1456
0.005 molar	90	3261, 2990
0.006 molar	30	1720, 1478
0.006 molar	60	1284

*Incubated in 0.1 microcurie of C^{14}-valine per ml of sea water at 20°C for 30 minutes.

TABLE XX.-The effect of varying the length of butyric acid treatment on C^{14}-valine incorporation by S. purpuratus eggs

Length of exposure in seconds to 0.0052 molar butyric acid in sea water	Counts per minute *
60	7994, 8107
90	16112, 16356
120	5272, 5345

*Incubated in 1.0 microcurie of C^{14}-valine per ml of sea water at 20°C for 10 minutes.

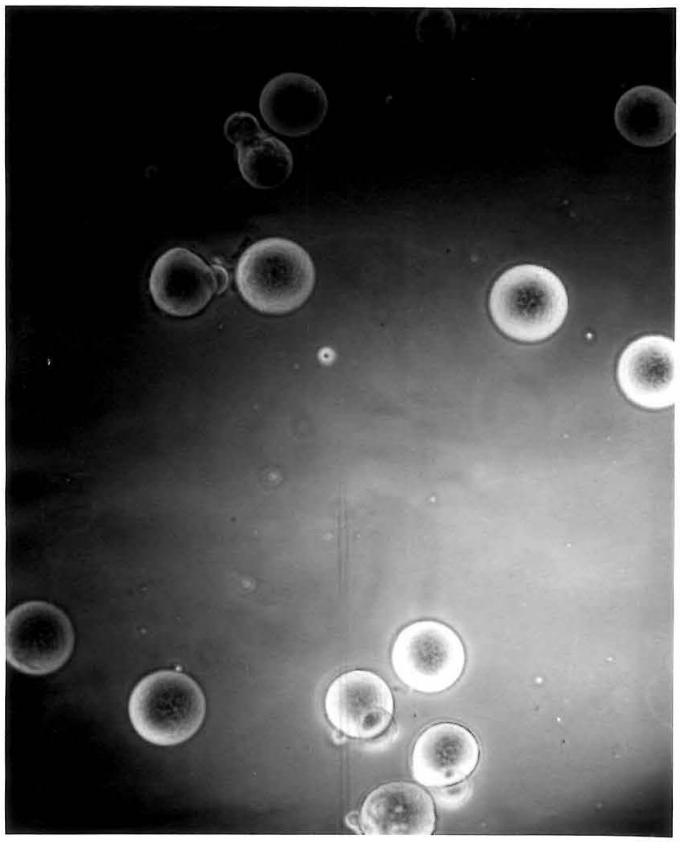

Fig. 18.-Non-nucleate fragments of L. pictus with have been treated with butyric acid and allowed to develop six hours. Treatment was with 0.0035 molar butyric acid in sea water for one minute. Note blebs on the surface of some of the fragments. Magnification is 150 times.

of the amino acid incorporating system so long as the optimum length of butyric acid treatment was not passed. If it was passed, amino acid incorporation decreased even though there was still 100% membrane elevation.

Polysome formation in artificially activated non-nucleate fragments

The non-nucleate fragment preparations of S. purpuratus eggs used for these experiments were approximately 99% pure by number and showed 80-90% fertilization membrane elevation upon artificial activation.

The sedimentation diagrams of untreated non-nucleate fragments do not show any indication of the presence of large numbers of ribonuclease sensitive aggregates (Fig. 19). In some experiments there are indications that the small amount of activity present is, however, due to the presence of a very small number of polysomes. As soon as 40 minutes after parthenogenetic activation, polysomes can be demonstrated in artificially activated fragments by virtue of both the presence of C^{14}-amino acid incorporated into hot TCA-precipitable material and an increase in the optical density at 260 mμ (Fig. 20). The transfer of the radioactivity to the monoribosome peak, and the reduction of the optical density following RNase treatment gives further support to the idea that ribosomal aggregates are present. The inability to demonstrate consistently a compensatory increase in optical densities of the monosome region of RNase treated samples is probably attributable in part to the small numbers of polysomes present compared to the number of monosomes and also to the low spatial resolving power introduced into the system

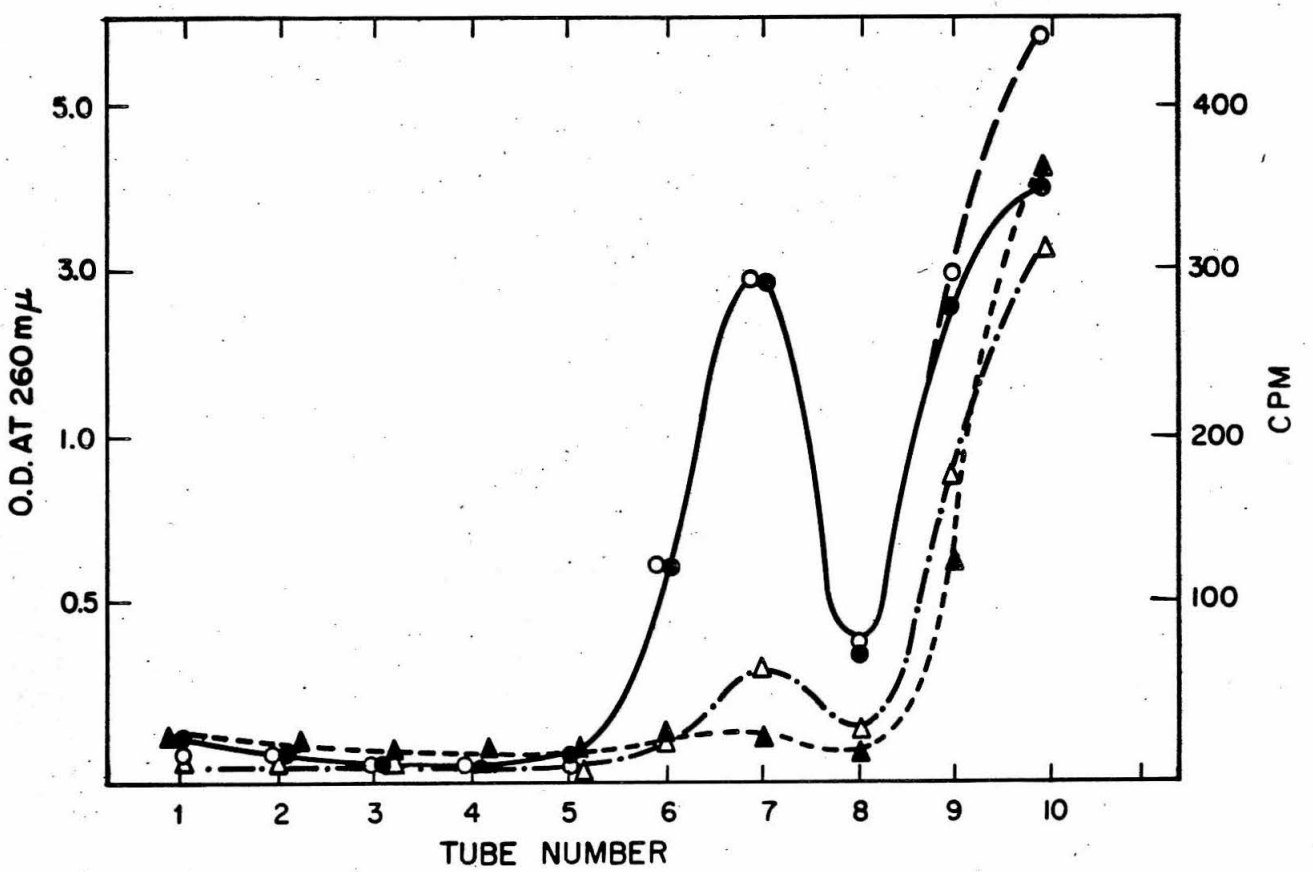

Fig. 19.-Sucrose gradient fractions of homogenate of unfertilized non-nucleate fragments of S. purpuratus eggs which were pulse labelled for five minutes with C^{14}-phenylalanine at a concentration of one microcurie per ml. The supernatant from a 10,000 rpm for 5 minutes centrifugation was layered over a sucrose gradient (60%, 30%, 25%, 20% and 15%) and centrifuged at 27,500 rpm for 105 minutes in a SW 39 rotor. RNase (0.001 mg per ml) treated samples were incubated at $4^{o}C$ for 45 minutes. The counts per minute are indicative of incorporation into hot TCA-insoluble material. Symbols: open circles - O.D. with RNase; closed circles - O.D. without RNase; open triangles - counts per minute with RNase; closed triangles - counts per minute without RNase.

