STUDIES ON NUCLEOSIDE AND AMINO ACID UPTAKE AND ON RNA AND PROTEIN SYNTHESIS BY GROWING OOCYTES, UNFERTILIZED AND FERTILIZED SEA URCHIN EGGS

Thesis by

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To Father

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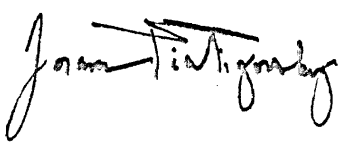

ABSTRACT

The principal theme of these investigations concerns the inhibited state of mature unfertilized sea urchin eggs with respect to uridine uptake and protein synthesis.

Part I demonstrates that unfertilized eggs are relatively impermeable to uridine. Fertilized eggs, however, develop during the first hour an energy-dependent, uptake mechanism for uridine accumulation. Labeled uridine assimilated by fertilized eggs is recovered as phosphorylated nucleosides, primarily triphosphates. Experiments support the idea that uridine penetration into sea urchin eggs depends upon the phosphorylation of the 5' carbon atom at the cell surface. Tests with puromycin show that protein synthesis is unnecessary for the generation of uridine uptake after fertilization. The evidence favors the view that uridine kinase is sequestered within the unfertilized egg and thus incapable of activity at the cell surface until after fertilization.

Parts II and III use biochemical and autoradiographic methods to show that growing oocytes of sea urchins, in contrast to many other organisms, undergo considerable RNA and protein synthesis. Protein synthesis in isolated oocytes occurs throughout the germinal vesicle and cytoplasm and takes place on polyribosomes. RNA synthesis is localized in the nucleolus. Mature eggs, however, synthesize only little protein even in mixed suspensions with oocytes. Long-term maintenance of spawned female sea urchins, after but one injection of labeled uridine, produces ripe unfertilized eggs possessing highly radioactive RNA. The distribution of label in the extracted RNAs is 70-80% ribosomal, 10-20% heterogeneous, and 5-10% soluble.

Part IV is an electron microscopic and biochemical examination of RNA-labeled mature unfertilized and fertilized eggs. The findings are correlated with the difference in protein synthesizing activity before and after fertilization. The results show that unfertilized eggs synthesize protein upon RNase-sensitive polyribosomes. The large increase in protein synthesis after fertilization occurs in association with the assembly of additional polyribosomes. Homogenates of unfertilized eggs also possess synthetically inactive, RNase-resistant, ribosomal aggregates. Evidence suggests that trypsin followed by RNase disperses the aggregates. Homogenates of fertilized eggs, however, contain very few RNase-resistant ribosomal aggregates. By forty minutes after fertilization, about 70% of the new protein synthesis can be attributed to the new polyribosomes. The weight of the evidence indicates that the remaining 30% of the stimulation of protein synthesis is due to the activation of "masked" polyribosomes.

Appendix 1 shows that, for unfertilized and fertilized eggs, competition for uptake of amino acids occurs primarily among those belonging to the same charge group. Appendix 2 demonstrates that one amino acid can displace another of the same category from intact eggs both before and after fertilization. By combination of these facts, then, it is possible to achieve greater labeling of egg-proteins than has been previously realized.

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GENERAL INTRODUCTION

The central theme of the present thesis concerns the dormancy of unfertilized sea urchin eggs. The principal aim of these investigations is the eventual understanding of metabolic repression at a cellular level. It is hoped that the development of this knowledge will contribute to greater control of biological processes.

A study of unfertilized eggs presents an especially interesting and unusual situation. On the one hand, the extreme suppression of mature unfertilized sea urchin eggs strikingly emphasizes metabolic inhibition and, in this respect, outlines the problems in question. On the other hand, one is faced, by necessity, with the paradox of performing measurements on an arrested system. Experiments presented in this thesis, however, show that some systems are considerably more inhibited than others and, in addition, that all processes studied operate to some extent before fertilization. Thus, it appears as if unfertilized eggs differ from fertilized eggs more quantitatively than qualitatively. The great stimulation in biochemical events that follows fertilization, then, has its most profound significance on developmental rather than metabolic changes.

The investigations presented in this thesis have explored unfertilized eggs by taking advantage of the cellular activity before maturation and after fertilization. Changes occurring during maturation of oocytes and after fertilization of ripe eggs have been followed and the results interpreted to relate to the suppression of unfertilized eggs.

The experiments are in agreement with those of many other investigators cited below; namely, unfertilized eggs possess preformed machinery held in rein until after fertilization. The present thesis demonstrates that the uptake of uridine by sea urchin eggs depends on the activity of uridine kinase on the surface of the cell but that this system is "masked" before fertilization.

Evidence is also provided indicating the existence of "masked" polyribosomes in unfertilized eggs. It is suggested that these represent the suppression of polyribosomes shown to be active in growing oocytes. The stimulation of protein synthesis after fertilization, however, depends primarily on the assembly of additional polyribosomes which form within minutes following insemination. From the studies of others (see below) it is apparent that the new polyribosomes result from the release of "masked" messenger RNA. Evidently, then, metabolic suppression of maturing oocytes is a complex process affecting separate sites within the egg. It might be of significance for future control of cellular processes that such events as nucleoside uptake and protein synthesis may be regulated at the level of fully developed systems.

1) Early evidence contributing to the concept of extra-nuclear control of early embryogenesis

In the fourth quarter of the nineteenth century the morphology of fertilization among animals was first accurately described and correctly interpreted. In 1875, Van Beneden, investigating fertilization of the rabbit egg, concluded that ". . . the first nucleus of the embryo would

be the result of union of male and female elements." He conservatively added, however, "I put forth this latter idea simply as a hypothesis, an interpretation which may or may not be accepted." (see Lillie, 1919).

It was accepted. The classical studies of Hertwig (1875; 1876; 1877; 1878) and Fol (1876; 1877; 1879) on fertilization in sea urchins (see Lillie, 1919; Wilson, 1925; Morgan, 1927 for reviews and all references prior to 1901) first established with demonstrative evidence that the egg-pronucleus and the sperm-pronucleus fuse to form the zygote nucleus. During this time, Schmitz (see Wilson, 1925, p. 9) described sperm-penetration in eggs of lower plants. Once the pronuclei have fused, cleavage and development follow.

With respect to cleavage, Boveri (1887; 1888) was the first to observe, while studying fertilization of the nematode Ascaris, that the fertilizing spermatazoon introduces into the egg a centriole which is utilized for mitosis. Wilson (1895) reinforced the observations of Boveri by stressing the spermatic origin of the cleavage centriole in his classic treatise An Atlas of the Fertilization and Karyokinesis of the Ovum. Boveri's discovery had the important impact of implying that the unfertilized egg lacked sufficient materials necessary for cleavage. But, the demonstrations of artificial parthenogenesis, which were soon to follow, dispelled the necessity of the sperm-derived centriole for the initiation of development.

Reviews and analyses of artificial parthenogenesis are given by Loeb (1913), Morgan (1927) and Tyler (1941). Apart from the phenomenon of natural parthenogenesis, experiments on eggs of annelids (Mead, 1896;

1897) and sea urchins (Hertwig, 1896; Morgan, 1896) provided the first demonstrations that spermatozoa were unnecessary for activation of the unfertilized egg. By 1900, Morgan had shown that sea urchin eggs could develop to the blastula stage if unfertilized eggs were temporarily exposed to an excess of magnesium chloride. By slightly modifying the conditions of the preliminary ionic treatment of unfertilized eggs, Loeb (1900) was able to raise sea urchin eggs parthenogenetically to the pluteus larval stage. A few years later Delage (1909) reported that two parthenogenetic larvae of sea urchins were cultured to sexual maturity after sixteen months of development. Subsequently, Loeb and Bancroft (1913) reported the metamorphasis and sexual maturation of a parthenogenetic frog. The sea urchins were males and the frog a female. It was recognized, then, that unfertilized eggs possess a complete intrinsic potential for development and differentiation.

Experiments by Delage (1899), showed that non-nucleate egg fragments of sea urchins, snails and polychaet worms, obtained by cutting unfertilized eggs, could be fertilized. Aster formation, cytoplasmic cleavage and development followed. It was thus established that the maternal nucleus is unnecessary for activation or early development.

It was not until 1901, however, that direct evidence was provided showing that the initiation of nuclear activity is not essential for parthenogenetic activation. Wilson (1901) produced anucleate fragments of unfertilized sea urchin eggs by vigorously shaking suspensions. Subsequent treatment of non-nucleate fragments thus obtained with magnesium chloride induced cytasters which were capable of division. Division of the aster, however, was not accompanied by any sign of cytoplasmic cleavage.

Since this method of enucleation occassionally disrupted intact nuclei, these experiments were more of an indication than a proof that nuclear material did not participate in the production of cytasters or in their subsequent cleavage.

Petrunkewitsch (1904) repeated experiments of this type on non-nucleate fragments of sea urchins and compared the effects of enucleation by shaking with that by cutting with a scalpel. Artificial activation with magnesium chloride was less successful than in Wilson's experiments. Nevertheless, some cytasters were seen in both preparations of activated fragments. The parthenogenetic merogones, however, did not divide. In the following year, Yatsu (1905) also cut fragments of unfertilized eggs of nemertean worms from their respective nuclei and showed that treatment with calcium chloride was followed by the appearance of cytasters in the activated fragment. The cytasters of the enucleated fragments possessed centrioles similar to those of whole eggs after parthenogenetic activation.

McClendon (1908) was the first to show that nuclei were unnecessary for the occurrence of cytoplasmic cleavage. He removed with a capillary pipette the nucleus of unfertilized starfish eggs, after emission of the first or second polar body, immersed the fragments in carbonated sea water for five minutes and then placed the non-nucleate cells in sea water. They divided irregularly into many smaller cells, each of which contained cytasters but lacked nuclei. A number of years later, Fry (1925) dissected nuclei with a glass needle from unfertilized eggs of sand dollars. A detailed study demonstrated that the nuclear membrane was not damaged

by the enucleation and that, therefore, one could be confident that the corresponding non-nucleate fragments were free of any nuclear contamination. Artificial activation of these fragments with butyric acid resulted in cytaster formation and, in eleven percent of the cases, cytoplasmic cleavage.

Nevertheless, Fry observed only sporadic and irregular cleavages. A few years later, Harvey (1932; 1936; 1940; see 1956) developed an effective method of sucrose density-gradient centrifugation for separating non-nucleate from nucleate fragments of unfertilized sea urchin eggs. She convincingly established that non-nucleate egg-fragments activated with hypertonicity may undergo cleavage and, in addition form blastulae, although often atypical in appearance. We are led then, to the unexpected conclusion of considerable significance that activation of the egg and early cleavage of the embryo do not depend on the immediate activity of the nucleus.

It is indeed impressive that Morgan, already in 1897 (T. H. Morgan, The Development of the Frog's Egg. The MacMillan Co., New York, N.Y. (1897), p. 131.) had the insight to interpret experiments on the development of egg-fragments and isolated blastomeres by writing, "There seems, therefore, no escape from the conclusion that in the protoplasm and not in the nucleus lies the differentiation power of the early stages of development." (Emphasis placed by Morgan.)

2) Evidence indicating a cytoplasmic regulation of messenger ribonucleic acid (RNA) activity upon fertilization.

More recent studies of fertilization of sea urchin eggs have provided a basis for understanding the nuclear and cytoplasmic factors influencing development. Experiments demonstrating a stimulation of protein synthesis after fertilization (Hultin, 1950; 1952; Hultin and Wessel, 1952; Hoberman, Metz and Graff, 1952; Nakano and Monroy, 1958; Nakano, Giudice and Monroy, 1958; Hultin and Bergstrand, 1960; Hultin, 1961; see Tyler, 1963; 1965a; Gross, 1964; Monroy and Maggio, 1964; Monroy, 1965a; 1965b; Grant, 1965; Tyler and Tyler, 1966b) were among the first to indicate the biochemical events underlying early development. Other experiments, described below, extended the tests to show that new protein synthesis after fertilization is initiated by cytoplasmic rather than by nuclear activity.

Briefly, chemical "enucleation" with dactinomycin (actinomycin D) (Gross and Cousineau, 1963; 1964; Gross, Malkin and Moyer, 1964) or physical enucleation by sucrose density-gradient centrifugation (Tyler, 1963, Brachet, Ficq and Tencer, 1963; Denny and Tyler, 1964; Baltus, Quertier, Ficq and Brachet, 1965; Tyler, 1966) does not prevent the stimulation of the incorporation of radioactively labeled amino acids into protein after fertilization or artificial activation. Furthermore, experiments in which fertilized eggs are made to develop in the presence of dactinomycin show that the pattern of newly synthesized soluble proteins during the early cleavage as well as later stages, obtained by disc gel electrophoresis (Spiegel, Ozaki and Tyler, 1965; Terman and Gross, 1965)

or by ion-exchange column chromatography (Ellis, Jr., 1966) is very similar to the pattern obtained by analysis of the control embryos. Moreover, fertilized, dactinomycin-treated eggs (Malkin, Gross and Romanoff, 1964) and artificially activated non-nucleate fragments (Burny, Marbiax, Quertier and Brachet, 1965; Denny, 1966) form active polyribosomes as do normally fertilized eggs (Monroy and Tyler, 1963; Stafford, Sofer and Iverson, 1964; Hultin, 1964).

Direct tests for template active RNA have shown that RNA extracted from unfertilized eggs of sea urchins stimulates the incorporation of labeled amino acids into acid precipitable material when tested in a cell-free system prepared from rat liver (Maggio, Vittorelli, Rinaldi and Monroy, 1964) and Escherichia coli (Slater and Spiegelman, 1966b). Additional evidence that unfertilized eggs contain significant quantities of messenger RNA is provided by other studies of RNA of mature eggs and embryos. Gross, Malkin and Hubbard (1965) labeled in vivo unfertilized eggs with RNA precursors during the final week of maturation and showed that labeled RNA elutes from a column of methylated albumin at an ionic strength higher than that sufficient to elute ribosomal RNA. This is consistent with the behavior of messenger RNA of other animals cells (Belitsina, Gavrilova, Ajtkhozin, Neyfakh and Spirin, 1964; Yoshikawa, Fukada and Kawade, 1964). It was also found that the labeled RNA from these eggs hybridized to homologous DNA in a manner not affected by a large excess of non-radioactive ribosomal RNA. Whiteley, McCarthy and Whiteley (1966) and Glisin, Glisin and Doty (1966) have also shown that RNA extracted from unfertilized eggs competes effectively with rapidly

labeled RNA, synthesized in the later stages of development, for hybridization to homologous DNA. The evidence obtained by a variety of methods, then, indicates that unfertilized eggs of sea urchins possess readily detectable messenger RNA which is presumably present in appreciable quantities.