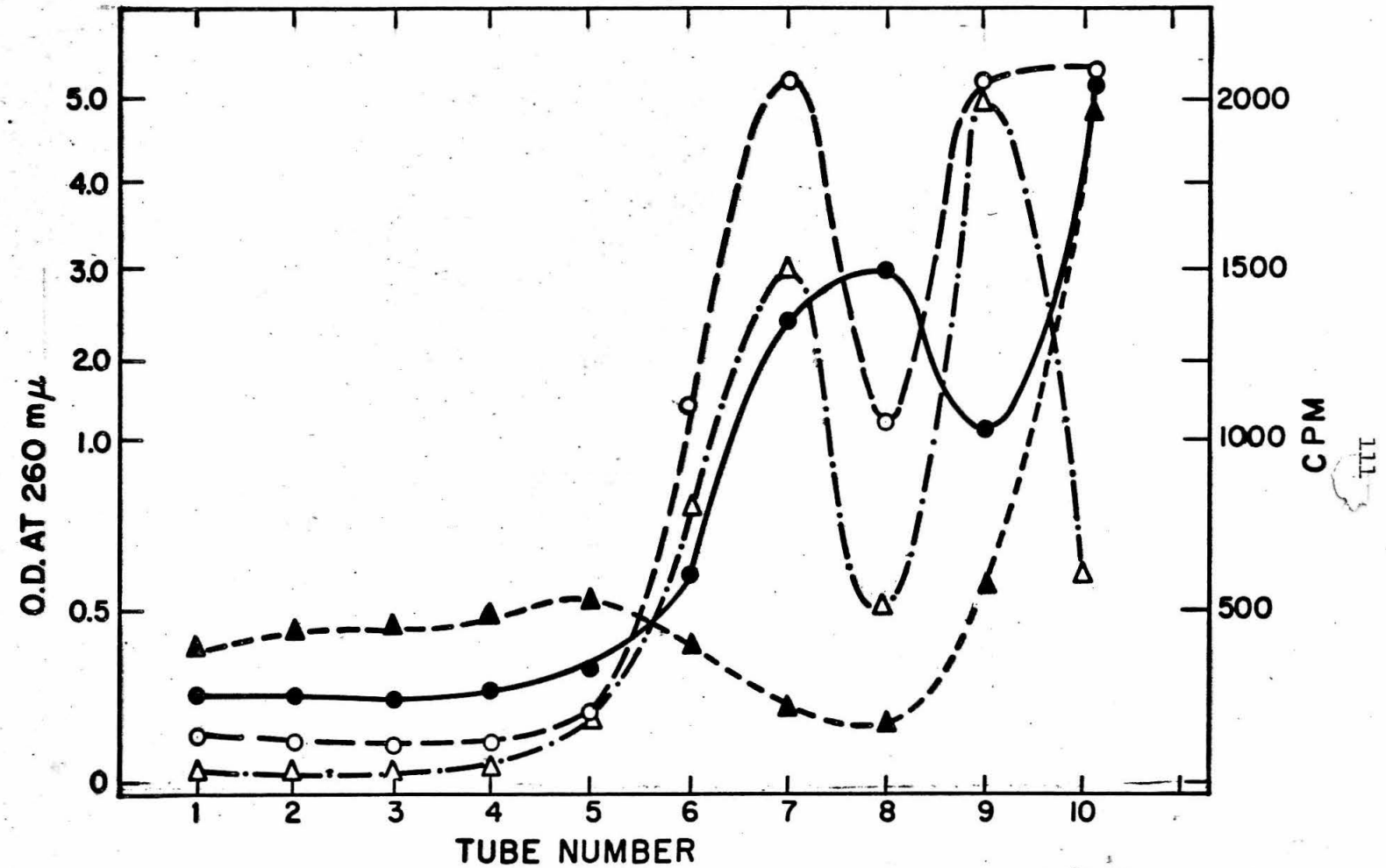

Fig. 20.-Sucrose gradient fractions of homogenates of butyric acid treated (0.005 molar for 90 seconds) non-nucleate fragments of S. purpuratus eggs. After developing for forty minutes, the fragments were pulsed for five minutes with one microcurie per ml of C^{14}-valine. Refer to Fig. 19 for additional conditions of preparation and centrifugation. Symbols: open circles - O.D. with RNase; closed circles - O.D. without RNase; open triangles - counts per minute with RNase; closed triangles - counts per minute without RNase.

by the collection of only 10 fractions from each gradient. Likewise, the lack of a peak or peaks in the polysome region of the gradients may be due to the collection of a relatively small number of fractions. On the other hand, it may be that the polysomes of sea urchin eggs are not homogeneous in size. In order to resolve this problem, more refined techniques will have to be used. Finally, if the specific activities (cpm/O.D. unit) of the polysome and monosome fractions are compared, it can be seen that the polysomes are considerably more active than the monosomes (Fig. 21).

RNA synthesis by non-nucleate fragments

In view of the evidence mentioned earlier that sea urchin eggs begin synthesizing RNA soon after fertilization, several experiments were performed to test for a similar synthesis in parthenogenetic non-nucleate fragments. The results of these experiments have been highly variable, and no conclusions can yet be made. This cannot be equated with any basic difference between these and the fertilized whole eggs because essentially the same lack of consistent results was obtained in experiments with them. The reason for this preponderance of inconsistent results probably stems from the paper strip method of counting in its present state of refinement, namely that in some cases a considerable amount of nonspecific absorption of the nucleotides to paper was observed, decreasing the sensitivity of the method drastically. In spite of this lack of results, both RNA synthesis and cytoplasmic DNA will be discussed later because of their inherent importance to the subject of this report.

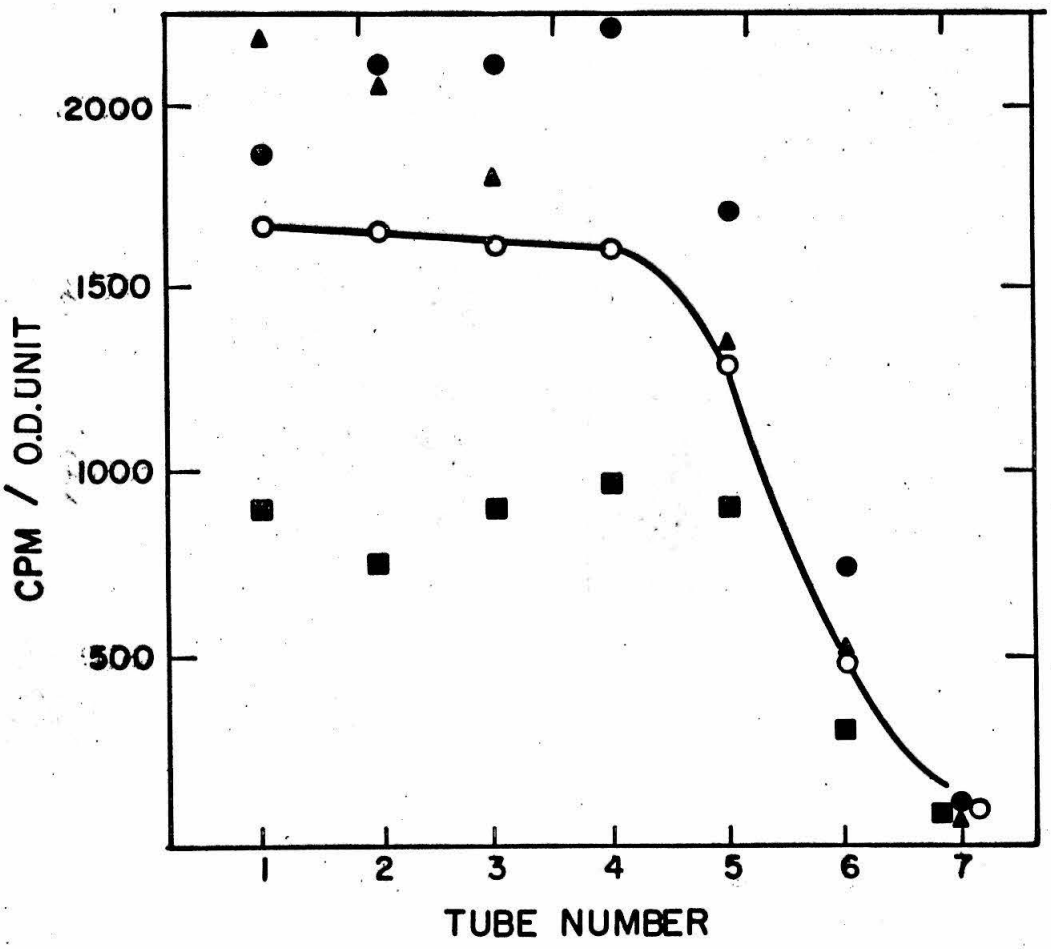

Fig. 21.-Specific activities of ribosome containing fractions from sucrose gradient separations of homogenates of butyric acid treated non-nucleate fragments of S. purpuratus eggs. Values in the polysome region are for RNase sensitive material only. Closed symbols represent values from three separate experiments, and the open circles represent the average of the three values.

DISCUSSION

Protein synthesis

Amino acid incorporation as an indication of true protein synthesis

The question of what extent amino acid incorporation into protein actually represents protein synthesis is especially pertinent since sea urchin eggs do not show any net increase in total protein during early development but on the contrary show a slight decrease (22, 23). One might then suspect that the incorporation being observed could be caused by terminal amino acid exchanges or other more non-specific binding of the amino acids to the proteins. On the other hand, it is felt that much of the experimental data presented previously is in disagreement with the above suggestion and, in fact, supports the alternative hypothesis that the amino acid incorporation is indicative of protein synthesis.

The first important observation is that the label remains associated with the protein fraction after rigorous purification procedures which often include dissolving with alkali and reprecipitation with acid several times. Secondly, the great difference in incorporation rates of identically treated homogenates of unfertilized and fertilized eggs suggests that the difference must be due to something more than just non-specific processes since the extent of their influence would be expected to be similar in both cases. The energy rich compound, PEP, stimulates incorporation activity of homogenates while incubation at 0°C completely inhibits it. The antibiotic puromycin whose mode of action is that of mimicking the sRNA at the ribosomal site (225) also inhibits the uptake (226).

Polysomes are the most active sites of incorporation both in the intact eggs and fragments and in cell-free preparations (126, 227). Finally, it has been shown that neither the ribosomal nor the supernatant fractions by themselves were capable of significant incorporation and that they had to be in combination before uptake took place at a rapid rate (228).

Thus, in summary, it has been shown that labeled amino acids are very tightly associated with the protein fraction and that their incorporation appears to be energy dependent. Incorporation is inhibited by a standard protein synthesis inhibitor. It occurs upon structures (polysomes) which have been for the most part characterized by various investigators as sites of active protein synthesis, and it requires accessory materials other than these structures before it can take place. On the basis of these observations it is felt justifiable to conclude that the amino acid incorporation observed is for the most part representative of protein synthesis and that there is, therefore, a great difference in the rate of synthesis taking place in the unfertilized and fertilized sea urchin egg.

The role of the proteins synthesized during early development

Amino acid incorporation increases rapidly during two periods of development, at fertilization and at the mesenchyme blastula stage (229, 230). From evidence reviewed elsewhere (p. 27) it appears likely that the latter burst of protein synthesis is directly mediated genetically and probably represents a synthesis of proteins necessary for the numerous differentiations which follow. The initial increase in amino acid incorporation, on the other hand, does not appear to be under the

direct control of the DNA of the cell nucleus and may represent only an increased rate of synthesis of proteins already existing in the fertilized egg. Evidence in favor of this is that, as mentioned earlier, new antigens were not immunologically detectable until the gastrula stage (46, 47). However, these findings do not necessarily mean that synthesis begins at this time. Since sperm antigens might be expected to be present in the newly fertilized eggs, and since they could not be detected by this method it might indicate that the time of appearance merely represents the increase of the new proteins to the lower limit of detection.

Methods of fractionation and detection such as electrochromatography, starch block electrophoresis and DEAE column chromatography also failed to show the appearance of any new proteins after fertilization though all of the previously existing components now showed the presence of labelled amino acid (231). Earlier studies relying upon such properties as water, ammonium sulfate and potassium chloride solubilities as well as sensitivity to urea, trypsin and heat treatments indicated that the proteins of fertilized eggs did somehow differ from unfertilized egg proteins (232-238). Electrophoretic analysis after many of these treatments, however, did not turn up any new proteins in the fertilized egg but rather suggested that the differences were probably due to changes in the secondary or tertiary structures of proteins existing in the unfertilized egg (236). Having previously concluded the likelihood that the amino acid incorporation observed in sea urchin eggs represents an actual synthesis of proteins, this structural rearrangement does not then appear to be related to the observed uptake of amino acids.

Even though there is no independently detectable synthesis of new proteins after fertilization it is not to say that the observed synthesis is unimportant to the well being of the embryo. From two lines of evidence this initial burst of protein synthesis appears to be essential to cleavage and to normal post-blastular development. The first line of evidence comes from studies of the effects of puromycin upon the developing sea urchin egg. This antibiotic is an inhibitor of protein synthesis which because of its structural similarities replaces sRNA from its binding site on the ribosomes (225). Contrary to the findings with the antibiotic actinomycin D (118) which had little effect upon sea urchin egg development until the gastrula stage, puromycin immediately caused cleavage to stop at a point just prior to the cell division (107, 226, 239). Accompanied by this inhibition was also a corresponding decrease in the rate of protein synthesis. The apparent correlation between the severity of cleavage inhibition and the decrease in amino acid incorporation into protein led Hultin (226) to propose that a certain amount of protein synthesis is necessary before cleavage can take place. Though puromycin had a very strong inhibitory effect on both the intact cells and the cell-free homogenates; until it has been shown that there is also an inhibition of amino acid incorporation in cell-free homogenates prepared from cleavage inhibited eggs, one might yet question at what level the puromycin is affecting the living cell. In other words, does puromycin even enter the cell in order to achieve this effect? This question is important to the validity of the Hultin's proposal especially

since inhibition of *in vivo* protein synthesis may represent only a decrease in amino acid permeability into the egg, such as appears to be the case for eggs reared in isotonic potassium chloride (53). In addition, cleavage can be inhibited by agents which apparently act at the level of the egg membrane and do not interfere with protein synthesis (240-242). Thus it would appear that the effects of puromycin treatment upon the intact eggs can be duplicated by agents which do not enter the cell or adversely affect the internal protein synthesizing machinery. Therefore, until more about the mode of action of puromycin upon intact cells is known this evidence cannot be viewed as a conclusive case for the dependency of cleavage upon protein synthesis. It has been stated that the unfertilized egg contains all of the spindle-associated proteins it will need for cleavage (243). However, autoradiograms of both isolated spindles (244) and whole cells (245) which had previously been allowed to incorporate labelled amino acids into protein *in vivo* showed a concentration of the radioactivity in this region. Since there was also a concentration of ribosomes in this region of the cell (246, 247) it cannot be concluded to what extent the spindle-associated incorporation represented a synthesis of mitotic apparatus proteins or just a synthesis of non-related proteins upon ribosomes trapped within the framework of this "organelle". Thus, from the foregoing even though there is the suggestion that synthesis of spindle-associated proteins is required for cleavage, it cannot be considered to be a definite conclusion.

Another possibility is that, as may be suggested from studies of hormone action (248, 249), the proteins being synthesized are instrumental in bringing about the increase in metabolic rate which takes place upon fertilization (250). Though there is no direct evidence supporting this possibility, it is not improbable.

There are also indications that at least some of the proteins synthesized during the initial burst of amino acid incorporation are important in later development. Bosco and Monroy (251) found that embryos cultured in the presence of the amino acid analogue ethionine shortly after fertilization cleaved normally but showed a very abnormal development of the primary mesenchyme cells. Presumably under the influence of certain ethionine containing compounds, the differentiation of these cells which contribute to the formation of the skeleton was inhibited in some cases to the point of complete degeneration; resulting at best in swimming blastulae filled with opaque granular material. The death of the embryos soon followed. Experiments in which the embryos were reared in ethionine containing sea water for only 30 minutes and then washed and placed in normal sea water for subsequent development showed that the most sensitive period began at the time of first cleavage. Sensitivity decreased considerably by the second cleavage and was almost negligible by the third and fourth cleavages. In keeping with the idea that there is a synthesis of some ethionine containing compound, the effect was found to be irreversible by methionine treatment. The true nature of this compound is not yet known. The large amount of evidence which indicated that beginning soon after fertilization

methionine was strongly incorporated into proteins (cf. 252) suggests that the ethionine containing compounds are also likely to be proteins. It is interesting to note that two other analogues, allyl-glycine and beta-phenyllactic acid, did not show any effects which could be interpreted as being specifically directed at any special stage of development, suggesting perhaps a special role for methionine containing compounds and methionine in particular in the differentiation of these cells.

A similar phenomenon though less specific was indicated from the observed effects of spermine on developing eggs. In addition to showing an inhibited rate of amino acid incorporation during incubation in the presence of the polyamine, eggs removed from it and washed thoroughly could no longer gastrulate though their development to this time was apparently normal (53).

In summary, it would appear that the protein synthesis initiated by fertilization is important for normal cleavage during the early stages and also for normal differentiation of the primary mesenchyme cells and their derivatives, thus having both an immediate and a delayed role in the development of the embryo.

Cell-free amino acid incorporation

Characteristics

The kinetic curves for incorporation of amino acid into proteins by homogenates of unfertilized and fertilized eggs are strikingly similar to those reported for incorporation by E. coli homogenates (253, 254). The reason for the lack of linearity in E. coli may very likely be due to the relatively short half life (two and one-half minutes) of the

endogenous mRNA (255). A similar situation might exist in the sea urchin egg homogenates if there were a significant amount of nuclease activity present even though the mRNA itself appears to be very stable in vivo (118). Since little is known about nuclease activity in developing sea urchin eggs this possibility cannot be given a great amount of consideration. From the studies in which the kinetics were compared between homogenates containing PEP, ATP and C^{14}-valine and those with only C^{14}-valine it can be seen that the availability of energy can be rate limiting especially during the initial phase of the incorporation. The step in the sequence of protein synthesis (cf. 203, 256, 257) responsible for this energy requirement is not known. However, since the shape of the curve is very similar in both cases it indicates that energy dependence is not likely to be the cause of the decrease in amino acid incorporation with time.

It has also been suggested (253) that decreases in amino acid incorporation such as this are due to a lack of release of the completed polypeptides from the ribosomes. This hypothesis has not been borne out in studies on protein synthesis in tissues from higher organisms (256-260). These studies consistently showed the release of completed polypeptides as well as the associated ribosome from the mRNA during cell-free incorporation. They also showed that ribosomal attachment to the mRNA took place at a slower rate than the release, possibly accounting for the observed decrease in activity. Additional studies have indicated that ribosomes released from a polyribosome are capable of reattachment, while it is the polyribosome itself which appears to

lose its affinity for taking on more ribosomes (257). This could be due to an easily damaged attachment point, to nuclease activity or perhaps even to some yet undiscovered labile enzyme necessary for attachment.