A relevant and important criticism to the conclusion that the utilization of messenger RNA by the unfertilized egg is specifically blocked at one or more sites in the sequence of steps of protein synthesis is that respiration, and, therefore, metabolism in general, is strongly suppressed in sea urchin eggs until after fertilization (Warburg, 1908; Tyler and Humason, 1937; Brock, Druckrey and Herken, 1938; Laser and Rothschild, 1939; Ballantine, 1940; Borei, 1948; Yasumasu and Nakano, 1963; Ohnishi and Sugiyama, 1963; Epel, 1964; see Rothschild, 1956; Monroy, 1965a; 1965b, for reviews). One might thus attribute suppressed protein synthesis by unfertilized eggs to an insufficient production of chemical energy such as adenosine triphosphate (ATP).

Although the regulation of messenger RNA activity in unfertilized eggs is still unclear, there is evidence favoring the hypothesis that the supression of protein synthesis is a specific and not a general metabolic block. First, unfertilized eggs of sea urchins possess large quantities of ATP (Whiteley, 1949) which decrease after fertilization (Rossi, Aiello and Scarano in Monroy, 1965a). Then, the joint addition of ATP and phosphoenolpyruvate to homogenates of unfertilized and fertilized sea urchin eggs does not affect the difference in the incorporation of labeled amino acids into protein (Hultin and Bergstrand, 1960; Hultin, 1961;

1964; Denny and Tyler, 1964; Denny, 1966; Candelas and Iverson, 1966). It is not the absence of ATP, then, that limits protein synthesis in unfertilized eggs.

More cogent information that general metabolic inertia of unfertilized eggs is not restricting protein synthesis comes from studies with the synthetic messenger RNA, polyuridylic acid. Polyuridylic acid stimulates homogenates of unfertilized eggs to incorporate C^{14}-phenylalanine into acid precipitable material as effectively as it does the corresponding homogenates of fertilized eggs and later embryos (Tyler, 1962; 1963; Nemer, 1962, Wilt and Hultin, 1962; Nemer and Bard, 1963). Furthermore, the post-microsomal supernatant fraction of homogenates of unfertilized eggs supports the incorporation of labeled amino acids into protein when supplemented with microsomes of fertilized or artificially activated eggs (Hultin, 1961). The demonstration that the activity of amino acid-activating enzymes remains constant after fertilization (Scarano and Maggio, 1957; Maggio and Catalano, 1963) is consistent with these observations and, in addition, suggests that amino acid incorporation by homogenates of unfertilized eggs is blocked at one of the later steps of protein synthesis. This is reinforced by the observation that C^{14}-amino acyl RNA is incorporated into protein by homogenates of fertilized but not of unfertilized eggs (Yasumasu and Koshihara, 1963; in Japanese; reference Monroy, 1965a).

In summary, then, unfertilized eggs are capable of incorporating amino acids into protein, but are apparently largely and specifically suppressed at one or more sites in the chain of events of protein synthesis.

The experiments with dactinomycin and artificially activated non-nucleate fragments, to which reference has been made earlier, show that the block to protein synthesis in the unfertilized egg is extra-nuclear. In addition the stimulation of protein synthesis after fertilization is not dependent upon DNA-primed RNA synthesis. One possibility receiving considerable support (Tyler, 1963, 1965a; Gross and Cousineau 1963, 1964; Wilt, 1966) is that messenger RNA is "masked" in the cytoplasm of the unfertilized egg. Thus, the significant advance in our knowledge since Morgan's statement of 1897, cited above, is that the "differentiating power" which lies "in the protoplasm" is most likely RNA.

3) Organization of thesis

During an investigation of the increase in the incorporation of labeled amino acids into protein by artificially activated non-nucleate egg fragments, Paul Denny, then a graduate student, and I were unable to demonstrate any significant incorporation of C^{14}-uridine into RNA by unfertilized, fertilized or parthenogenetically activated sea urchin eggs. We considered it possible, however, that the unfertilized or just-fertilized egg is relatively impermeable to uridine rather than not synthesizing RNA. It was already known at that time that unfertilized sea urchin eggs are largely impermeable to phosphate (Whiteley, 1949; Litchfield and Whiteley, 1959; Whiteley and Chambers, 1961). It was considered of interest, then, to explore the extent to which uridine penetrates the unfertilized egg and to investigate possible permeability changes that occur after fertilization.

A preliminary experiment indicated that, in fact, unfertilized eggs are only slightly permeable to uridine. One hour after fertilization, on the other hand, labeled uridine readily penetrates the eggs and is concentrated by them. Intrigued by the large and unmistakable difference between unfertilized and fertilized eggs with respect to uridine uptake, I went during the summer of 1964 to the Friday Harbor Laboratories of the University of Washington to study this phenomenon more extensively under the guidance of Dr. Arthur Whiteley. The results of this exploration are presented in Part I of this thesis.

Due to the relative impermeability of unfertilized and just fertilized-eggs to RNA precursors, and due to the accumulated evidence (see above) favoring the presence of "masked" messenger RNA, I resorted to labeling the RNA of unfertilized eggs during oogenesis. Thus the nature of the "masked" messenger RNA and the changes occurring after fertilization which may be correlated with the stimulation of protein synthesis could be advantageously explored. Parts II and III, then, present studies on oocyte biosynthesis, both in vitro and in vivo, respectively. The in vivo investigations demonstrate that long-term labeling of spawned sea urchins, after an initial injection of radioactive uridine, effectively yields large batches of mature unfertilized eggs possessing highly labeled RNA. The in vitro experiments show, by scintillation counting and autoradiographic methods, that intact isolated oocytes undergo considerable intrinsic protein and RNA synthesis. The RNA labeled in vivo, therefore, can be assumed to have been synthesized primarily by the oocytes themselves during the course of maturation.

Part IV of this thesis presents studies conducted with the unfertilized eggs labeled in vivo during oogenesis. The types of RNA's synthesized by the oocytes and the subsequent compartmentalization of the labeled RNA in the mature egg has been examined, the formation of polyribosomes after fertilization quantitated and their protein-synthesizing capacity determined. Parallel investigations utilizing electron microscopy supplement and extend the results obtained by the biochemical procedures.

Appendix 1 and 2 of this thesis are additional explorations which are apart from the principal theme of metabolic suppression of unfertilized eggs. The two appendices present a study of amino acid uptake and displacement in unfertilized and fertilized sea urchin eggs. These experiments are a digression stimulated by observations made in the course of the tests presented in the four main parts of the thesis. They have been included primarily on account of the methodological role they played in the successful accomplishment of several experiments of Part IV.

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PART I

Section 1

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A CHANGE IN PERMEABILITY AND UPTAKE OF [^{14}C]URIDINE IN RESPONSE TO FERTILIZATION IN *STRONGYLOCENTROTUS PURPURATUS* EGGS

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SUMMARY

1. The extent to which the unfertilized *Strongylocentrotus purpuratus* egg is permeable to uridine has been investigated by experiments measuring the partitioning of [^{14}C]uridine between the inside and outside of the cell while the eggs remained suspended in the radioactive uridine and after the eggs were washed with ice-cold sea water. It was found that very little uridine entered the unfertilized egg. After fertilization, the label became concentrated in the egg and depleted from the environment. The label was not lost from the eggs by washing with sea water.

2. Uridine uptake after fertilization has been in part characterized by its time course, dependence on exogenous uridine concentration and response to 2,4-dinitrophenol. A lag phase, acceleration phase and constant accumulation phase was found in the rate of uridine uptake which reached a plateau within the first hour of development. Once the maximal rate of accumulation was attained, fertilized eggs were shown to be very efficient in concentrating very small amounts of exogenous [^{14}C]uridine. As the concentration of uridine was raised beyond 0.5–1.0 μM in the sea water, there was no further increase in the rate of uptake. 2,4-Dinitrophenol-inhibited uridine uptake. At $-3°$ only a very small amount of uridine penetrated into the eggs, and they did not reach equilibrium with the outside concentration, indicating penetration involves more than diffusion.

3. Most of the radioactivity taken up by the fertilized egg remained soluble in ice-cold 0.2 M perchloric acid. No appreciable free [^{14}C]uridine was detected inside the fertilized eggs. The soluble radioactivity was identified on the basis of anion-exchange column chromatography to be in the form of phosphorylated nucleosides, primarily triphosphates.

4. The observations are believed to indicate that fertilized eggs accumulate uridine by phosphorylating the nucleoside at the surface of the cell, a process which may be deficient in unfertilized eggs because of lack of phosphate donors or of the necessary enzymes.

INTRODUCTION

One of the problems of early development concerns the synthesis of new RNA. It is now known from experiments with actinomycin D (refs. 1,2), and non-nucleated egg fragments[3–5] that the protein-synthesizing activity that occurs upon fertilization and early development is mostly independent of the activity of the nucleus. Therefore, new RNA synthesis is not necessary for the initiation of protein synthesis. However, there is also evidence that some new RNA is synthesized within 30 min after fertilization in sea urchins[6–8] and by the four-cell stage in amphibians[9] (see GROSS[10] for review).

Investigation of the time and extent of such RNA synthesis in intact eggs is complicated by possible changes in permeability of the egg to the labeled precursors. Preliminary tests[11] with [^{14}C]uridine showed that the unfertilized sea-urchin egg was relatively impermeable to this substance and that upon fertilization there was a great increase in uptake, in fact to the extent that the surrounding fluid could be considerably depleted of this material. This suggested the possibility of an active transport mechanism such as has been described for many other systems and has been studied extensively by one of us[12] for phosphate uptake in sea-urchin eggs. In this communication, the change in permeability to uridine upon fertilization in sea-urchin eggs has been explored with attention given to the question of the possible occurrence of an active transport mechanism.

MATERIALS AND METHODS

Living material

Eggs and sperm were shed from the sea urchin *Strongylocentrotus purpuratus* by 0.55 M KCl injection[13]. The eggs from one female were strained through bolting silk, washed twice, stripped of their gelatinous coat by a short treatment with sea-water at pH 5.2 and finally washed again two or three times. Fertilized eggs with less than 98 % membrane elevation were discarded. Excess sperm were washed away unless otherwise stated.

Uridine uptake

Equal aliquots of eggs were measured by mixing a dilute egg suspension in filtered sea water and quickly transferring an aliquot with a wide-mouth syringe to polystyrene test tubes containing [^{14}C]uridine in sea water. The temperature was controlled by a water bath at 15.0°±0.1°. After exposure to the labelled uridine, the eggs were chilled to 0°, washed four times with ice-cold sea water, placed on Millipore HA filters (0.45 μ pore size) and washed twice with sea water. A direct test with [^{14}C]uridine in sea water resulted in considerable radioactivity adhering to the Millipore filters. Very thorough washing of the eggs was, therefore, necessary. Duplicate samples were assayed from any one tube. Radioactivity was determined with a Nuclear–Chicago gas-flow micromil-window geiger counter to an accuracy of ±4.4 % or, more often, ±1.4 %.

When an egg count was desired, an aliquot of eggs was suspended in sea water and 1.0 ml sucked into a wide-mouth pipette. Ten such samples totalling 500–1000 eggs were counted.

[^{14}C]Uridine-partitioning experiment

To determine the permeability of the unfertilized and fertilized egg to uridine while avoiding the loss of uridine from the cell during the washings described above, a modification of the method described by TYLER AND MONROY[14] for ^{42}K was used. Eggs were either exposed to the labelled uridine directly in a sedimentation tube containing a narrow, calibrated stem or first exposed in a separate test tube and then an aliquot transferred to the sedimentation tube to determine the egg volume. The egg suspensions were stirred by bubbling air for a given length of time, tightly packed by centrifugation and chilled to 0°. Duplicate aliquots of supernatant sea water containing the label were removed and dried on stainless-steel planchets. The remaining radioactive sea water was now reduced until the eggs occupied a significant and known percentage of the suspension. The eggs were resuspended and two more aliquots were assayed. Finally the eggs were thoroughly washed in cold sea water, the volume re-established and a third pair of aliquots dried on planchets. Self-absorption was compensated by appropriate quenching with non-radioactive eggs and sea water.

Averaging the duplicate samples of the data obtained from the partitioning measurements, one can calculate the counts/min of ^{14}C acquired by the unfertilized or fertilized eggs as follows,

$$\text{counts/min eggs} = \text{counts/min suspension} - \text{counts/min supernatant} \times (1 - V_e/V)$$

where counts/min eggs = counts/min in eggs in a unit volume of egg suspension; counts/min suspension = counts/min in a unit volume of egg suspension; counts/min supernatant = counts/min in a unit volume of supernatant containing [^{14}C]uridine after exposure of the eggs to the label; V_e = volume of eggs in a unit volume of suspension (V); V_e/V = volume fraction of eggs in egg suspension during the partitioning measurement.

The actual amount comprising a unit volume varies in different experiments but is consistent within any one series of measurements.

One can also compute the ratio of counts/min of label inside the eggs to that remaining outside the eggs in an equivalent volume of surrounding supernatant as follows,

$$\frac{^{14}C \text{ inside eggs}}{^{14}C \text{ outside eggs}} = \frac{\text{counts/min eggs}}{\text{counts/min supernatant} \times V_e/V}$$

Dowex chromatography

Ion-exchange column chromatography was performed using the ammonium formate system described by HURLBERT[15]. Dowex-50 H^+ form (California Biochemical Corp., AG 50W X8, 200–400 mesh) and Dowex 50 Cl^- form (California Biochemical Corp., AG 1 X8, 200–400 mesh) were freed of fine particles and exchanged with 1 N NaOH and 1 N HCl. Dowex-1 Cl^- form was converted for chro-

matography to the formate form by washing first with sodium formate followed by concentrated formic acid. In all cases the column was washed with distilled water until the eluate was neutral. The dimensions of the columns were 0.6 × 4.0 cm and the flow rate was 0.5 ml/min. The eluate was collected in 10.0-ml fractions and each fraction was assayed for radioactivity to locate the label and for absorbance at 260 mμ to locate markers added to the extract. The details of the experiment are given with the results.

Chemicals

[2-^{14}C]Uridine (New England Nuclear Corp., specific activity 24.2 mC/mmole); 2,4-dinitrophenol (Eastman Organic Chem., three times recrystallized with ethanol); uridine 5'-monophosphate (Sigma Chemical Co.); uridine 5'-triphosphate (Sigma Chemical Co.).

RESULTS

[^{14}C]Uridine-partitioning experiment in fertilized and unfertilized eggs

Since the fertilized egg is known to accumulate much more radioactive uridine than the unfertilized egg[6,8], an investigation was undertaken to test the extent to which the latter is permeable to the nucleoside. Table I lists the data obtained for the partitioning type of measurements (columns 7 and 8; 11 and 12) and for the washed-eggs type of measurements (columns 9 and 13). Table II lists the values for [^{14}C]uridine uptake by the eggs calculated from the data of the partitioning measurements (columns 3 and 7) by the formula given in the section on MATERIALS AND METHODS. In this table the corresponding values from the washed-eggs measurements (columns 4 and 8) are given for comparison. Ratios of ^{14}C inside to ^{14}C remaining outside the eggs in an equivalent volume of supernatant after the eggs have been incubated with labelled uridine were calculated from the values obtained by both these types of measurements. They are listed for the unfertilized eggs (columns 5 and 6) and for the fertilized eggs (columns 9 and 10).

The values recorded for ^{14}C inside the eggs by the two types of procedures accord very well particularly for the fertilized eggs where uptake is high and relative experimental error correspondingly low.

Within the limits of accuracy of the method, it is clear that the unfertilized eggs accumulated very little label. The radioactivity was not significantly reduced by the copious washings. If the unfertilized egg cortex had been freely permeable to the [^{14}C]uridine, assuming the nucleoside to be readily soluble in the fluid of the egg cytoplasm which is approx. 80 % of the egg volume[16,17], the ratio of counts in a unit volume of eggs to that in a unit volume of supernatant containing the [^{14}C]-uridine would be 0.8 or very close to it. Table II lists the results of this calculation for the partitioning (column 5) and the washed-eggs (column 6) types of measurement for the unfertilized egg. There is some variation in the ratios, ranging from 0 to 0.311 for the partitioning measurements and from 0.029 to 0.515 for the washed-eggs measurements. Nevertheless, even the high values in each case are considerably less than 0.8 which would be predicted from free diffusion of the label across the

TABLE I

UPTAKE OF [^{14}C]URIDINE BY UNFERTILIZED AND FERTILIZED EGGS

[^{14}C]Uridine-partitioning experiments in unfertilized and fertilized eggs. Expt. 1 was done at the Division of Biology, California Institute of Technology, with the use of a Packard TriCarb liquid scintillation counter, Model 500D. Expts. 2–11 were done at Friday Harbor as described in the MATERIALS AND METHODS section.

						Unfertilized eggs			*Fertilized eggs*			
Temp. (°C)	*Expt.*	[^{14}C]-*Uridine* (μC/*ml*)	*Pulse duration* (*min*)	V_e/V *during pulse*	V_e/V *during partitioning*	*Counts/min supnt.*	*Counts/min susp.*	*Counts/min eggs (washed)*	*Begin pulse (min after fertilization)*	*Counts/min supnt.*	*Counts/min susp.*	*Counts/min eggs (washed)*
21	1	0.05	60	0.025	0.385	5025 5007	3663 3708	179 176	12	2716 2865	25508 21459	23047 23845
15	2	0.10	40	0.370	0.370	1237 1149	807 702	18.2 10.2				
	3	0.04	30	0.049	0.320	332 325	201 236	3.3 2.6				
	4	0.10	40	0.210	0.210	1497 1537	1187 1326	34.7 16.8				
	5	0.10	45	0.130	0.130	1432 1409	1299 1268	70.8 12.0	25	72.3 198	1242 1097	1149 1207
	6	0.05	45	0.026	0.260	571 490	384 398	57.8 43.2	25	36.5 36.5	3947 3797	3297 3297
	7	0.04	10	0.014	0.500	218 202	116 122		40	39.6 39.8	747 613	
	8	0.03	20	0.032	0.500	313 161	128.5 138	5.2 6.2				
	9*	0.03	19	0.033	0.500	196 219	119.9 106.2	18.5 45.9	45	94 79	2162	2257 2137
−3	10	0.03	15	0.013	0.330	526 493	239 273	0.7 0.8	6	411 408	306 259	29 26.3
	11	0.05	15	0.011	0.400				4	384 369	327 277	17.2 12.9

* Trypsin membrane removal

TABLE II

CALCULATIONS ON DATA GIVEN IN TABLE I

The data summarized in Table I have been applied to the formulae given in the MATERIALS AND METHODS section. See text for discussion.

Temp. (°C)	*Expt.*	*Unfertilized eggs*				*Fertilized eggs*			
		Counts/min eggs		^{14}C *inside eggs* / ^{14}C *outside eggs*		*Counts/min eggs*		^{14}C *inside eggs* / ^{14}C *outside eggs*	
		Partitioning	*Washed*	*Partitioning*	*Washed*	*Partitioning*	*Washed*	*Partitioning*	*Washed*
21	1	600.7	177.5	0.311	0.092	21767	23446	20.26	21.82
	2	2.9	14.2	0.007	0.032				
	3	−4.9 = 0	3.0	0	0.029				
	4	58.1	25.8	0.182	0.081				
15	5	47.7	95.4	0.261	0.515	1051.9	1178	59.87	67.05
	6	−1.6 = 0	50.5	0	0.039	3845	3297	405.16	347.42
	7	14		0.134		660.2		33.26	
	8	14.8	5.7	0.124	0.048				
	9*	9.3	32.2	0.090	0.310	2118.8	2207	48.49	51.03
−3	10	−85.4 = 0	0.8	0	0.003	8.1	27.7	0.059	0.205
	11					76.1	15.1	0.505	0.100

* Trypsin membrane removal

membrane. The average ratio of ^{14}C inside the eggs to that remaining outside the eggs in an equivalent volume of supernatant after a specified time was 0.117 and 0.143 for the partitioning type and washed-eggs type of measurement, respectively. In Expt. 9, the vitelline membrane was digested with trypsin (EC 3.4.4.4) so that a fertilization membrane was not formed after insemination even though 95 % of the eggs in a fertilized aliquot from this suspension cleaved. The low ratio demonstrates, however, that a resistance to the penetration of [^{14}C]uridine existed in these eggs as in the non-trypsin-treated eggs. There is no apparent correlation in these experiments between the amount of label accumulated by the unfertilized eggs and the length of time the eggs were incubated with the [^{14}C]uridine or the amount of radioactivity to which the eggs were exposed. The spread in the ratios expressed in Table II may be a consequence of the sum of experimental errors or perhaps may represent a differential competence of different batches of eggs to take up uridine.

The fertilized eggs gave a markedly different result. The label was concentrated within the eggs and the culture solution became appreciably depleted of radioactivity. As in the unfertilized eggs, the ^{14}C that penetrated the fertilized eggs was not washed out of the cells by thorough washing with ice-cold sea water. The ratio of the counts/min per unit volume of eggs to the counts/min per unit volume of radioactive supernatant was much greater than 0.8, varying in different experiments from 20.26 to 405.16. This variability in the ratio may be in part accounted for by its sensitivity to small changes in the denominator.

An attempt to distinguish between possible changes in membrane permeability and changes in uridine metabolism within the egg was made by reducing the temperature to $-3°$ during exposure of the fertilized eggs to the radioactive isotope. The low temperatures should slow uridine metabolism but might not severely affect the diffusion rate across the membrane. The results of the calculations given in Table II for Expts. 10 and 11 show that the chilled eggs took up very little label. These eggs had been pre-chilled 5 min after fertilization for a period of 15 min before administration of [^{14}C]uridine, and would not yet be incorporating uridine at a rapid rate. Nonetheless, the fact that the ratio of ^{14}C inside to that outside the eggs while the eggs remained suspended in the labelled uridine solution was clearly less than 0.8, as in the unfertilized eggs, indicates that membrane changes at fertilization have not made the eggs freely accessible to uridine by diffusion. A temperature-dependent mechanism other than diffusion seems, therefore, responsible for uridine entry into the fertilized egg. The unfertilized eggs at low temperature accumulated virtually no [^{14}C]uridine. Fertilized eggs, chilled to $-3°$ for 30 min and then warmed to 15°, retained their ability to accumulate [^{14}C]uridine.

Time course of the rate of [^{14}C]uridine uptake

A representative experiment, done by the washing method, which demonstrates the change in the rate of uridine uptake during early development, is shown in Fig. 1. The eggs were exposed at the indicated stages for 4 min to [^{14}C]uridine. During approximately the first 10 min there was a lag in uridine accumulation which was followed by a period of rapid acceleration in the rate of uptake. At the end of the first hour, or shortly thereafter, the rate of uptake reached a plateau. As the eggs cleaved the rate of uptake remained essentially constant. At the end of the

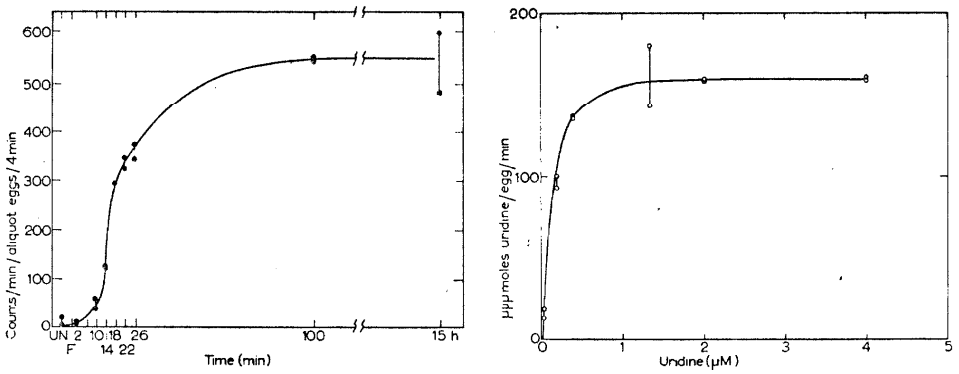

Fig. 1. Time course of [^{14}C]uridine uptake after fertilization. Eggs were fertilized and 1.0-ml aliquots containing approx. 10^4 eggs were transferred to [^{14}C]uridine (0.083 μC/ml) at the designated times. Sperm were not washed away until after the 4-min exposure to the label. UN, unfertilized and F, fertilized eggs.

Fig. 2. Dependence of [^{14}C]uridine uptake on uridine concentration. Fertilized, washed eggs at the two-cell stage were exposed to increasing concentrations of [^{14}C]uridine. Duplicate test tubes each containing $6.8 \cdot 10^4$ eggs were exposed to the [^{14}C]uridine in a total volume of 2.0 ml. After 15 min, the eggs were quenched with a large excess of non-radioactive uridine and processed as described in the MATERIALS AND METHODS section.

experiment in Fig. 1 enough label remained in the sea water to saturate the uptake mechanism as judged by the results reported in the next section. In one experiment in which uridine uptake was followed until the early prism stage, the rate of accumulation remained nearly constant.

Dependence of [^{14}C]uridine uptake on uridine concentration

Eggs at the two-cell stage, the maximal rate of uridine uptake having been reached, were exposed to increasing concentrations of the radioactive isotope (Fig. 2). Not only were the developing eggs extremely efficient in accumulating label from very low concentrations of uridine ($2.0 \cdot 10^{-9}$ M uridine) in the sea water, but also a concentration of uridine was reached which saturated their mechanism of uptake. In several experiments this occurred at 0.5–1.0 μM uridine. Beyond this point, the rate of uptake remained constant as tested with concentrations of uridine in the sea water up to 75 μM.

Effect of 2,4-dinitrophenol

It was considered of interest to investigate to what extent uridine uptake after fertilization was dependent on a continuous supply of energy. 2,4-Dinitrophenol uncouples phosphorylations from oxidations[18] and so interferes with energy-requiring processes. Table III summarizes the results. 2,4-Dinitrophenol, when added to a dilute egg suspension concurrently with [^{14}C]uridine, appreciably reduced uptake of the nucleoside by the egg. The 2,4-dinitrophenol was used at a concentration which proved to inhibit uridine uptake and cleavage reversibly (0.1 mM). 2,4-Dinitrophenol added within 1 min after fertilization caused greater inhibition than when the eggs were treated 5 min or more after fertilization.

TABLE III

EFFECT OF 2,4-DINITROPHENOL ON [^{14}C]URIDINE UPTAKE BY FERTILIZED EGGS

Radioactive suspensions contained few eggs. [^{14}C]Uridine concentrations were: Expt. 1, 0.008 μC/ml; Expt. 2, 0.033 μC/ml; Expt. 3, 0.10 μC/ml; Expt. 4, 0.10 μC/ml.

Expt.	*2,4-Dinitrophenol concentration (mM)*	*[^{14}C]Uridine and inhibitor added (min after fertilization)*	*Pulse duration (min)*	*Control eggs (counts/min)*	*Treated eggs (counts/min)*	*Percentage inhibition (averaged values)*
1	0.1	1	30	1367 1269	129 155	89.2
2	0.1	85	5	945 964	235 212	76.6
3	0.1	6	15	102 107	22.2 26.9	76.6
		35	15	393 426	127.5 155	65.4
4	0.1	1	30	359 351	14.6 14.6	95.9
		60	30	1012 1137	228 233	78.5
		90	30	949 886	282 293	68.7
		120	30	910 923	280 303	68.2

Fig. 3 is the graphic representation of Expt. 4. It illustrates the extent to which 2,4-dinitrophenol reduced uridine accumulation by the fertilized egg during a 30-min exposure to the radioactive isotope at different times shortly after fertilization. In all cases enough [^{14}C]uridine remained in the sea water so as not to decrease the rate of uptake during the experiment.

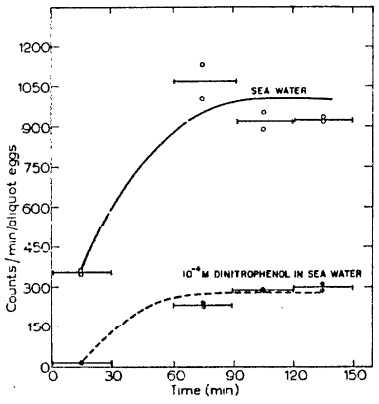

Fig. 3. Effect of 2,4-dinitrophenol on [^{14}C]uridine uptake. Eggs were fertilized and dilute aliquots transferred to 2,4-dinitrophenol and [^{14}C]uridine (0.10 μC/ml) at the times indicated. Sperm were not washed away until after the exposure to the label. The horizontal lines represent the length of time the eggs were in contact with the 2,4-dinitrophenol and [^{14}C]uridine.