A third possible explanation for this phenomenon in sea urchin egg homogenates was suggested by the observation of Kavanau (261) that autolysis was a real source of error in amino acid pool measurements even if all extractions were carried out at below freezing temperatures. Because this could be shown to occur at low temperatures, the probability of significant proteolysis taking place at the incubation temperature (25°C) for amino acid incorporation is very great. If this is the case, then the observed decrease in protein synthesis merely represents a dilution of the C^{14}-amino acid pool by C^{12}-amino acids in a manner analogous to the usual quenching procedure in which an excess of C^{12}-amino acid was added to the sample to stop the uptake of the labelled precursor at a specific time. Calculations made on the basis of Kavanau's measurements of valine or phenylalanine in the free amino acid pool and proteins of the eggs of the sea urchin S. purpuratus (22) indicate that the breakdown of 10 to 15% of the total protein could bring about a ten-fold increase in the free amino acid present. An increase such as this could theoretically decrease the amount of C^{14}-amino acid incorporated to one-tenth its initial value. On the other hand, measurements of amino acids in the pool and proteins of P. lividus eggs (23) showed that the pool was much too large to be greatly altered in size by extensive proteolysis.

The extent of proteolytic activity in the homogenates in which amino acid incorporated was investigated is not known. On the other hand, the relatively small increase in amount of C^{14}-amino acid incorporated resulting from a forty-fold increase in the specific activity, as mentioned earlier, does indeed suggest that the free amino acid pool in these homogenates was quite large.

It is not being suggested that the observed difference in activities of unfertilized and fertilized homogenates might be solely due to large differences in pool size. The similarity of the kinetic curves as well as the recombination experiments of Hultin (28) give no such indication. On the other hand, until a uniform incorporation rate can be achieved for longer periods, it would appear that the in vitro incorporation experiments are primarily a measure of the amino acid incorporating capacity of the ribosomal complex, assuming that all other factors are equal. Hultin's experiments (28) did indeed indicate that conditions for amino acid incorporation were very similar in unfertilized and fertilized egg homogenates. In keeping with this observation it has been shown that amino acid activating enzymes were active in unfertilized egg homogenates (262), and that these enzymes as well as sRNA were in excess in both unfertilized and fertilized eggs (98). If it can be assumed that the hot acid soluble material from egg homogenates is C^{14}-amino acyl-sRNA (Fig. 13) then the results go one step further to indicate that the concentration of this complex is also very similar before and after fertilization.

Magnesium requirements

The role of magnesium in amino acid incorporating systems is very complex. It is essential to the functional integrity of the ribosomes (263-269) and necessary for the attachment to the ribosomes of both the sRNA (270, 271) and the mRNA (123, 271, 272).

As pointed out earlier even though the magnesium ion optima for both the unfertilized and fertilized homogenates are very similar for L. pictus and S. purpuratus eggs, there are indications of a slight difference. This shift can be interpreted most simply as indicating a difference in the concentration of available magnesium ions in unfertilized and fertilized eggs and may be either due to a loss of magnesium to the surrounding medium as suggested by Monroy-Oddo (206) or to an increase of the bound form within the cell. This latter possibility could be accounted for at least in part by the formation of polysomes which occurs upon fertilization.

In comparing the magnesium requirements for amino acid incorporation in cell-free systems of E. coli or rat liver with that of sea urchin eggs it appears that in general these systems have a higher optimum than the sea urchin eggs (253, 258, 273, 274). Even when taking into account the endogenous magnesium of the eggs which then gives an optimum of 0.005 molar, the value is still lower than that for the others. It is interesting to note that the addition of polyamines to cell-free systems generally lowers the magnesium requirement (273, 274). The addition of 0.001 molar spermine to the rat liver system, besides stimulating incorporation, shifts the optimum magnesium ion concentration

down to where it is very similar to the observed optimum for sea urchin egg homogenates. Thus a possible explanation for the low optimum observed in homogenates of these eggs is that they already contain polyamines. Though bacteria are known to contain polyamines (267), they have not been demonstrated in sea urchin eggs. The addition of spermine to homogenates of sea urchin eggs consistently causes an inhibition of incorporation as well as a slight shift in the magnesium optimum (53). Though several concentrations were used, the inhibition still may have been due to the use of supraoptimal amounts since the stimulatory effect in the rat liver system occurred at a concentration of added spermine lower than any tested in this series of experiments.

It should also be pointed out that in all of the magnesium ion requirement studies the measure of activity was the incorporation of C^{14}-valine into protein. Since there is no evidence to the contrary, the possibility remains that the optimum magnesium concentration might be different when other amino acids are used. If this were the case, it could be an explanation for some of the differences between what has been observed for incorporation by sea urchin egg homogenates and other systems.

Potassium requirements

The value of potassium ions to cell-free amino acid incorporating systems has been known for some time (204, 205, 275). Though changes in the concentration of this ion resulted in altering the sedimentation pattern of E. coli ribosomes (125, 272), it would appear to function primarily in aiding the attachment of the amino acyl-sRNA complexes to the ribosome (276, 277). These studies as well as the one by Lubin

(254) also showed that at least for the E. coli system the ammonium ion could be used in place of potassium often with an additional two- to three-fold increase in activity. Sodium and lithium were antagonistic to either of these ions, and it was shown that this antagonism was most pronounced if the lithium was added prior to the onset of incubation (254). Addition of the inhibitory ion only three minutes after the start of incubation had almost no effect upon the incorporating activity. Conjecturing from these findings it might be suggested that of the two sRNA molecules attached to actively incorporating ribosomes (278) the one to which the polypeptide is linked remains the same for the entire synthesis of the protein, and it is this association to the ribosome which is dependent upon potassium or ammonium ions. This may in part by why the ribosome-sRNA-polypeptide complex is resistant to dissociation by the removal of magnesium ions from the medium (279-281).

The optimum concentration of potassium or ammonium ions in homogenates of E. coli is 0.16 to 0.20 molar (276, 277). This value is surprisingly close to that reported here for sea urchin egg homogenates (0.18 molar). Whether or not this represents a similar function at the level of the amino acid incorporating system is not known but it is highly suggestive. The effects of ammonium ions upon incorporation in sea urchin egg homogenates have not been tested.

In concluding this portion of the discussion it must be pointed out that even though sea urchin egg homogenates showed a requirement for both magnesium and potassium for active amino acid incorporation, in no cases were the unfertilized egg homogenates stimulated to incorporate at a rate

comparable to the homogenates prepared from fertilized eggs. Thus it seems unlikely that changes in the concentration of either of these ions is directly responsible for the activation of the amino acid incorporating system upon fertilization. This is not to imply that either or both may not be important at some point in the activation procedure, only that they are apparently not solely responsible.

Incorporation by anucleate egg fragments

The activation of protein synthesis in parthenogenetic non-nucleate sea urchin egg fragments is conclusive evidence that the nucleus is not needed for such a phenomenon to take place. However, the relative amount of stimulation in these fragments was so much less than what was observed in fertilized whole eggs that it was at first thought that perhaps the two phenomena might be different. This now appears to be very unlikely in view of the comparable activities of fertilized fragments, the kinetic curves and the polysome forming abilities. Furthermore Tyler (289) has found that the pattern of incorporation of each of the twenty amino acids into fertilized whole eggs is very similar to the pattern obtained from artificially activated non-nucleate fragments. This not only indicates a similar uptake and pool composition, but also a similar utilization of the pool amino acids in both cases.

The effect of cold treatment upon L. pictus eggs is curious especially since the eggs and fragments of S. purpuratus do not seem to be similarly affected. Though no specific study has been given to this problem, the following suggestions are nevertheless put forward. The first is that

the increased activity is due to a parthenogenetic activation resulting from the relative shock of the treatment. Another is that cold treatment affects the plasma membrane in a way, possibly by the slowing down of transport reactions, that allows amino acids and other materials to leak from the cell. In this way the increase in labelled amino acid incorporation could reflect an increase in the specific activity of the endogenous pool rather than an increase in the absolute amount of incorporation. Another question which was not pursued further was why the incorporation activities of non-nucleate fragments of L. pictus eggs was greater than nucleate fragments. It might be suggested in this case that the inactive protein synthesizing machinery might be concentrated into the more dense region of the egg or that the pool might be displaced during the centrifugation procedure. Fragments of S. purpuratus do not show this pronounced difference, and it may well be because the entire egg is considerably more dense than that of L. pictus.

The kinetic studies while giving similar patterns for all of the eggs and fragments studied, have indicated certain differences which, however, appear to stem from the method of activation rather than the presence or absence of a nucleus. The first difference is that butyric acid treated eggs and fragments usually showed a more rapid rate of activation than did their fertilized counterparts. This may in part be explained if the difference between the two methods can be visualized as being that sperm activation is initiated at a single point while butyric acid activation probably initiates from points all over the surface of the egg. Thus one would expect that though the final rate of incorporation was very similar

in each, the activation which proceeded from several points reached this level sooner than the single point activation. The other difference is that the butyric acid treated eggs and fragments began to show a decrease in activity sometime later than two hours after activation. On the other hand, the fertilized samples continued to show a gradual increase. This difference may indicate a need for the nucleus after this time or it may merely be due to the fact that the parthenogenetic cells seldom divided, and that if they were given the additional treatment necessary to bring about division (139) they would then show this increase in activity as well.

The formation of polysomes in the parthenogenetic fragments ties the phenomenon of protein synthesis activation in fragments closer to that in fertilized whole eggs. Their formation in non-nucleate fragments would appear to give strength to the idea of a cytoplasmically localized activation mechanism in these eggs which has as its final step polysome formation. It is felt, however, that the importance of these findings should be qualified in view of a recent hypothesis with supporting evidence which has been put forth by Haselkorn and Fried (283, 284). Their hypothesis in the form of an equilibrium equation states that the mRNA-ribosome-sRNA complex is in a state of equilibrium with its components until polypeptide synthesis has begun; at which time it is stabilized. In addition to their evidence which comes largely from competence studies between different ribonucleic acids and synthetic polynucleotides, there is considerable additional support for the idea that the nascent polypeptide not only stabilizes the polysomal structure but also the structure of the

ribosomes themselves (279-281). If the above equilibrium hypothesis is correct, it stands to reason that polysomes which are unable to synthesize proteins could exist under certain conditions and that they very likely would not be detected by sucrose gradient centrifugation since the centrifugation from the presence of the uncomplexed members would shift the equilibrium drastically in that direction. Though there is no evidence to suspect that such a condition exists in the unfertilized egg, it remains a possibility which must be considered when viewing the significance of the phenomenon of polysome formation.

The nature and role of cytoplasmic DNA

In addition to having a counterpart in the cytoplasm of the amphibian egg (83), the evidence favoring the presence of DNA in the cytoplasm of the sea urchin egg was given strength by the recent reports of DNA existing in chloroplasts (185) and mitochondria (186, 187). Whether or not these DNAs play any role in the metabolism of cells is not yet known. In eggs its most likely role is that of a storage form of DNA precursors; however, the stimulation of protein synthesis in non-nucleate fragments by parthenogenetic activation possibly suggests a more active role of supplying mRNA to the protein synthesizing system. This possibility must first be considered in light of experiments with actinomycin D treated eggs (118) which, as mentioned earlier, continued to incorporate amino acid into protein at a rate comparable to the controls. Since, however, cytoplasmic and nuclear DNAs may not have the same properties as suggested by Durand (285) and Ficq (184), there is little by which

to judge the effectiveness of the antibiotic. On the other hand, it is well known that actinomycin D inhibits RNA synthesis templated by another special type of cytoplasmic DNA, namely that of the DNA viruses (286).

The inability to demonstrate consistently an increase in incorporation of uracil or uridine into RNA by the artificially activated fragments was not suggestive of an active role by the cytoplasmic DNA. However, since this increase was very difficult to show consistently even in the whole eggs, the results cannot be given a great deal of weight.

Though the idea of a cytoplasmic DNA which may be important in one capacity or another in early development has been explored, there are still strong reservations as to its actual existence at least in sea urchin eggs.

Probably the greatest hinderance to a reliable study of cytoplasmic DNA was that even though it was measurable, the amount was still a very small percent (ca. 0.005%) of the total dry mass of the egg cell; thus preventing its direct isolation. The specificities of the methods used for its detection were undoubtedly severely taxed by the rich variety of compounds within the egg itself. For instance, it is known that the Dische method persists in giving an aberrant color reaction on DNA extracts of unfertilized and fertilized sea urchin eggs throughout several cleavages even though extracts of later stage embryos and spermatozoa develop the usual color which absorbs at 595 mμ (190, 287). Since Elson and Chargaff (190) ran simultaneous microbiological assays

on these extracts and found only one-tenth the DNA that they found by the other method, it does not seem likely that the color development was specific for the DNA. The microbiological assay methods admit to a great deal more specificity than that of the color reactions; however, the possibility cannot be definitely ruled out that some hithertofore unknown or unsuspected active substitute or cofactor might be present to give misleading growth rates. In addition, misleading results could come from the presence of thymidine containing compounds within the egg (183, 288) that might possibly also exist in a polymeric form.

As mentioned previously, the early incorporation of labelled precursors into DNA indicates a small pool. This is also suggested from studies on fertilized sea urchin eggs using 5-fluorodeoxyuridine (FUDR), the inhibitor of thymidylate synthetase (289). Since this enzyme catalyses the conversion of deoxyuridylate to thymidylate, its inhibition effectively stops DNA synthesis in the absence of endogenous thymidine. Curiously enough, this analogue by itself stopped sea urchin development at the 8 to 16 cell stage (38, 39) whereas in the presence of exogenous thymidine the embryo could develop on to the blastula stage.

The amount of endogenous precursors theoretically necessary to allow the egg to go to the 8 to 16 cell stage coincided with the amounts of cytoplasmic DNA reported to be present (190, 191, 290). In agreement with this, incorporation studies using labelled glycine or acetate indicated that most of the DNA synthesized during the early cleavage stages came from an unlabelled source of precursors (291). Equally consistent, however, were the findings that the free thymidine pool

was also large enough to support DNA synthesis through the 8 to 16 cell stage (38, 292). It is also interesting that the egg contains a pool of "masked" deoxynucleotides such as deoxycytydine diphosphate choline which is 10 to 15 times larger than the thymidine pool (183, 288). On the basis of the FUDR studies it would appear, however, that this class of thymidine containing compounds is unavailable in the capacity of a DNA precursor at least during the early cleavage stages.

The enzyme for converting 5-methyl-5-deoxycytidylic acid (MedCMP) to thymidylic acid is present and active in the sea urchin egg (293); however, the substrate appears to be largely lacking since exogenous MedCMP can indeed reverse the effects of FUDR at the 16 cell stage (38). The indication is then that the eggs rely very heavily upon their endogenous thymidine pool and its replenishment by way of conversion of deoxyuridylate to thymidylate during the early stages of development. It is definitely not clear that cytoplasmic DNA is contributing anything to the pool; in fact the high degree of agreement in values for the pool nucleotides and for cytoplasmic DNA suggests that possibly they are the same and that DNA such as that found in the nucleus does not really exist in the cytoplasm of these eggs. To conclude this, however, would take more comprehensive studies than have been done so far.

An alternate interpretation of the increase in uridine incorporation upon fertilization

As mentioned earlier, the very existence of RNA synthesis in sea urchin eggs is still questioned; however, those that were able to demonstrate it also found that it increased after fertilization.

A point in question is whether or not this increased incorporation of precursors into RNA which seemed to be partly messenger actually represented an increased rate of synthesis of the RNA. Though the answer cannot be conclusively reached, there is evidence that it may not. Of the investigators that analysed the sedimentation patterns of fertilized egg RNA only Nemer (110) was able to effectively look at the pattern from unfertilized eggs. He found that it was identical to the pattern of labelled RNA from fertilized eggs in all ways except the amount of radioactivity present. This then indicates that the unfertilized egg is also participating in a small amount of RNA synthesis and that upon fertilization there is only a quantitative increase in incorporation. Thus, in addition to being interpreted as a true increase in the rate of synthesis, this increase in incorporation could easily be achieved if there was a decrease in size of the endogenous pool of RNA precursors or an increase in the rate of uptake of the labelled exogenous precursors. Concerning the first possibility nothing is known; however, an increase in the rate of uptake of labelled precursors upon fertilization was first noted by Nemer (38) and later confirmed by Piatigorsky and Denny (294). The characteristics of this increase resemble those of an active transport stimulated uptake: One-hour fertilized eggs showed as much as a fifteen-fold increase in the amount of precursor (uridine-2-C^{14}) transported into the eggs over that in unfertilized eggs (295). Assuming that the endogenous pool sizes in the unfertilized and newly fertilized eggs are equivalent, one could expect to find as much as a ten-fold increase in the amount of labelled precursor incorporated without any actual increase in the rate

of synthesis of RNA. The limits of this value very easily include the two- to three-fold increase in incorporation at one hour after fertilization reported by Gross, Spindel and Cousineau (111) and the increase of approximately three times for a 20-minute pulse and four-hour chase of newly fertilized eggs reported by Nemer (110).

The consistency of the above observations thus makes it very unlikely that there is any true change in the rate of RNA synthesis upon fertilization of the sea urchin egg and that what has been observed is a change in permeability to the precursors.

The RNA synthesis found in both the unfertilized and newly fertilized eggs may represent a turnover of nuclear RNA which is of little consequence to early development. Recently a report by Hultin (227) has come out which presents evidence in support of the above hypothesis. He showed that when unfertilized eggs were incubated in P^{32}-phosphate, a small amount of label was incorporated into RNA. Of this, 85% was associated with the nuclear fraction and only 2.5% with the RNA of the 12,000 X g supernatant. Fertilization of these eggs did not affect this ratio. Furthermore, the pattern was not any different in eggs which had been fertilized then incubated for 30 minutes in the label. It was also shown that the specific activities of polysomes were no different than the specific activities of the unfertilized egg ribosomes. In addition, from other reports, it appears highly unlikely that any significant amount of this nuclear RNA leaves the nucleus much before the blastula stage (106, 108, 296).