Partial identification of the label by Dowex chromatography

Since the fertilized egg concentrates [^{14}C]uridine many times over the radioactivity in an equivalent volume of the culture solution, an investigation was made of the extent to which the label remains as free uridine inside the egg. Fertilized eggs which had reached their maximal rate of uridine uptake were exposed to the radioactive isotope for a specified time. The labelled eggs were then thoroughly washed, chilled to 0° and sonicated for 30–60 sec in an equal volume of ice-cold 0.4 M perchloric acid. All subsequent steps were performed at 0–4°. The sonicate was centrifuged at 27 000 × *g* for 15 min. The supernatant fraction was neutralized with KOH, recentrifuged to clear the potassium perchlorate precipitate from the fluid and assayed for radioactivity. The pellet from the homogenate was either dissolved in 2 M NaOH after washing in 0.4 M perchloric acid or was suspended directly in perchloric acid to the original volume and its radioactivity assayed. Table IV shows

TABLE IV

DISTRIBUTION OF [^{14}C]URIDINE TAKEN UP AFTER FERTILIZATION INTO ACID-SOLUBLE AND ACID PRECIPITABLE FRACTIONS

Expts. 1 and 2: the acid-insoluble pellet was washed once in 0.4 M perchloric acid, and 50 µl assayed for radioactivity. Expts. 3 and 4: the acid-insoluble pellet was resuspended in cold 0.4 M perchloric acid without washing and 50 µl assayed directly for radioactivity.

Expt.	*Begin pulse (min after fertilization)*	*Pulse duration (min)*	[^{14}C]*Uridine (µC/ml)*	*Counts/min per 50 µl*		
				Homogenate	*Cold perchloric acid-soluble fraction*	*Cold perchloric acid-precipitable fraction*
1	40	32	0.025	357	332	1.2
				430	339	1.3
2	62	53	0.025		628	15.5
					635	24.1
3	67	30	0.10	360	366	39
				378	381	50
4	75	30	0.025	464	503	15
				385	518	16.4

that most (88–99.6 %) of the radioactivity within the egg was soluble in ice-cold perchloric acid. The high values of acid-precipitable materials are from the insoluble pellets which had not been washed and were probably contaminated with slight residual soluble label.

An aliquot of the soluble extract was now layered on a Dowex-50 (H^+ form) column and washed through with an equal volume of distilled water. Virtually all of the radioactivity descended through the column. An aliquot of the eluate was adsorbed on a Dowex-1 (Cl^- form) column, washed with distilled water and then with 0.03 M NH_4Cl. No radioactivity passed through the column indicating [^{14}C]uridine accumulated by the egg was no longer free uridine. A direct test with labelled uridine on both Dowex-50 (H^+ form) and Dowex-1 (Cl^- form) confirmed that the nucleoside is not adsorbed by these resins under the present conditions.

The chemical forms of the labelled compounds into which uridine had been metabolized were in part established by means of Dowex-1 (formate form) column chromatography. A 0.5-ml aliquot of the radioactive Dowex-50 (H^+ form) eluate was neutralized with NH_4OH, supplemented with 5'-UMP and 5'-UTP markers, adsorbed on a Dowex-1 (formate form) column and washed with 10.0 ml of distilled water. All the label was retained on the column until after elution with solutions (0.6 M and 1.35 M) of ammonium formate at pH 5.0. The eluate was collected in 10-ml fractions. The results are shown in Fig. 4. The small peak of radioactivity which

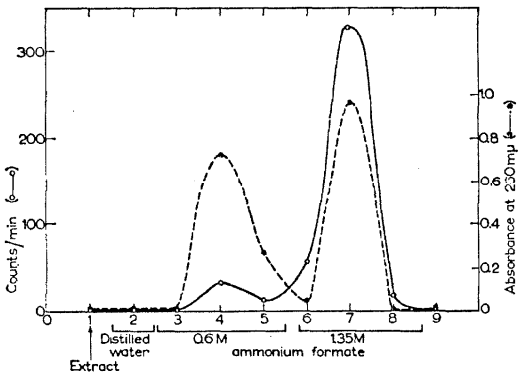

Fig. 4. Dowex-1 (formate form) column chromatography of the acid-soluble label accumulated by the fertilized eggs. Fertilized eggs were exposed to [^{14}C]uridine, and the radioactive acid-soluble fraction of the homogenate was passed through a Dowex-50 (H^+ form) column and eluted from a Dowex-1 (formate form) column, with ammonium formate at pH 5.0 as indicated. Fractions of 10.0 ml were collected and assayed for ^{14}C and absorbance at 260 mμ to locate the nucleotide markers. Further details given in the text.

descended with the 0.6 M ammonium formate elution coincided with the UMP marker. The remaining label was recovered by elution with 1.35 M ammonium formate and found to coincide with the UTP marker. UTP chromatographed alone appears only in this fraction. In the absence of markers there was no significant absorption at 260 mμ. The ratio of the radioactivity in the peak containing the UTP to that containing the UMP was 8.5 and the total recovery of radioactivity from the column in the experiment shown in Fig. 5 was 92.0 %. In five experiments including the present the average total recovery of the label in these two peaks was 99.8 %.

The method of chromatography employed is useful for separating UMP from UTP. Theoretically, in a gradient elution UDP comes off the column at a time just when the ammonium formate concentration was increased in the present experiment. For this reason one cannot be certain which of the two peaks, if either, contains labelled UDP. Furthermore, this method does not resolve the phosphorylated forms of uridine from the phosphorylated forms of other nucleosides. However, it seems established that the soluble label was almost exclusively in the form of phosphorylated nucleosides, probably primarily in the form of nucleoside triphosphates. Only a very small amount of the radioactivity had been incorporated into polynucleotides which precipitate with cold perchloric acid.

DISCUSSION

Previous studies of the incorporation of labelled precursors into RNA or acid-precipitable materials by the unfertilized sea-urchin egg have given divergent results. A number of investigators were unable to detect any RNA synthesis at all. FICQ *et al.*[19] exposed unfertilized eggs of *Paracentrotus lividus* for 20 min to [^{3}H]-cytidine and for 2 h to [^{3}H]uridine in separate experiments and found no incorporation of label into RNA on the basis of autoradiography. BRACHET *et al.*[20] were also unable to show any incorporation of [^{32}P]phosphate into acid-precipitable RNA of unfertilized Arbacia eggs that had been exposed to large quantities of the labelled phosphate for 10 min. WILT[8,21], using *Strongylocentrotus purpuratus* eggs, reported essentially no incorporation of [^{14}C]- or [^{3}H]uridine into RNA even after long exposures of the eggs to the radioactive precursors. GLISIN AND GLISIN[22] have recently reported that unfertilized *Lytechinus pictus* eggs incubated with [^{32}P]phosphate for 5 h in sterile sea water did not contain any label in the 4-, 18- or 28-S RNA. MARKMAN[23] reported very little incorporation of [^{14}C]adenine into RNA of unfertilized *Paracentrotus lividus* eggs during a 20-min exposure of the eggs to the label. GROSS *et al.*[24], however, exposed unfertilized eggs of *Arbacia punctulata* to [^{14}C]-uridine for time intervals up to 2 h and reported slight incorporation into materials precipitated by acid. Autoradiograms, though, showed no incorporation of tritiated precursors into RNA. Some RNA synthesis by the unfertilized egg was reported by NEMER[25] who incubated *Paracentrotus lividus* eggs for 5 h with ^{14}C-labelled uridine and cytidine and noted some incorporated radioactivity into acid-precipitable materials. Later, NEMER[6] reported that unfertilized *Strongylocentrotus purpuratus* eggs after a 20-min incubation with [^{3}H]uridine exhibited the label in 4-, 17- and 22-S RNA's. After a 4-h chase with unlabelled uridine, which followed the 20-min radioactive pulse, the pattern of the distribution of radioactive RNA persisted with an increase in the specific activity of the different species of RNA. Furthermore, it has been reported by TOCCO *et al.*[26] that the unfertilized egg of *Sphaerechinus granularis* incorporated [^{32}P]orthophosphate into the RNA of the nuclear, mitochondrial and, to a lesser extent, microsomal fractions during a 2.5-h exposure of the eggs to the radioactive precursor. HULTIN[27] has reported some time ago very low incorporation of $^{15}NH_4Cl$ by *Paracentrotus lividus* eggs exposed for 5 h to the isotope, but recently[28], using [^{32}P]phosphate exposures of 3 h, he reported higher incorporation of label into the RNA with 85 % of the RNA radioactivity in the nuclear fraction and about 2.5 % in ribonucleoprotein particles.

There is more agreement as to the incorporation of labelled precursors into RNA shortly after fertilization. VILLEE *et al.*[29], HULTIN[27], SCARANO AND KALCKAR[30], MARKMAN[23], GROSS AND COUSINEAU[2], NEMER[6], WILT[8], TOCCO *et al.*[26] and GROSS *et al.*[7] have reported that RNA is being synthesized in the fertilized sea-urchin egg. It may be mentioned, however, that BRACHET *et al.*[20] were unable to find [^{32}P]-phosphate incorporation into RNA or cytochemically to detect RNA synthesis after fertilization. Also, though SCARANO AND KALCKAR[30] found labelled glycine incorporated into RNA after fertilization, exposure of the eggs to [^{14}C] adenine during the second 2 h of development proved only to label the nucleotide pool. Finally GLISIN AND GLISIN[22] have reported that [^{32}P]phosphate is incorporated only in the terminal pCpCpA grouping of 4-S RNA up to the four-cell stage when sterile conditions are employed.

The present experiments introduce another factor into investigations utilizing labelled uridine to measure RNA synthesis before and after fertilization. The unfertilized sea-urchin egg showed an exceedingly low permeability to radioactive uridine while the fertilized egg actively concentrated the label into the cell. The result is that the labelled nucleotide pool used as a source by the cell for RNA synthesis has a lower specific activity in the unfertilized egg than in the fertilized egg assuming that the size of the nucleotide pool remains constant.

The results of the experiments presented here should be considered in terms of (1) the generation of an active transport system for uridine after fertilization and (2) the initiation of cellular uridine metabolism at fertilization. These two mechanisms of accumulating uridine are not mutually exclusive.

The existence of an active transport mechanism for [^{14}C]uridine entry into the fertilized egg has the following support. The shape of the curve obtained when uridine accumulation was plotted against the concentration of uridine to which the eggs were exposed is consistent with a carrier or enzymatically controlled mechanism of uptake. Second, the uptake was sensitive to 2,4-dinitrophenol and so had an energy requirement which is common to most transport mechanisms. It may be mentioned, however, that this is distinct from the phosphate active transport system that starts at fertilization. LITCHFIELD AND WHITELEY[31] and WHITELEY AND CHAMBERS[32] have shown that in the case of phosphate 2,4-dinitrophenol blocked the generation of the transport system, but after its establishment phosphate accumulation proceeded when energy metabolism was strongly suppressed. In these experiments the labelled uridine which did accumulate in the presence of 2,4-dinitrophenol is probably explained by a drain of high-energy phosphate reserves in the cell. A third factor consistent with an active transport mechanism for uridine uptake is the small amount of label which was able to penetrate the fertilized egg chilled to $-3°$ 5 min after fertilization. Unless the lag phase of uridine uptake after fertilization represents an opening of pores on the surface slowly allowing more uridine to enter the cell, one might expect more radioactivity to have entered the fertilized egg cytoplasm in the form of free uridine even if its subsequent metabolism was blocked by the low temperature. But very little label did penetrate the egg under these conditions implying a temperature-dependent step necessary to carry the molecule into the cell. The small amount of [^{14}C]uridine which was taken up by the fertilized egg probably represents an incomplete temperature block. Finally, indirect evidence which might suggest an active transport mechanism is the striking ability with which the fertilized egg concentrated the ^{14}C over the amount of label in an equivalent volume of sea water. The radioactivity acquired could not be washed out of the eggs.

On the other hand, cellular uridine metabolism commencing after fertilization should also be considered as the driving force for the entry of uridine into the fertilized egg during exposure to radioactive uridine. Since the label accumulated by the fertilized egg was not in the form of free uridine but had been phosphorylated, the nucleoside possibly did not enter against a concentration gradient and could have diffused into the egg and become quickly metabolized. The energy requirement for uridine uptake and the suggestion of an enzymatically controlled mechanism of accumulation could both be a consequence of cellular metabolism of uridine that entered by diffusion.

However, when the partitioning of ^{14}C between the inside and outside of the cells subjected to $-3°$ was measured as the eggs remained suspended in the radioactive sea water, all of the label was excluded from the unfertilized eggs and much of the label was excluded from the fertilized eggs. This result lends support to the possibility that a temperature-dependent surface reaction is necessary for the uptake of uridine. A hypothesis consistent with the data is that an enzymatically controlled phosphorylation of uridine occurring on the cell surface is the mechanism by which uridine entered the fertilized egg. No free labelled uridine would then be found in the egg which proved to be the case. Apparently an important factor that limited uridine uptake in the unfertilized egg was a depressed ability to convert the nucleoside to a nucleotide.

In any event uptake of radioactive uridine occurred to a very small extent before fertilization and to an appreciable extent shortly after fertilization. The amount of label accumulated after fertilization cannot be accounted for by the initiation of RNA or DNA synthesis since the evidence indicates only slight quantities of nucleic acids were being made during this period, the bulk of the incorporated [^{14}C]uridine being recovered as nucleoside triphosphates. On the other hand, uridine uptake may be a consequence of the synthesis or activation, upon fertilization, of a transphosphorylating kinase which catalyzes the phosphorylation of uridine. This could be because of the *de novo* synthesis of such a kinase, as appears to be the case for phosphate transport (WHITELEY AND CHAMBERS[32]; WHITELEY AND YANAGISAWA[33]). Alternatively, it has been known for some time from the work of a number of investigators (see WHITELEY[12]) that ATP is present in the unfertilized echinoid egg. A mobilization of arginine phosphate occurs immediately after sperm entrance evidently at the expense of ATP (CHAMBERS AND MENDE[34]). Also, a decrease in ATP after fertilization has been noted by others (AIELLO *et al.*[35]). One might propose then, that a change after fertilization rendering available for use ATP, or other phosphate donors, already present in the unfertilized egg is responsible for the increased uptake of radioactive uridine after fertilization. The nucleoside can now be phosphorylated. Our evidence suggests that the phosphorylation might occur at the cell surface and thus can serve not only as the first step in the metabolism of uridine but also as the mechanism by which uridine enters the cell.

NOTE ADDED IN PROOF

Recent experiments[36] utilizing puromycin have shown that protein synthesis is unnecessary for the initiation of uridine uptake upon fertilization.

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PART I

Section 2

FURTHER STUDIES ON THE INITIATION AND MECHANISM OF C^{14}-URIDINE UPTAKE AFTER FERTILIZATION OF STRONGYLOCENTROTUS PURPURATUS EGGS.

INTRODUCTION

After publication (Piatigorsky and Whiteley, 1965, see Section 1 of Part I of this thesis) of the experiments on uridine uptake by unfertilized and fertilized sea urchin eggs, other investigators confirmed that nucleoside uptake is strongly stimulated after fertilization (Mitchison and Cummins, 1966; Siekevitz, Maggio and Catalano, 1966).

Mitchison and Cummins (1966) studied the uptake of H^3-cytidine before and after fertilization of eggs of the sea urchin Paracentrotus lividus. They found that unfertilized eggs accumulated very little label. After fertilization, however, the rate of cytidine uptake gradually increased during the first hour of development. The rate of uptake reached a plateau shortly before the first cleavage, and remained constant thereafter. Cytidine uptake was not studied beyond the blastula stage.

Mitchison and Cummins also showed that the uptake of tritiated cytidine by fertilized eggs was greatly inhibited by the presence of an excess of non-radioactively labeled uridine or thymidine. On the other hand, excess cytosine or uracil only slightly suppressed the uptake of labeled cytidine. These results are consistent with the hypothesis (Piatigorsky and Whiteley, 1965, see Section 1 of Part I of this thesis) that nucleoside uptake depends on the phosphorylation of the ribose moiety of the pyrimidine.

Further studies have been made on the development and mechanism of uridine uptake in fertilized eggs of Strongylocentrotus purpuratus.

The results have been published in an abstract (Piatigorsky, 1965) and are presented below.

EXPERIMENTS

Uptake of C^{14}-uridine nucleotides after fertilization

It has been established earlier that C^{14}-uridine only slowly penetrates the unfertilized sea urchin egg. In addition it was determined that C^{14}-uridine accumulated after fertilization is principally in the form of nucleoside-5'-monophosphate and triphosphate, primarily the latter. No free C^{14}-uridine can be found inside the fertilized eggs.

These observations were interpreted to mean that uridine is converted to uridine-5'-monophosphate at the cell surface and that this serves as the mechanism by which uridine enters the egg. One might predict, then, that labeled uridine-2'- or 3'-monophosphate would be taken up by fertilized eggs but that uridine-5'-monophosphate would be rejected since the "uptake phosphorylation" site, namely, the 5' carbon atom of uridine, is already occupied.

This prediction was tested with fertilized eggs that had reached their maximal rate of uridine uptake. The rate of uptake of C^{14}-uridine by the fertilized eggs was compared with that of C^{14}-uridine-2'- and 3'-monophosphate, 5'-monophosphate, 5'-diphosphate and 5'-triphosphate. The results are illustrated in Fig. 1.

Fig. 1. UPTAKE OF C^{14}-URIDINE NUCLEOTIDES BY FERTILIZED (60 MINUTES) EGGS OF STRONGYLOCENTROTUS PURPURATUS. Five equal samples of a fertilized egg-suspension in artificial sea water, prepared as described previously (Piatigorsky and Whiteley, 1965, see Section 1 of Part I of this thesis), were distributed to C^{14}-2-uridine (sp. act., 30 c/M), C^{14}-2-uridine-2'- and 3'-monophosphate (sp. act., 15 c/M), C^{14}-2-uridine-5'-monophosphate (sp. act., 22 c/M), C^{14}-2-uridine-5'-diphosphate (sp. act. 19 c/M) and C^{14}-2-uridine-5'-triphosphate (sp. act., 25 c/M). Each isotope was at a concentration of 0.05 μc/ml in a total volume of 2.0 ml containing approximately 3×10^4 eggs. Incubation was for 10 min at 20°C. Uptake was stopped by the addition of 5.0 ml of ice-cold artificial sea water followed by thorough washing by centrifugation with iced sea water. Duplicate aliquots of the washed egg-suspensions were transferred to filter paper strips, were air-dried and assayed directly for radioactivity by scintillation counting (Tri-Carb spectrometer) in a toluene scintillation fluid with about 50% efficiency.

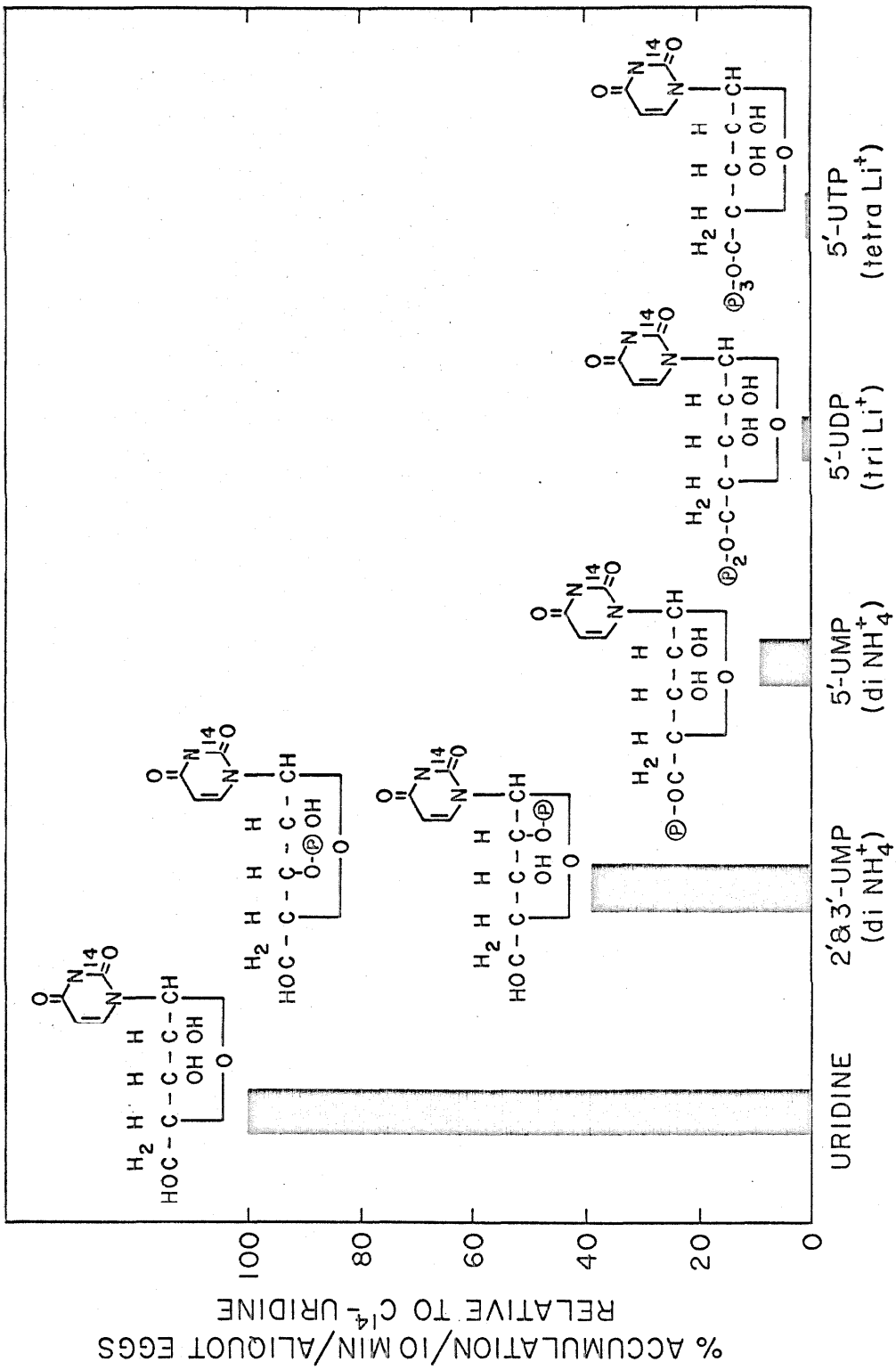
% ACCUMULATION/10 MIN/ALIQUOT EGGS
RELATIVE TO C14- URIDINE
0
20
40
60
80
100
URIDINE
2'&3'-UMP
(di NH4+)
5'-UMP
(di NH4+)
5'-UDP
(tri Li+)
5'-UTP
(tetra Li+)

Figure 1.

The height of the vertical bars of the figure represents the amount of labeled isotope accumulated relative to the amount of C^{14}-uridine taken up by the fertilized eggs. It is clear that the mixture of C^{14}-uridine-2' and 3'-monophosphate was accumulated by the eggs more effectively than the C^{14}-uridine-5'-monophosphate. Barely detectable quantities of C^{14}-uridine-5'-diphosphate or triphosphate entered the eggs.

In two experiments of this type, one of which is shown in Fig. 1, labeled uridine-5'-monophosphate was accumulated only 7.7 to 9.8% as readily as C^{14}-uridine, while the mixture of labeled uridine-2'- and 3'-monophosphate was taken up in amounts ranging from 36.9 to 39.5% that of C^{14}-uridine. Thus, phosphorylated uridine is taken up less effectively than uridine. However, the position of the phosphate groups attached to uridine makes a significant difference in the ability of fertilized eggs to accept and accumulate the nucleotide.

Effect of puromycin on the development of uridine uptake after fertilization.

The evidence, then, strongly indicates that uridine uptake is dependent upon uridine kinase activity. One possibility limiting the rate of uridine uptake before fertilization is that very little uridine kinase is present in unfertilized eggs. Another possibility, however, is that unfertilized eggs possess uridine kinase, but that it is largely inactive in uridine uptake until after fertilization.

To distinguish between these two possibilities, the generation of the uridine uptake system in normally fertilized eggs was compared with

that in eggs fertilized in the presence of puromycin which is known to inhibit protein synthesis (Yarmolinsky and de la Haba, 1959). If the initiation of uridine uptake after fertilization is unaffected by the inhibition of protein synthesis, it is likely that uridine kinase synthesis is unnecessary for the development of the system responsible for nucleoside uptake. The results of the experiment are shown in Fig. 2.

The upper graph of Fig. 2 demonstrates that the eggs fertilized in the presence of puromycin were reversibly suppressed with respect to protein synthesis. The puromycin was also effective in reversibly inhibiting cleavage. Nevertheless, the lower graph of Fig. 2 shows that the puromycin-treated eggs accumulated C^{14}-uridine at a rate identical to that of the controls. Puromycin did not adversely affect the lag phase, acceleration phase or constant accumulation phase of uridine uptake after fertilization. Protein synthesis is, evidently, unnecessary for the initiation of uridine uptake.

DISCUSSION

The present experiments provide additional evidence that uridine uptake by fertilized sea urchin eggs involves an enzymatic phosphorylation of uridine, at the cell surface, converting uridine to uridine-5'-monophosphate. The failure of uridine-5'-diphosphate to be accumulated by the eggs indicates that uridine-5'-monophosphate is converted to the corresponding triphosphate only within the cell rather than at its surface.

Fig. 2. EFFECT OF PUROMYCIN ON THE INCORPORATION OF C^{14}-VALINE INTO PROTEIN (UPPER GRAPH) AND ON THE UPTAKE OF C^{14}-2-URIDINE (LOWER GRAPH) BY EGGS OF STRONGYLOCENTROTUS PURPURATUS. Eggs were obtained, washed and stripped of their gelatinous coat as described previously elsewhere (Piatigorsky and Whiteley, 1965, see Section 1 of Part I of this thesis). A suspension of unfertilized eggs was divided into two parts, one of which was incubated with puromycin at 10^{-4}M for 30 min. at 20°C. The other remained in artificial sea water. The egg-suspensions were fertilized and duplicate aliquots were incubated at the indicated times after fertilization in the presence and absence of puromycin (10^{-4}M), either for 10 min. with C^{14}-2-uridine (sp. act., 30 c/M) at 0.05 μc/ml or for 30 min. with C^{14}-valine (sp. act., 208.5 c/M) at 0.25 μc/ml. Each incubation tube contained approximately 3×10^4 eggs in a total volume of 1.0 ml at 20°C. C^{14}-uridine uptake and C^{14}-valine incorporation was stopped by addition of an excess of C^{12}-uridine and C^{12}-valine, respectively, in ice-cold artificial sea water. The eggs were thoroughly washed with iced sea water by centrifugation, transferred to filter paper strips and air dried. C^{14}-uridine uptake was assayed as given in the legend to Fig. 1. C^{14}-valine incorporation into protein was measured by processing the papers with 5% trichloroacetic acid and alcohol followed by scintillation counting as described elsewhere (Tyler, 1966).

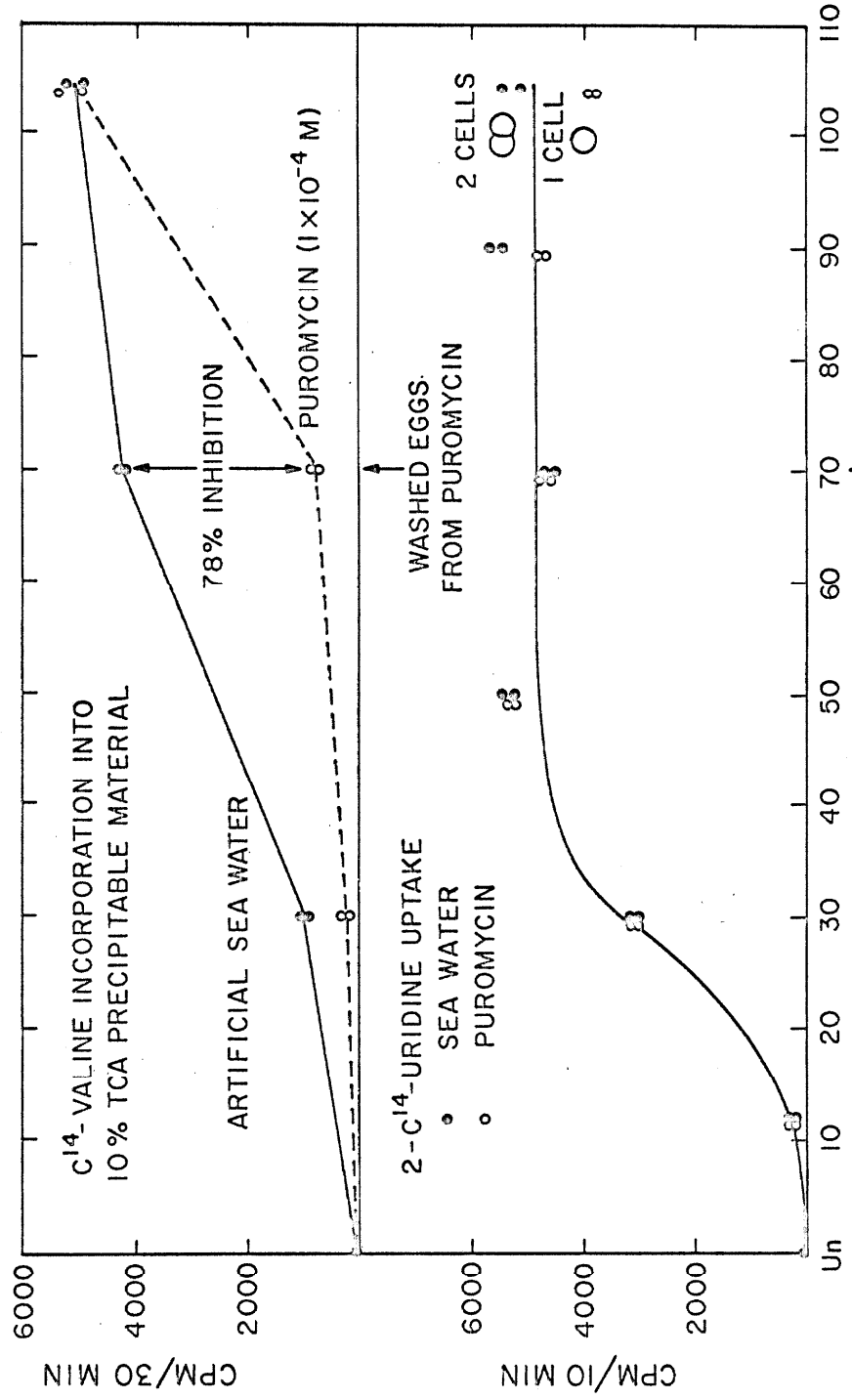
C14- VALINE INCORPORATION INTO
10% TCA PRECIPITABLE MATERIAL
ARTIFICIAL SEA WATER
78% INHIBITION
PUROMYCIN (1×10-4 M)
CPM/30 MIN
6000
4000
2000
2-C14-URIDINE UPTAKE
SEA WATER
PUROMYCIN
WASHED EGGS
FROM PUROMYCIN
2 CELLS
1 CELL
CPM/10 MIN
Un
10
20
30
40
50
60
70
80
90
100
110
WASHED EGGS
FROM PUROMYCIN
MINUTES AFTER FERTILIZATION

Figure 2.