Mechanisms for the activation of the protein synthesizing system

Location of the "mask"

The stimulation of amino acid incorporation by artificial activation of non-nucleate fragments suggests that the unfertilized sea urchin egg cytoplasm contains all of the components necessary to undertake an active protein synthesis. This finding also implies that even though the unfertilized egg cytoplasm does have this potential, the amino acid incorporating system is somehow maintained in an inactive state until the proper stimulus is given. This inactive state seems likely to be due primarily to a lack of protein synthesizing units, the polysomes. The formation of polysomes upon fertilization might have easily been explained as a synthesis of new messenger RNA had it not been that actinomycin D had no effect upon protein synthesis (118) or that non-nucleate egg fragments were as active as whole eggs. Thus, on the basis of the poly U experiments many investigators have come to believe that the bulk of the ribosomes of the unfertilized egg are capable of attaching to messenger RNA and infer from this that there is a species of messenger in the cytoplasm which is "masked" in the sense that it does not let ribosomes attach. The evidence, however, does not yet exclude the possibility that the ribosomes may in themselves be unable to attach to the messenger. As suggested by Tyler (100) the extent of stimulation by poly U in both the unfertilized and fertilized homogenates may be misleading since the poly-nucleotide will theoretically be twenty times more selective for the incorporation of phenylalanine than will the endogenous mRNA. This observation took on more than theoretical significance

when experimental evidence indicated that approximately one-twentieth of the total amino acids being incorporated into fertilized sea urchin egg proteins by endogenous mRNA was phenylalanine (98). Thus it could be expected that if the poly U stimulated homogenates were synthesizing polyphenylalanine then a twenty-fold increase in incorporation would only represent a net polypeptide synthesis equal to that of the endogenous system. Since poly U has been shown to inhibit the incorporation of amino acids other than phenylalanine (124, 297) it would appear either to be able to replace endogenous mRNA from the actively incorporating complex or to effectively compete with it for the reattachment of active ribosomes. In support of this latter possibility it has recently been shown that poly U can compete with other messengers for ribosomes and in fact appears to be considerably more capable of doing so than either polycytidylic acid or TYMV-RNA (283, 284). This finding suggests that as long as there is a rate limiting number of ribosomes and poly U is in excess, one might expect to find an eventual replacement of nearly all of the endogenous messenger with poly U in the active complex. This, then, points out the possibility that unless a stimulation of more than twenty times is observed one may not be obtaining any net increase at all. Since most of the experiments reported thus far with unfertilized sea urchin egg homogenates have shown poly U or poly UC stimulations in the neighborhood of twenty times (97, 100) or less (101) very little can be concluded on this basis with respect to the bulk of the monoribosomes present. The outstanding deviations from this pattern are those experiments reported by Nemer and Bard (98). They show a

100-fold stimulation by poly U in the unfertilized homogenate and approximately a five-fold increase in endogenous incorporation upon fertilization. When the poly U stimulated activities were converted and compared to the endogenous activities they were equal, clearly indicating potentially active ribosomes and a lack of mRNA in the unfertilized eggs. These experiments still are subject to certain limitations in their interpretation. As shown earlier, there was a rather clear cut optimum concentration of magnesium required for amino acid incorporation by the endogenous system. When calculated to include the magnesium contained within the egg (214, 215), the optimum came to a value close to one-half that used in the Nemer and Bard experiments. Therefore, it is likely that their measurements of endogenous activity were made on inhibited homogenates, and it follows that if the magnesium optimum for poly U stimulation was either higher or broader than that of the endogenous system, misleading amounts of stimulation would result. Though this possibility is only hypothetical, it is instrumental in pointing out that before a completely valid comparison of increases by poly U stimulation and fertilization can be made, the comparative ionic requirements should be known. The need for additional caution along these lines is called for by the recent observations that changes in magnesium concentration could affect poly U by broadening its specificity to amino acids other than phenylalanine (298).

In keeping with some of the above observations the recent work of Maggio, Vittorelli, Rinaldi and Monroy (299) can be interpreted to suggest that both the mRNA and the ribosomes of unfertilized eggs are masked.

Phenol extracted RNA from unfertilized eggs stimulated rat liver ribosomes to incorporate a great deal more amino acid into protein that it did when added back to unfertilized egg ribosomes. Even though this latter combination was ten times more active than the unfertilized ribosomes by themselves, it was still at an activity level approximately one-tenth that of the mesenchyme blastula ribosomes. It is also interesting to note that the absolute amount of stimulation which was observed by addition of the extracted RNA to unfertilized egg and mesenchyme blastula ribosomes was very similar even though it represented a 1000% increase for the unfertilized egg ribosomes and only a 16% increase for the mesenchyme blastula ribosomes. This finding may be indicative of a very small population of available ribosomes in the later stages of development, possibly so low as to be rate limiting. Finally, it was found that RNA extracted from the microsomal pellet was much more active with the rat liver ribosomes than was the extracted total RNA. Until more extensive RNA fractionations are undertaken, it cannot be said that this represented the intracellular location of the masked mRNA since the small molecular weight RNA (sRNA) present in the total RNA extract may have obscured any chance for locating a non-microsomal mRNA. Unfortunately, the interpretation of any of the above experiments at face value may not be valid. This is due to the fact that there is not positive indication that there were equal amounts of ribosomes in samples that were compared. Even with this in mind, these experiments are felt to be sufficient evidence to warrant the continued study of both the messenger RNA and ribosomes as possible causes of the inactivity of the unfertilized egg.

Possible analogies

There are now several cases reported in which protein synthesis has been activated, apparently in the absence of a corresponding increase in messenger RNA synthesis. The tendency to draw analogies between these and what is observed in the sea urchin egg upon fertilization is very great. However, as each system is studied in more and more depth, it would appear that no one is truly analogous.

The regenerating rat liver has been shown to be considerably more active in protein synthesis than the normal liver (300). Though the increase in the activity of the regenerating liver may in part be due to a synthesis of messenger RNA, it has now been shown that the microsomal fraction of the normal liver contains a heat-labile, detergent dissociable inhibitor of protein synthesis which is apparently lost when the tissue undergoes regeneration (301). So far as is known, the inhibitor does not destroy messenger nor interfere with its attachment to the ribosomes. It is antagonized by guanosine triphosphate (GTP) which in this case is apparently not functioning as an energy source. This association of protein synthesis inactivity with the microsomes themselves is admittedly similar to that found in the unfertilized sea urchin egg. It differs, however, in that homogenates of the unfertilized eggs did not respond to GTP and they could not be activated by detergent treatment (28). Though this does not rule out the possibility of an inhibitor being present, it does suggest that it is not the same type as that found in normal liver microsomes.

The action of hormones on certain tissues is, among other things, that of stimulating protein synthesis. In the case of the thyroxine effect on rat tissue, this increase in protein synthesis appears to be the forerunner of the classical response, namely an increase in the basal metabolism rate (248, 249). The actual mechanism by which the thyroxine stimulates protein synthesis is, however, the subject of a fair amount of controversy. The effect was reported to be actinomycin D-sensitive and accompanied by an increase in RNA synthesis and RNA polymerase activity (302, 303). Thyroxine added to cell-free homogenates of rat liver also stimulates amino acid incorporation (304). However, this increase was dependent upon the presence of mitochondria and was not sensitive to the addition of actinomycin D or DNase. It could not be brought about by the addition of nuclear RNA polymerase. Furthermore, a poly U stimulation of phenylalanine incorporation in these homogenates was further enhanced when the hormone was added. These findings have led the latter authors (304) to propose that the thyroxine induced effect is basically an increase in ribosomal activity, while the former authors (303) suggested that its action is primarily that of derepressing DNA. Though there is no hope of conclusively resolving these differences as yet, they may in part be due to differences in experimental procedure. Tata and his coworkers (303) have for the most part studied the effect of thyroxine on thyroidectomized rats. It is not inconceivable that thyroid hormone deficiency might cause a general decrease in all cellular processes including RNA synthesis and that thyroxine treatment merely results in the restoration of all processes to normal levels. The

values for RNA polymerase activity under the different conditions does indeed suggest this possibility. Its activity in the thyroidectomized animals was six-tenths that of normal animals, and upon administration of exogenous hormone the final activity was only one-tenth higher than the normal controls (303). On the other hand, none of the animals used by Sokoloff and his coworkers (304) were thyroidectomized and thus possessed normal hormone levels at all times except when additional thyroxine was administered. In addition, they have based their studies primarily on the in vitro effects of the hormone. It thus appears possible that the two groups were studying two different phenomena and that their results were equally valid. Since the in vitro thyroxine effect (304) and a protein synthesis inhibitor (301) have been demonstrated at the ribosomal level in homogenates of rat liver, one wonders if both phenomena are related. Though speculation on this point is immaterial to this discussion, it appears that problems in protein synthesis regulation are rapidly coming to a place where the identification of specific products and their functions is a necessity.

Hormone treatment of ovariectomized rats showed that within four hours after the injection there were increases in RNA, nucleotides, protein and phospholipids in the uterine tissues (305). Careful time sequences and the use of actinomycin D seemed to indicate that the estrogen was capable of inducing protein synthesis without a corresponding RNA synthesis; leading the above author to suggest that RNA polymerase was the first thing to be synthesized. More recently it has been shown that the number of ribosomes also increased as a result of this treatment (306). This increase was

both actinomycin D and puromycin sensitive, again suggesting that protein synthesis is necessary initially. In fairness, it should be added that this same author and his coworkers now feel that the action of the hormone is that of derepressing DNA to allow RNA and consequently protein synthesis. This then sets the stage for more RNA and protein synthesis. The use of estrogen-deficient overiectomized rats in these experiments, however, again points to the possibility mentioned above that treatment of deficient animals may bring about widespread responses not because they are the specific actions of the hormone but because the entire tissue now returns to its normal levels of activity.