It is likely that the reduced rate of uptake of the mixture of C^{14}-2'- and 3'-monophosphate, relative to the rate of uridine uptake, can be attributed to the negative charge of the attached phosphate group as well as to steric interference of uridine kinase activity. The slight uptake of uridine-5'-monophosphate by the eggs possibly indicates that the cell surface possesses some uridylic acid kinase activity.

Uridine kinase is not usually employed in the biosynthesis of RNA, but rather is considered as a "salvage pathway" (Kornberg, 1957; Reichard, 1959; Brockman and Anderson, 1963) in the conversion of uracil to uridine triphosphate. Thus the low activity of uridine kinase at the cell-surface of unfertilized eggs does not necessarily reflect an absence of RNA synthesis. The unfertilized sea urchin egg may be synthesizing, or turning over, more RNA than was earlier believed (see Discussion to Section 1 of Part I). In support of this contention, Siekevitz, Maggio and Catalano (1966) have recently reported that unfertilized eggs of Paracentrotus lividus may be synthesizing RNA at an even faster rate than fertilized eggs if one considers relative differences in uptake of the labeled precursor.

It is of interest to note that rapidly proliferating cells, such as those present in regenerating rat liver, mammalian intestine and growing ascites cells show high activity for uridine kinase and other "salvage pathway" enzymes. Cells from tissues with low growth rates, on the other hand, such as heart muscle, brain and liver show low "salvage pathway" enzyme activities (Skold, 1960). Fertilized eggs, naturally, represent cells belonging to the former, and unfertilized

eggs to the latter category.

An important conclusion from the experiment on the initiation of uridine uptake after fertilization in the presence of puromycin is that uridine kinase undergoes a large increase in activity rather than net synthesis after fertilization. Other enzymes are also known to be inhibited until after fertilization (see Monroy, 1965a, for review). Aldolase (Ishihara, 1957; 1958a, b; 1963), glucose-6-phosphate dehydrogenase (Bäckström, 1959; Isono, 1963a, b; Isono, Tsusaka and Nakano, 1963), 6-phosphogluconate dehydrogenase (Bäckström, 1963) and possibly glucose-6-phosphate phosphorylase (Aketa, Bianchetti, Marrè and Monroy, 1964) are all activated at fertilization. The experiments by Ishihara on aldolase activity and by Isono on glucose-6-phosphate dehydrogenase activity, before and after fertilization, are of particular interest in the present connection and possibly represent specific examples that have general application.

According to Ishihara, aldolase activity can be released from a low-speed pellet (five minutes at 400xg) of an homogenate of unfertilized sea urchin eggs by a brief treatment with deionized water. Five minutes after fertilization or artificial activation, however, aldolase activity cannot be demonstrated in this pellet, but rather is found in the corresponding supernatant fraction. Isolation of the cortex of unfertilized eggs by treatment with 0.01 N HCl has provided evidence that aldolase may be bound at the egg surface until after fertilization.

Similarly Isono and his collaborators have shown that glucose-6-phosphate dehydrogenase activity is transferred from the low-speed pellet to the supernatant fraction of sea urchin egg-homogenates within five

minutes after fertilization. The enzyme may be released from the pellet either by the addition of salts to the homogenate or by elevation of the pH above 8. The enzyme released by high ionic strength of the homogenate of unfertilized eggs can be made to bind again to the sediment by appropriate dilution of the homogenate. Following the example of Ishihara, Isono and his collaborators suggest that glucose-6-phosphate dehydrogenase is sequestered by attachment to relatively large particles in the unfertilized egg and is, therefore, largely restrained in enzymatic activity. In addition, these investigators speculate that after fertilization the rapid exchange of potassium (Tyler and Monroy, 1959) provides ionic conditions at the site of attachment of the enzyme conducive to the liberation of glucose-6-phosphate dehydrogenase which is then free to act catalytically.

Runnström (1933), attempting to account for the low respiratory rate of unfertilized eggs, suggested that cytochrome oxidase and cytochrome C were physically separated until after fertilization. Later, Runnström, among others (Runnström, 1949, 1956; Runnström,and Immers, 1956; Monroy, 1957) have perpetuated the idea of a barrier between enzyme and substrate as one mode of inhibiting enzyme activity in unfertilized eggs. More recently, however, experiments (Maggio and Monroy, 1959; Maggio, Aiello and Monroy, 1960; see Monroy 1965b) on the respiratory rate before and after fertilization have indicated that homogenate systems of unfertilized eggs may possess a soluble inhibitor of cytochrome oxidase and that this disappears after fertilization. Physical separation of enzymes from their substrate then, may not be the only

mechanism employed by unfertilized eggs to restrain enzymatic activity. The examples of aldolase, glucose-6-phosphate dehydrogenase and uridine kinase (presented in this thesis) would, thus, represent specific cases of the separation theory.

Another mechanism of inhibition of enzyme activity that is especially well documented for microorganisms (see Cold Spring Harbor Symposium Quant. Biol. 26, 1961) is that of end-product, or feed-back, inhibition of enzyme activity. Anderson and Brockman (1964) have demonstrated that the phosphorylation of uridine to its nucleotide derivative by enzyme systems from mouse tumor cells and human epidermoid carcinoma cells may be inhibited by both uridine triphosphate and cytidine triphosphate, as may also the phosphorylation of cytidine. Furthermore, Mitchison and Cummins (1966) showed that fertilized sea urchin eggs are sensitive to feed-back inhibition of cytidine uptake when continuously in the presence of labeled cytidine. End-product inhibition, then, may contribute to the control of uridine kinase activity.

There is, however, no evidence at present that feed-back inhibition suppresses enzymatic activity in unfertilized eggs. One argument against the release of feed-back inhibition of uridine kinase occurring after fertilization is that uridine kinase activity increases as uridine triphosphate accumulates in the egg during the acceleration phase of the rate of uridine uptake. The favored alternative is that uridine kinase is held within the cell until after fertilization. Uridine, therefore, cannot be phosphorylated at the cell surface by unfertilized eggs and thus cannot penetrate into the egg.

PART II

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RNA- and Protein-Synthesizing Capacity of Isolated Oocytes of the Sea Urchin *Lytechinus pictus*[1]

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INTRODUCTION

Due to the increasing number of investigations concerning the activation of protein synthesis at fertilization in sea urchins (e.g., Nakano and Monroy, 1958; Hultin, 1961, 1964; Gross and Cousineau, 1963, 1964; Tyler, 1963, 1965; Monroy and Tyler, 1963; Brachet *et al.*, 1963; Nemer, 1963; Wilt, 1964; Denny and Tyler, 1964; Gross, 1964; Monroy, 1965; Spirin and Nemer, 1965), the types of RNA's synthesized and the process by which the egg suppresses biosynthetic activity during oogenesis, has taken a renewed interest. One of the difficulties of studying the cellular activities involved in oogenesis of most animals is that the growing oocyte is lodged within the ovary where it interacts with somatic ovarian, and other, cells of the organism. Investigations of the metabolic activity of oocytes during oogenesis have, then, been largely restricted to an examination of these cells while still in the whole animal or the excised ovary.

There is evidence (cf. Tyler, 1955; Zalokar, 1960; Brachet, 1960; Raven, 1961; Beams, 1964; Roth and Porter, 1964; Grant, 1965) that, in many species of animals, growth of the oocyte occurs largely through the accumulation of substances that are synthesized in the follicle cells or even in more distant tissues of the body. The present investigation with sea urchins was undertaken because, on occasion, a number of animals upon artificial shedding provided egg suspensions containing unusually high percentages of isolated oocytes. This permitted exploration of the intrinsic synthetic capacity of these cells with

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[2] Damon Runyon Cancer Research Fellow.

respect particularly to RNA and protein. A combination of quantitative and autoradiographic methods was used for this purpose.

A number of studies have been made on the biosynthetic activities of growing oocytes in echinoderms. Starfish oocytes, after being shed from the ovary, have been found to be capable of RNA synthesis (Ficq, 1953; Ficq and Errera, 1955; Geuskens, 1961, 1965) and of protein synthesis (Ficq, 1953; Ficq and Errera, 1955; Monroy and Tolis, 1964). In sea urchins the accumulation of RNA and of protein by oocytes within the ovary has been shown by cytochemical studies (Cowden, 1963; Esper, 1965), autoradiographic investigations (Immers, 1961; Ficq, 1964; Gross *et al.*, 1965b), and direct isolation of RNA made during oogenesis (Gross *et al.*, 1965b; Piatigorsky, in preparation). The labeling of macromolecular constituents of mature eggs of sea urchins has proved feasible by the injection of radioactive precursors into females in process of ripening oocytes (Tyler and Hathaway, 1958), and this applies also to protein (Nakano and Monroy, 1957; Immers, 1959), RNA (Gross *et al.*, 1965b; Piatigorsky, 1965; Tyler and Tyler, 1966), and DNA (Holland and Giese, 1965, for young oocytes).

MATERIALS AND METHODS

Living material. The batches of eggs with large percentages of oocytes were obtained during a 3-week period toward the end (November-December) of the fall spawning period of *Lytechinus pictus*. The eggs were shed by the usual procedure of injection of 0.55 *M* potassium chloride into the perivisceral cavity of the sea urchin. They were passed through bolting silk to remove any debris that may have been released from, or come off, the animals, washed several times in artificial sea water, adjusted to a known volume, and an aliquot counted to determine the total number (Tyler and Tyler, 1966). The experiments on the mature eggs were done at other times when, as is customary during most of the season, very few, if any, oocytes appear in the batches of eggs that are shed.

RNA and protein synthesis. The eggs in artificial sea water were distributed to polystyrene test tubes and preincubated with or without dactinomycin (actinomycin D) at 30 μg/ml. After a specified time, L-valine-^{14}C or uridine-2-^{14}C was added to the eggs. The final concentration of dactinomycin was 22 μg/ml, and the eggs were suspended in a total volume of 0.20 ml. The suspension was incubated at 20°C for various lengths of time as noted in the individual experiments. The

test was terminated by the addition of 1.0 ml of ice cold 0.1 *M* valine-^{12}C or uridine-^{12}C. The eggs were then washed, by centrifugation, three times with ice cold artificial sea water, resuspended in 3 drops of artificial sea water, and placed on filter papers to dry. The papers were then processed for incorporation of uridine-^{14}C into RNA and valine-^{14}C into protein as described previously (Tyler, 1966). Measurements of radioactivity were made with a Packard, TriCarb Scintillation Counter at an efficiency of approximately 50%.

Autoradiography. Radioactive eggs were fixed for 1 hour with several changes of 95% ethanol:glacial acetic acid (3:1). The eggs were then repeatedly washed with 70% ethanol, stained with 0.5% eosin Y in 70% alcohol to facilitate their subsequent identification in the paraffin, dehydrated, and embedded in duPont Paraplast. The cells were sectioned at 5 μ in thickness and mounted on slides coated with albumen. After removal of the paraffin and rehydration of the sections, the slides were dipped in Kodak Nuclear Track Emulsion type NTB 3. Development of the emulsion was done after 4 days for the valine-^{14}C slides and after 21 days for the uridine-^{14}C slides. Sections were then stained through the emulsion with Giemsa stain according to Schmid (1965). Finally, the autoradiograms were mounted in Permount and examined for the localization and number of reduced silver grains.

RESULTS AND COMMENTS

Measurements of Incorporation of Valine-^{14}C and Uridine-^{14}C

Table 1 lists the data obtained in twenty experiments in which incorporation of both valine-^{14}C and uridine-^{14}C were measured and nine experiments in which only valine-^{14}C incorporation was tested. Experiments 1 through 9 were done on suspensions that contained more than 99% mature, fertilizable eggs. In experiments 10 through 20 the suspensions contained large numbers of oocytes, as indicated in the table.