Cytoplasmic mechanisms for the initiation of protein synthesis

Since the studies of protein synthesis activation in sea urchin eggs seem to point toward the unmasking of messenger RNA or the activation of the ribosomes at fertilization, several conceivable mechanisms for accomplishing this purpose will be considered.

The conversion of natural messenger RNA from a form which does not accept ribosomes to a form which does has not yet been demonstrated in vitro. It has, however, been brought about in certain synthetic polynucleotides by treatment with formaldehyde (307). The most plausible explanation seems to be that the polynucleotide is prevented from attaching to ribosomes because of the configuration of its secondary structure and that it is this structure which is altered upon formaldehyde oxidation of the amino groups. The secondary structure of synthetic

polynucleotides has also been altered by changes in temperature and magnesium ion concentrations (298). In each case it was the least amount of stable configuration in the messenger which corresponded to the greatest amount of amino acid incorporation. In keeping with the above studies on formaldehyde treated messenger, these latter authors suggested that ordered secondary structure interferes with ribosomal attachment; resulting in reduced amino acid incorporation. Competence studies of ribosomal attachment to synthetic polynucleotides, TYMV-RNA and TMV-RNA, have indicated that the rate of ribosomal attachment to messenger RNA also depends largely upon its secondary structure (283, 284). Thus, one possibility might be that certain changes occur within the egg upon fertilization which tend to reduce the amount of ordered secondary structure of the messenger RNA. The likelihood of a temperature change occuring upon fertilization is remote, whereas as shown earlier, there is evidence of a change in magnesium ion concentration. There is, however, from these studies no indication that this change in any way affects the over all activity of the protein synthesizing system. A change in ionic strength would also be a possible mechanism for altering the amount of hydrogen bonding within RNA molecules. Though the potassium studies might have indicated such a mechanism if it existed, they cannot be thought of as being primarily studies of ionic strength largely because potassium very likely has a specific function in maintaining the integrity of the active complex (276, 277). Thus, very little can be said regarding this possibility.

Another suggested mechanism for the activation of protein synthesis in sea urchin eggs would be a change in the complexing ability of the messenger RNA or ribosomes. This property of nucleic acids appears to be influenced by the same factors which can bring about changes in the secondary structure, though often with opposing results when related to amino acid incorporation into protein (298). Not only does complexing ability apply to the stability of the active protein synthesizing complex but also to the stability of other complexes involving RNA and DNA. It may well be that complexing of this latter type is responsible for the masking of components necessary for protein synthesis. It is noteworthy that two of the most common inhibitors of protein and RNA synthesis; puromycin and actinomycin D, act by this very principle (225, 308). The existence of DNA-RNA and RNA-RNA complexes has been demonstrated numerous times under in vitro conditions (309-313). It seems likely that at least in some cases these in vitro findings are representative of conditions within the living organism.

In addition to the above known complexes, it has been shown that purines, pyrimidines and other physiological compounds can bind to nucleic acids and in doing so apparently affect the secondary structure (314, 315). Studies with poly U have shown that stable complexes can be formed with complementary polynucleotides which are no more than two residues long (316). Depending upon the magnesium ion concentration and ionic strength, either three stranded or two stranded complexes may be produced. It has also been demonstrated that even short adenylic acid polynucleotides which contain a few uridylic acid residues can be made to complex with

the poly U. With these findings in mind it may be suggested that the messenger RNA or even the ribosomes of the unfertilized egg are masked with small polynucleotides in areas of close complimentarity. Furthermore, if the messenger of these eggs has specific ribosomal attachment sites as has been suggested for other RNAs which act as messengers (284, 317) only these small portions would need to be complexed in order to mask the entire molecule. Masking of messenger RNA or ribosomes in this manner could well be the explanation for why mild alkaline degradation of TMV-RNA generates inhibitors of amino acid incorporation (284). A large percentage (up to 90%) of the ribosomes from E. coli have been shown to be unable to attach to added poly U or TYMV-RNA (123, 272). It is tempting to suggest that such ribosomes are indeed masked by small molecular weight polynucleotides especially since E. coli ribosomes which have been dissociated by dialysis against a low magnesium ion concentration and then reconstituted no longer show this high degree of inactivity (307).

There is also the possibility that the mRNA and the ribosomes contained in the cytoplasm of the unfertilized egg are both capable of combining and carrying out protein synthesis but are restricted from each other by some barrier. Though this possibility has not been investigated, it is known that there are particles and vesicles within cells which apparently rupture during the normal pattern of cellular events and release their contents either intra or extra-cellularly as the case may be (61). Citing as an example, the situation of corticle vesicle breakdown upon fertilization in sea urchin eggs,

Hultin (227) has pointed out that a mechanism for breaking down particles or vesicles which might contain mRNA is present in the sea urchin egg. However, the identity of such a particle, if it exists, is as yet unknown.

It has recently been suggested by Glišin and Glišin (318) that the mechanism of activation of protein synthesis in sea urchin eggs at fertilization might be the addition of the three terminal nucleotide residues (-cytidylic acid -cytidylic acid -adenylic acid) to the sRNA. Though this hypothesis is based largely upon their experimental evidence, it is difficult to see how a mechanism such as this could be operating, in view of the results of the homogenate fractionation studies mentioned earlier (28).

The histones are another class of compounds which at this point could possibly be acting as inactivators of protein synthesis in unfertilized sea urchin eggs. They are known to be potent inhibitors of DNA-primed RNA synthesis (319), by virtue of their ability to form stable complexes with DNA. In addition to being found in nuclei where one might suppose that they could perform in a regulatory capacity, histones have also been identified in association with ribosomes (cf. 320). Some indication of their function in the cytoplasm may come from the finding that histone added to a cell-free protein synthesizing system which was not DNA-dependent greatly inhibited the incorporation of amino acids (321). Such a mechanism has not yet been demonstrated in vivo. Furthermore, it is not known that histones exist in sea urchin eggs. However, they have been demonstrated in the cytoplasm of amphibian oocytes and have been shown to decrease as development progresses (322).

SUMMARY AND CONCLUSIONS

The amount of stimulation, kinetics of activation and ability to form polysomes is essentially the same for parthenogenetically activated and fertilized non-nucleate and nucleate fragments and whole eggs. Thus it seems likely that the phenomenon which is taking place in the parthenogenetic non-nucleate fragments is identical to that which occurs at fertilization in whole eggs. It may be concluded from these studies that the activation which takes place at fertilization is not dependent upon the production of nuclear mRNA. It is also felt that the production of a cytoplasmic mRNA is very unlikely especially since the existence of cytoplasmic DNA is at present on a questionable basis.

Taking all of the studies on protein synthesis activation in sea urchin eggs into account, it seems that the most likely explanation is that there is cytoplasmic masked mRNA which is unmasked at fertilization. However, this does not yet preclude the possibility that the ribosomes of the unfertilized egg are also inhibited. Concerning this masked condition, very little can be said except that from the potassium and magnesium studies it appears that unmasking at fertilization does not simply involve a shift in the concentration of either of these ions.

APPENDIX

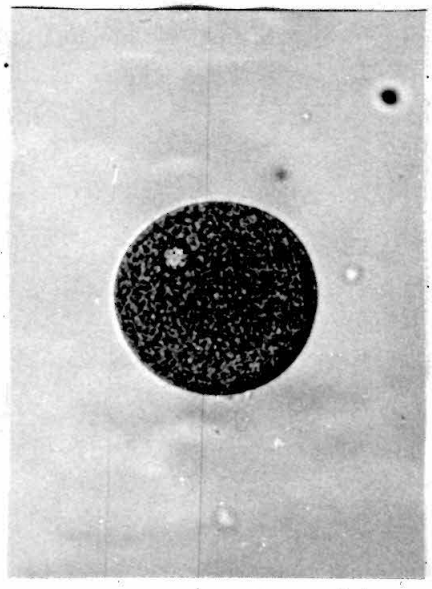

Fig. 22.-Unfertilized egg. Mag. 250 X.

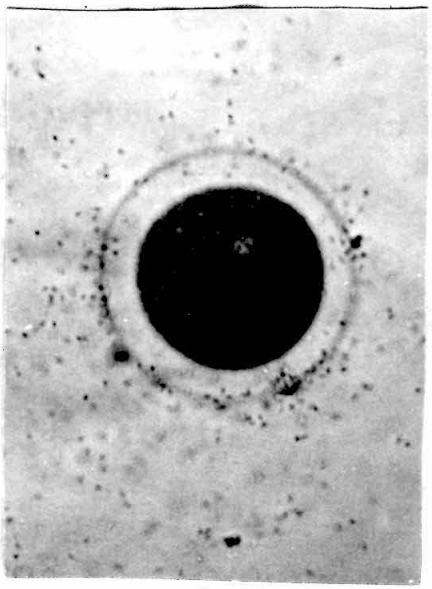

Fig. 23.-Fertilized egg at 5 minutes after fertilization. Note fertilization membrane and surrounding spermatozoa. Mag. 250 X.