The experiments are listed in an order corresponding to increasing numbers of oocytes in the suspension. The measurements were all done in duplicate, and both determinations for each test are listed in the table. The average value is also given and is placed in parentheses. Column 2 specifies the proportion of oocytes present in a given experiment. The criterion used for scoring oocytes was the presence of a germinal vesicle, irrespective of size of the egg. (In

TABLE 1

INCORPORATION OF VALINE-^{14}C[a] AND URIDINE-^{14}C[b] INTO PROTEIN AND RNA, RESPECTIVELY, BY VARIOUS MIXTURES OF MATURE AND IMMATURE EGGS OF *L. Pictus* IN PRESENCE AND ABSENCE OF DACTINOMYCIN

Expt. No.	Proportion of oocytes in suspension (%)	Labeling time (min)	Preincubation in dactinomycin (min)	Incorporation of valine-^{14}C			Incorporation of uridine ^{14}C		
				Conc. (μC/ml)	Counts per minute per 10^4 eggs in 15 min		Conc. (μC/ml)	Counts per minute per 10^4 eggs in 15 min	
					Control	Dactinomycin		Control	Dactinomycin
1	<1	60	—	0.83	482; 489 (486)[c]	—	—	—	—
2	<1	60	—	0.83	689; 792 (741)	—	—	—	—
3	<1	60	—	0.83	430; 445 (438)	—	—	—	—
4	<1	60	—	0.83	473; 578 (526)	—	—	—	—
5	<1	15	—	0.36	268; 400 (334)	—	—	—	—
6	<1	15	—	0.36	366; 372 (369)	—	—	—	—
7	<1	15	30	2.0	420; 439 (430)	1551; 1659 (1605)	—	—	—
8	<1	60	60	0.72	484; 768 (626)	360; 512 (436)	2.0	5; 2 (4)	0; 0 (0)
9	<1	30	60	0.38	526; 851 (689)	539; 657 (598)	—	—	—
10	(3–5)[d]	30	60	0.50	4168; 4617 (4393)	5265; 6062 (5664)	—	—	—
11	10–15	20	60	2.0	11514; 10998 (11256)	8178; 10905 (9542)	—	—	—
12	15–20	15	60	0.70	3762; 5404 (4593)	3532; 3499 (3516)	—	—	—

13	25	20	60	2.0	6413; 6823 (6618)	4352; 5187 (4770) 6950; 5647 (6299)	—	—	—
14	30	15	60	0.72	3877; 3420 (3649)	3309; 3962 (3636)	2.0	95; 108 (102)	3; 17 (10)
15	50	20	60	2.0	13111; 12647 (12879)	13709; 13051 (13380)	—	144; 252 (198)	8; 7 (8)
16	(50)	60	40	2.0	3157; 3147 (3152)	11706; 9730 (10718)	1.0	19; 18 (19)	12; 17 (15)
17	(50)	60	30	2.0	1137; 1355 (1246)	3860; 4443 (4152)	—	20; 11 (16)	8; 5 (7)
18	(50)	30		2.0	1573; 4026 (2800)	7370; 7020 (7221)	2.0	89; 55 (72)	33; 36 (25)
		30	360		8012; 11749 (9881)	8213; 10184 (9199)	—	287; 149 (218)	72; 64 (68)
19	(50)	15	40	2.0	19713; 20089 (19901)	15809; 14875 (15342) 17974; 19720 (18847)	2.0	87; 60 (74)	43; 32 (40) 8; 20 (14)
20	(50)	15	70	2.0	10967; 13286 (12127)	6821; 6215 (6518) 10962; 8420 (9691)	2.0	106; 143 (125)	30; 26 (28) 16; 9 (13)

[a] Experiment 1 to 4, sp. act. 208.5 cpm; experiment 5, 6, and 17, sp. act. 200 cpm; experiment 7–16 and 18–20, sp. act. 185 cpm.

[b] Sp. act. 30 cpm except experiment 17, sp. act. 25.2 cpm.

[c] Average values given in parentheses in columns 6, 7, 9, and 10.

[d] Estimated values are given in parentheses in column 2.

some experiments the percentage of oocytes in the suspension was estimated, rather than numerically determined by counting eggs, and is, therefore, placed in parentheses.) For purposes of comparison the data have been normalized to incorporation values corresponding to 10^4 eggs labeled for 15 minutes. The actual length of time that the eggs were exposed to the radioactive material for each experiment is given in column 3. In all cases, the eggs treated with dactinomycin were preincubated before exposure to the valine-^{14}C or the uridine-^{14}C for the amount of time designated in column 4. Dactinomycin was present, then, for the sum of the times indicated in columns 3 and 4.

The data listed in column 6 show that incorporation into protein occurs in the various types of suspensions, but to different extent depending upon the proportion of oocytes present. The amount of incorporation of valine-^{14}C by the suspensions containing almost entirely mature eggs was substantially less than in those with oocytes. The average incorporation for the group of experiments (1–9) with predominantly mature eggs, is 2.37 $\mu\mu$moles for 10^4 eggs for 15 minutes while the corresponding average value for the group of experiments (10–20) with suspensions containing various amounts of oocytes is 37.9 $\mu\mu$moles, an increase of approximately sixteenfold. In the mature eggs, in this group of experiments, the amount of incorporation of valine-^{14}C was very much the same in the different experiments, the average values of the experiment with the least incorporation (experiment 5) being 1.52 $\mu\mu$moles and that of the experiment with the highest incorporation (experiment 2) being only 3.23 $\mu\mu$moles. The oocyte suspensions on the other hand, differed considerably in the amount of valine-^{14}C incorporated into protein in the different experiments. For instance, experiment 17 was a suspension of eggs containing about 50% oocytes which incorporated only 6.12 $\mu\mu$moles of valine-^{14}C while experiment 19, which also had about 50% oocytes incorporated 97.8 $\mu\mu$moles of valine-^{14}C. In fact, experiments 10–14 all were suspensions of eggs containing less than 50% oocytes, yet their incorporation values for valine-^{14}C were each greater than experiments 16, 17, or 18 which contained substantially more oocytes. It should be noted that even in experiment 17, which had the lowest valine incorporation of all the tests on the oocyte group, the values were appreciably higher than those of experiment 2, which had the highest value for valine incorporation in the mature egg group. The variation in the ability of the different suspensions of eggs to incorporate valine-^{14}C into protein cannot, then, be explained simply by

the number of oocytes present. Furthermore, it is not explained by differences in time of exposure to the labeled valine or its concentration. The varying amount of incorporation into protein observed in the different experiments in the oocyte group could possibly represent variations in the physiological state of the eggs. This would include variations in the size of the free amino acid pool which, of course, would result in differences in labeling of the new protein rather than differences in synthesis. Other possible interpretations would relate to differences in the size distribution, and of corresponding stage of growth of the oocytes in the different suspensions. Quantitative data pertaining to this possibility were not obtained in these experiments.

In order to obtain further information concerning the question of whether or not the incorporation of the valine-^{14}C into protein represents actual synthesis as contrasted with other kinds of exchange or absorptive processes, sucrose density gradient centrifugation was done with an homogenate of an oocyte suspension. The experiment is illustrated in Fig. 1. The details of the experiment are given in the legend to the figure. The results shown in the upper graph of Fig. 1 demonstrate that radioactivity is associated with particles which sediment faster than single ribosomes in the sucrose gradient. Mild treatment of a portion of the 10,000 *g* supernatant fraction with ribonuclease (lower graph of figure), however, caused this labeled material to disappear from the polyribosome region, and much of it then to coincide in sedimentability with the single ribosomes, which were located in the gradient by their absorbancy at 260 mμ. Thus, the labeled amino acids are incorporated by the eggs upon structures which sediment in a sucrose gradient in a fashion similar to that of the amino acid incorporating structures in fertilized sea urchin eggs (Monroy and Tyler, 1963; Stafford *et al.*, 1964) and other types of cells (Warner *et al.*, 1962; Wettstein *et al.*, 1963; see Rich *et al.*, 1963, for references), namely, polyribosomes. Polyribosomes have been interpreted by the cited authors, as well as by many other investigators, as the principal site of protein synthesis.

Very little, if any, incorporation of uridine-^{14}C into RNA was found in the test with mature eggs as is shown by the values listed in column 9 of Table 1. Only one such test (experiment 8) was done with mature eggs since it has already been shown that mature unfertilized eggs are relatively impermeable to the nucleoside (Piatigorsky and Whiteley, 1965). This impermeability can account for the very low degree of labeling of the RNA by eggs of the mature group. The pos-

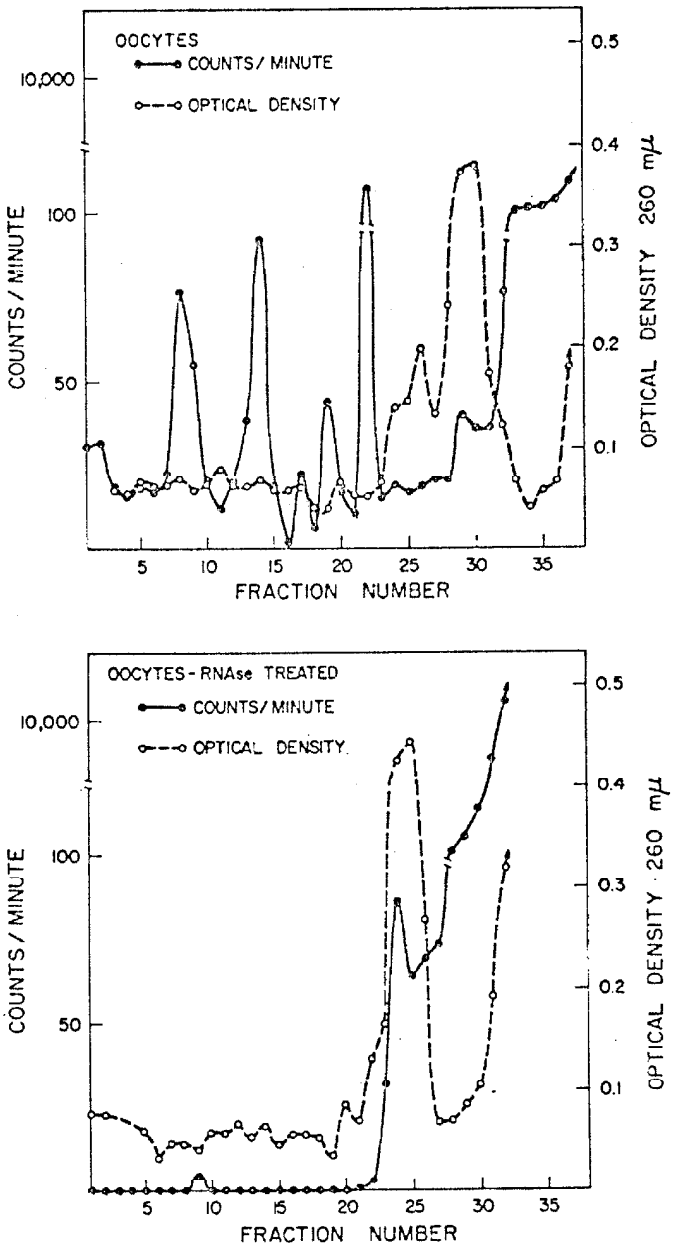

FIG. 1. A suspension of about 5×10^4 eggs of which 50% were oocytes, was exposed to phenylalanine-^{14}C (0.5 μC/ml; specific activity 360 c/M) and valine-^{14}C (0.5 μC/ml; specific activity 185 c/M) for 10 minutes in a total of

sibility still exists that the mature eggs can synthesize RNA. In the suspensions containing the oocytes, however, there was appreciable incorporation of uridine-^{14}C into RNA. There is no direct correspondence in these experiments between the amount of uridine-^{14}C incorporated into RNA and the amount of valine-^{14}C incorporated into protein.

As in valine-^{14}C incorporation, a correlation between the amount of uridine-^{14}C incorporation and the number of oocytes present or the length of time the eggs were incubated with the uridine-^{14}C is not evident. Once again, in addition to fluctuations in nucleoside pools, the variation observed in incorporation into RNA may be due to differences in the size distribution of the oocytes in the various experiments.

In the experiments listed in Table 1 tests were also made of the ability of dactinomycin to interfere with RNA and protein synthesis. The results are listed in columns 7 and 10. In the one experiment on uridine-^{14}C incorporation performed on the mature egg group, dactinomycin had an inhibitory action. However, since the values for incorporation of the label in the controls are very low one cannot be certain if the small suppression is meaningful. In the oocyte group dactinomycin consistently inhibited uridine-^{14}C incorporation into RNA, although it did not abolish it completely. Continuation of the incorporation into RNA in the presence of dactinomycin has been

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5.0 ml of artificial sea water at 20°C. The eggs were then washed twice in ice cold 0.55 *M* KCl, resuspended in 10 volumes of hypotonic homogenization buffer (0.004 *M* $MgCl_2$, 0.025 *M* KCl, 0.005 *M* β-mercaptoethanol, and 0.05 *M* Tris, pH 7.6), allowed to cytolyze at 0–4°C with occasional gentle shaking over a period of 30 minutes, and centrifuged for 10 minutes at 10,000 *g*. The supernatant fraction was removed and divided into two equal samples. To one (lower graph) ribonuclease (Sigma Chemical Corp., bovine pancreas RNase, 5 times crystallized) was added to give a final concentration of 2 μg/ml. To the other an equivalent volume of homogenization buffer was added. The samples were incubated at 4° for 75 minutes. Sodium deoxycholate (0.5% final concentration) was added to both samples. The material was layered on top of a 15–30% (w/v) sucrose gradient in homogenization buffer, lacking mercaptoethanol, and centrifuged at 28,000 rpm for 90 minutes in a Spinco SW 39 rotor. After centrifugation, the bottom of the tube was punctured and 2-drop fractions were collected, diluted with 8 drops of distilled water and absorbancies at 260 mμ measured with a Beckman DU spectrophotometer. Direct measurements of radioactivity were then made on the same samples by scintillation counting. The difference in the location of the peak of the single ribosomes in the two graphs is due to differences in the total number of fractions collected.

shown to occur also for cytidine-^{3}H and methylcytosine-5-^{3}H in oocytes of *Paracentrotus lividus* (Ficq, 1964). This possibly represents terminal labeling of transfer RNA as has been reported for fertilized sea urchin eggs (Glisin and Glisin, 1964; Malkin *et al.*, 1964; Gross *et al.*, (1965a).

Dactinomycin had no appreciable inhibiting effect on valine-^{14}C incorporation into protein in the 14 tests listed in Table 1 with either mature or immature egg suspensions. But dactinomycin suspensions induced a three- to fourfold stimulation of incorporation into protein on one occasion with the mature eggs (experiment 7) and in three separate tests with the oocyte-suspensions (experiments 16, 17, 18). Autoradiographs, not presented in this paper, of the eggs of experiment 16 confirmed the increased incorporation of valine-^{14}C into protein as a result of dactinomycin treatment. In another experiment done during the time interval when we were observing the erratic dactinomycin stimulation effect, we preloaded a suspension of eggs containing about 50% oocytes with valine-^{14}C and exposed half of these eggs to dactinomycin for varying lengths of time while the other half remained in artificial sea water. Again, the dactinomycin-treated eggs incorporated approximately 3 times more valine-^{14}C into protein than did the control eggs if the dactinomycin was present longer than 20 minutes. Evidently, then, the stimulation by dactinomycin could not be accounted for by a change in the permeability of the eggs to valine. It should also be noted that in our experiments that showed a stimulating effect of dactinomycin on incorporation of valine, as well as in those experiments in which no stimulation occurred, there was the usual marked inhibition of incorporation of uridine into RNA.