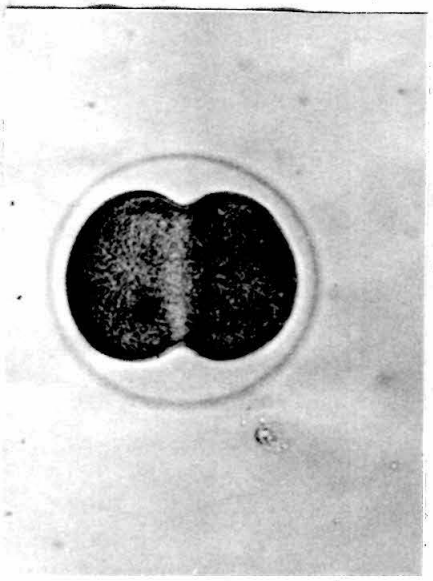

Fig. 24.-Fertilized egg in first cleavage at about one hour after fertilization at 21.5°C. Mag. 250 X.

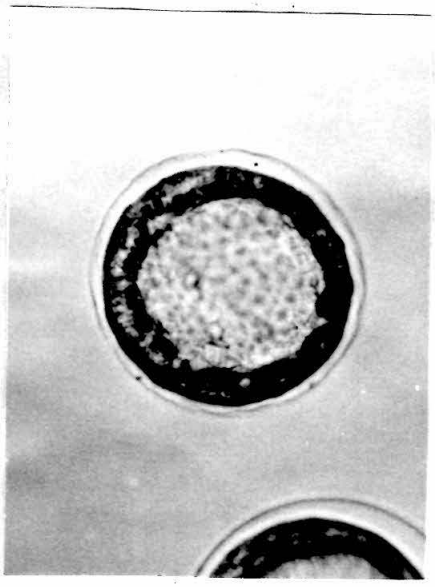

Fig. 25.-Blastula immediately prior to hatching, after about 10 hours of development at 21.5°C. Note blastocoel. Total cell number approximately 1000 (24). Mag. 250 X.

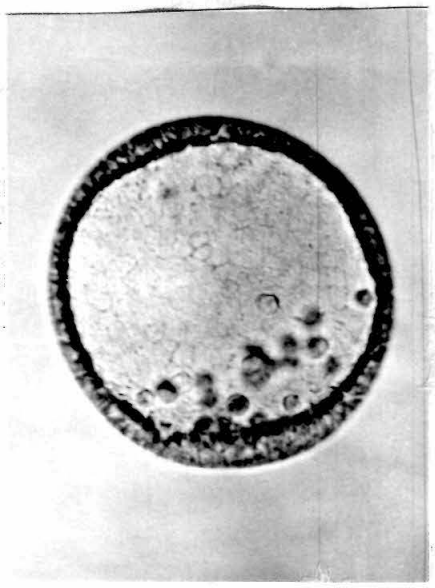

Fig. 26.-Mesenchyme blastula after about 13 hours of development at 21.5°C. Note primary mesenchyme cells which are in the process of migrating up the inside of the blastocoel wall. Mag. 325 X.

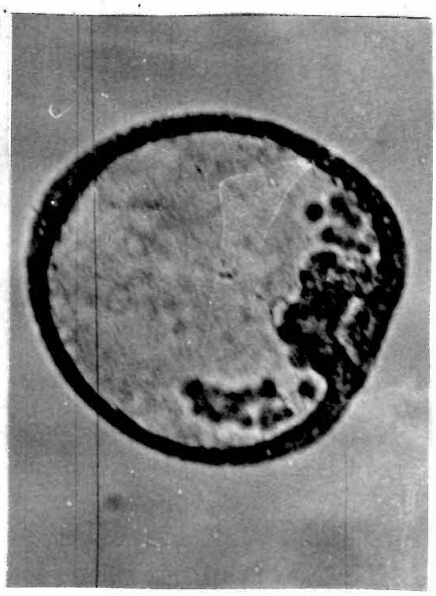

Fig. 27.-Early gastrula after about 17 hours of development at 21.5°C. Note clumps of primary mesenchyme cells and invagination of the forming gut. Mag. 325 X.

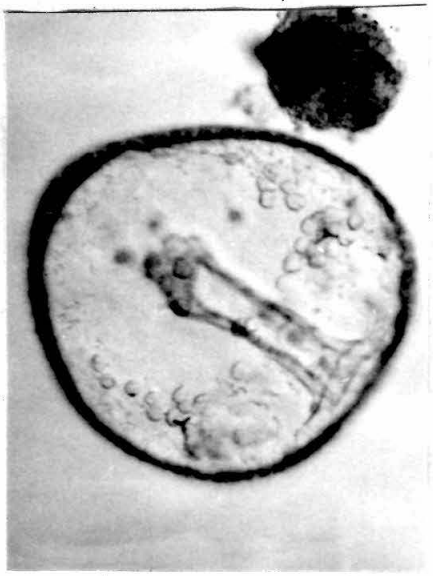

Fig. 28.-Mid gastrula after about 20 hours of development at 21.5°C. Note gut and large mesenchyme cells. Small tri-radiate spicules are the beginnings of the skeleton. Mag. 325 X.

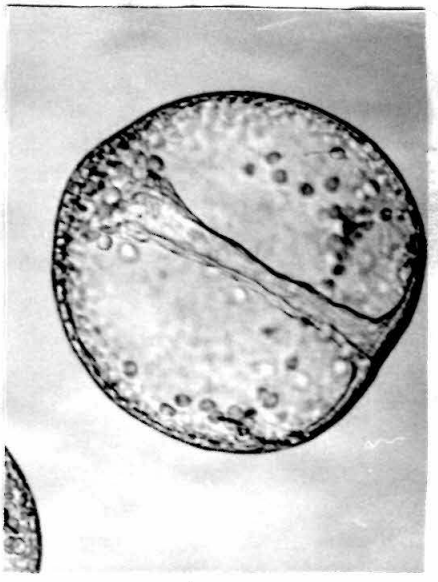

Fig. 29.-Completed gastrula after about 24 hours of development at 21.5°C. Note gut, mesenchyme cells and spicules. Mag. 325 X.

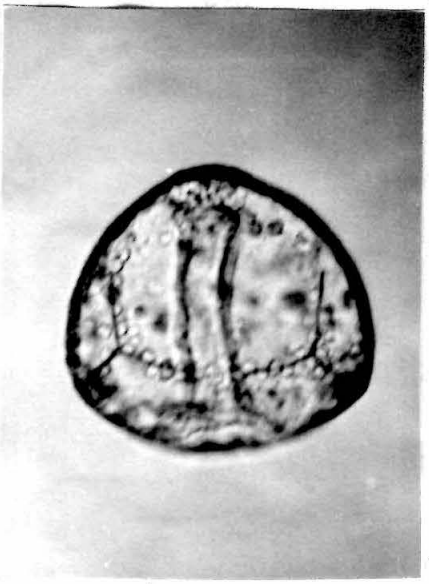

Fig. 30.-Late gastrula to early prism after about 30 hours of development at 21.5°C. Note thickening of gut and enlarged spicules. Mag. 275 X.

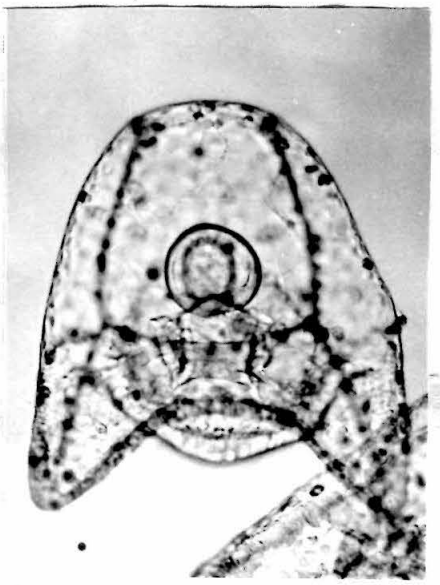

Fig. 31.-Early pluteus larva after about 40 hours of development at 21.5°C. Note skeletal arrangement and circle in center which is the outline of the stomach. Total cell number 2000-3000 (24). Mag. 325 X.

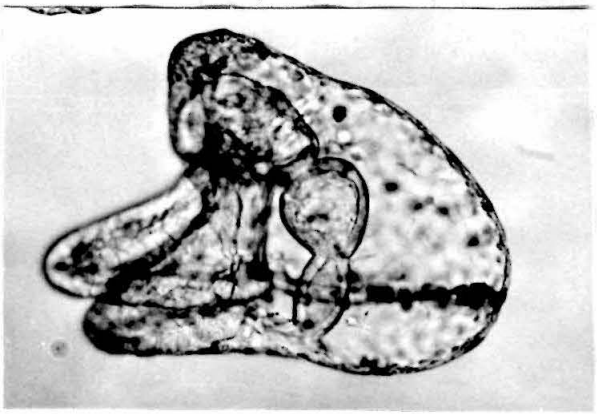

Fig. 32.-Early pluteus larva (side view) at the same stage as above. Note the gut regions (from top to bottom), esophagus, stomach and intestine. Mag. 325 X.

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