The experiments (7, 16, 17, and 18 of the table) in which stimulation by dactinomycin occurred were the first ones that were done in this series. We have attempted to recapture this phenomenon by repeating the tests under a variety of conditions affecting the nutritional status of the eggs. Since there has been a report (Honig and Rabinovitz, 1965) that glucose can relieve dactinomycin inhibition of protein synthesis in sarcoma-37 cells, we incubated eggs in the presence and absence of glucose while testing for the dactinomycin stimulation. Glucose had no effect on either RNA or protein synthesis nor on the effect of dactinomycin on these processes. Next we explored the possibility that the size of the free amino acid pool might have been a factor in the dactinomycin stimulation of protein synthesis. These experiments proved also to be negative, but they did demonstrate the

existence of a competition among amino acids for entrance into the cell and this study has been reported elsewhere (Tyler *et al.*, 1966). While we cannot assess at this time the significance of the stimulation of protein synthesis by dactinomycin that we have observed, it should be noted that there have been a number of other publications reporting increased protein and enzyme synthesis (Pollock, 1963; Coleman and Elliot, 1964; Rosen *et al.*, 1964; Garren *et al.*, 1964; Papaconstantinou *et al.*, 1966). In the sea urchin, a slight increase by dactinomycin, rather than the normal developmental decrease, in deoxycytidylate aminohydrolase activity has been demonstrated (Scarano *et al.*, 1964). This has been interpreted by the investigators as due to the suppression of the synthesis of an inhibitor.

Autoradiographic Examination

Samples of eggs from experiment 16 were prepared for autoradiography as described under Materials and Methods. Illustrative sections are shown in Fig. 2. In the examination of the slides the mature eggs could be easily distinguished from the oocytes by the large size of the nucleus in sections of the latter type. The results of scoring these preparations are consistent with those reported above. Figures 2b, 2d, and 2f show mature eggs, with small nucleus, while Figs. 2a, 2c, and 2e show oocytes, with large germinal vesicle. These representative sections show very little radioactivity in a mature egg treated with valine-^{14}C (Fig. 2b) in contrast to the heavy labeling of the cytoplasm and nucleolus, in an oocyte (Fig. 2a). Since mature eggs are known to be adequately supplied with sRNA and activating enzymes (Scarano and Maggio, 1957; Hultin, 1961; Maggio and Catalano, 1963) and fail to show radioactivity it is unlikely that the labeled material in the oocytes is valyl-sRNA, but rather protein, as is the customary interpretation of the results of this procedure. Also the section of a mature egg treated with uridine-^{14}C (Fig. 2d) shows no detectable labeling while that of an oocyte (Fig. 2c) is heavily labeled in the nucleolus. The labeling of the oocyte by uridine-^{14}C is almost entirely eliminated by dactinomycin (Fig. 2e).

It should be noted that not all the oocytes examined in either the uridine or valine series were intensely labeled. Furthermore, some mature eggs showed considerable incorporation of valine-^{14}C. The following figures give the results of scoring the intensity of radioactivity of a number of eggs in which the different types could be readily distinguished. For the present purpose no attempt was made

Fig. 2. Autoradiographs of sections of mature, unfertilized eggs (b, d, and f) and oocytes (a, c, and e) of Lytechinus pictus exposed to valine-^{14}C (a and b) or uridine-^{14}C in the presence (e and f) or absence (c and d) of dactinomycin. The details of the procedure used are given under Materials and Methods.

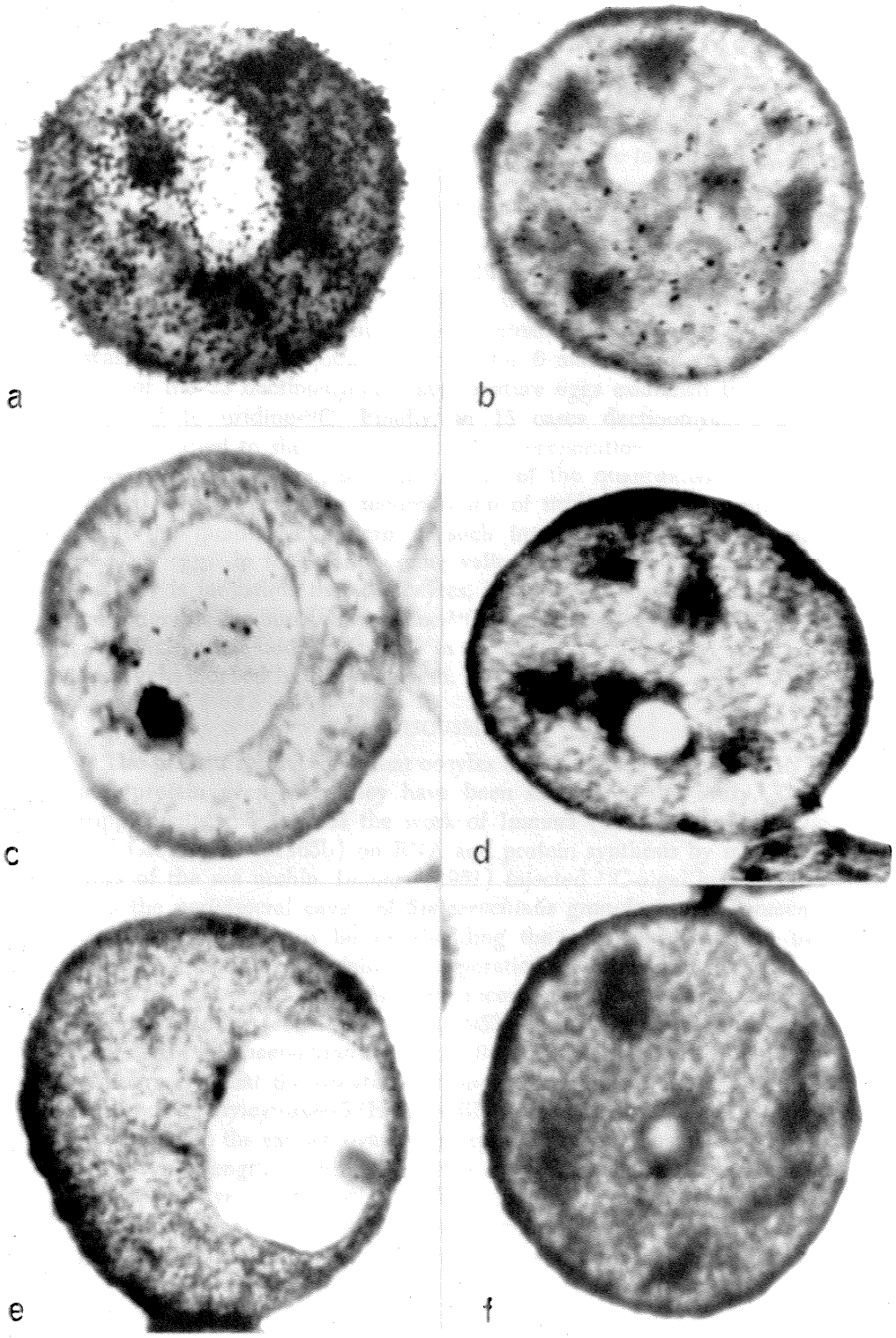

Figure 2.

to distinguish among oocytes of various sizes. In 24 oocytes, 5 did not incorporate any valine-^{14}C, 7 showed moderate incorporation, and 12 were very radioactive. Examination of 14 mature eggs revealed 4 eggs with significant incorporation of valine-^{14}C, 8 eggs with some radioactivity, and only 2 eggs without any ^{14}C incorporation. An examination of 15 oocytes of the uridine-^{14}C set showed that all were highly radioactive and that the label was localized in the germinal vesicle. Of these, 7 were labeled predominantly in the nucleolus. There was no radioactivity found in any of the 6 mature eggs scored or in any of the 23 dactinomycin-treated mature eggs examined that were exposed to uridine-^{14}C. Finally, in 15 cases dactinomycin-treated oocytes failed to show any uridine-^{14}C incorporation.

It is apparent, then, by combination of the quantitative data obtained (Table 1) on the incorporation of uridine-^{14}C and valine-^{14}C with the qualitative pattern of such incorporation (Fig. 2), that mature eggs do incorporate some valine-^{14}C into proteins, but their activity is far below that of oocytes. In addition, oocytes incorporate considerable amounts of uridine-^{14}C into RNA, principally in the germinal vesicle and particularly in the nucleolus. This RNA synthesis is largely blocked by dactinomycin.

DISCUSSION

The present results show that oocytes of sea urchins synthesize RNA and protein even after they have been shed from the ovary. This supplements and extends the work of Immers (1961), Ficq (1964), and Gross *et al.* (1965b) on RNA and protein synthesis by maturing eggs of the sea urchin. Immers (1961) injected ^{14}C-algal hydrolyzate into the perivisceral cavity of *Sphaerechinus granularis* and *Paracentrotus lividus* 4 hours before shedding the eggs and observed, by autoradiography, appreciable incorporation of labeled materials by the growing oocytes but only slight incorporation by the mature eggs of *Paracentrotus* (see also Immers, 1959) and no incorporation by the ripe eggs of *Sphaerechinus*. Ficq (1964) demonstrated, also by autoradiography, that the oocytes of *Paracentrotus* incorporated cytidine-^{3}H and methylcytosine-5-^{3}H into RNA and phenylalanine-^{14}C into protein when the excised ovary was exposed to the radioactive isotopes for varying lengths of time. The mature eggs, in contrast, were much less radioactive. Gross *et al.* (1965b) studied the pattern of incorporation of uridine-^{3}H into the RNA of maturing oocytes of *Arbacia punctulata* which had been injected with the isotope 1 week before

examination. Autoradiography of the sectioned ovaries from the radioactive females showed that the small oocytes were intensely labeled while most of the mature eggs had not incorporated the uridine into RNA. Furthermore, eggs closer to the ovarian wall were more radioactive than those found at an intermediate position in the acinus of the ovary or in the central lumen. The kinds of labeled RNA's that were extracted from eggs that were shed from these animals were characterized as 28 S and 18 S ribosomal RNA, 4 S transfer RNA as well as a third type sedimenting in a sucrose gradient between the 28 S and 18 S species, which was interpreted as messenger RNA on the basis of its high specific activity. It is presumed that these RNA's become labeled during the terminal stages of maturation of a small proportion of the eggs which occurred during the time that the uridine-^{3}H was present. The results of experiments of this type, which have been going on here for some time and which are the subject, so far, of only brief reports (Piatigorsky, 1965; Tyler and Tyler, 1966, p. 1232) largely confirm the findings of Gross *et al.* (1965b).

The present quantitative results with the suspensions containing isolated oocytes accord with other investigations on sea urchins, cited above, with respect to the synthetic activity of the oocytes in the ovary, the relative quiescence of the mature eggs, and the response of the oocytes to dactinomycin. In studies on oocytes of other groups of animals, suppression of RNA synthesis by dactinomycin has been observed by Izawa *et al.* (1963) in isolated oocytes of *Triturus viridescens* and by Geuskens (1965) in isolated oocytes of *Asterias rubens.*

Our autoradiographic results also are largely in agreement with those of Immers (1961), Ficq (1964), and Gross *et al.* (1965b). Immers (1961), however, found that incorporation of ^{14}C-algal hydrolyzate was confined to the nucleolus, especially the outer rim of the nucleolus, and the germinal vesicle. In riper oocytes, though, the label occurred also in the cytoplasm. In the present experiments, the autoradiographs of oocytes that had incorporated uridine-^{14}C or valine-^{14}C have shown uridine incorporation to be primarily within the nucleolus and valine incorporation to be principally in the cytoplasm and nucleolus. The mature eggs, on the other hand, exhibit very little incorporation of the radioactive materials.

Incorporation of radioactive isotopes into macromolecules does not constitute a proof that synthesis is taking place. However, in these experiments uridine-^{14}C incorporation into the nucleolus was effec-

tively prevented by dactinomycin and valine-^{14}C incorporation occurred on polyribosomes, which is additional evidence that the incorporation, in fact, represents actual synthesis.

Some of the most extensive recent investigations of macromolecular synthesis in oocytes have been done in amphibians. Although with one exception (Izawa *et al.*, 1963) the experiments were done *in vivo* or with pieces of ovary, the results are similar to those obtained with echinoderms and one may consider that the statements pertain to synthetic activity of the oocytes themselves. Thus, RNA is synthesized primarily in the nucleus, especially in the nucleolus (Ficq, 1961, 1962, 1964; Geuskens, 1961; Ozban *et al.*, 1964), and along the loops of the lampbrush chromosomes (Brachet and Ficq, 1956; Gall and Callan, 1962; Izawa *et al.*, 1963). Furthermore, it has been shown (Brown and Littna, 1964; Davidson *et al.*, 1964) that the RNA made during the course of oogenesis is principally 28 S and 18 S ribosomal RNA along with some 4 S transfer RNA and slight quantities of heterogeneously sedimenting, DNA-like RNA. Amphibian oocytes are correspondingly active in protein synthesis (Ficq, 1961, 1964; Pantelouris, 1958; Geuskens, 1961). These studies have also shown that neither RNA nor protein synthesis occur to any appreciable extent after the oocyte has matured.

The intense labeling of the oocyte nucleolus with RNA precursors appears to be a general phenomenon and has been noted in many species (Vincent, 1955; Ficq, 1953, 1961, 1964; Zalokar, 1962, 1965; Ozban *et al.*, 1964). There is now considerable information about the synthetic activity of the nucleoli of various types of cells (Ficq, 1955a,b, 1966; Vincent, 1954, 1957; Pantelouris, 1958; Vincent and Baltus, 1960a,b; Immers, 1961; Sirlin, 1962; Sirlin *et al.*, 1963; Birnstiel *et al.*, 1963; Brandt and Finamore, 1963; Brown and Gurdon, 1964; Gurdon and Brown, 1965; Perry, 1965). The subject cannot be reviewed here, but it may be noted that the oocyte nucleolus is rather special in being of exceptionally large size. One possibility is that it is a trap for most of the RNA that is being synthesized during oogenesis.

A comparison of the average value of the incorporation of valine-^{14}C into protein of the suspensions of eggs containing almost exclusively mature eggs with those containing varying proportions of oocytes shows an approximately sixteenfold decrease in protein synthesis upon maturation. This means that the oocytes are, on the average, at least as active in valine incorporation into protein as are

the fertilized eggs of *Lytechinus* which show from three- to tenfold (Denny and Tyler, 1964; Timourian and Denny, 1964) to as much as twenty-threefold (Tyler *et al.*, 1966) enhancement of incorporation of this amino acid upon fertilization. One of the experiments (experiment 19) listed in Table 1 of this communication, however, shows almost 40 times more valine incorporation by the oocytes than by the average incorporation for the mature eggs. This represents a suspension of eggs containing only approximately 50% oocytes. Comparing equal numbers of cells, then, the oocytes in this experiment were about 80 times as active as the mature eggs. In fact, per unit volume the oocytes incorporated valine even more actively than 80 times the rate of the mature eggs. Evidently, oocytes of sea urchins go through stages during their growth of extremely intense protein synthesis, significantly greater than that of fertilized eggs.

These comparisons neglect possible variations in the size of the free amino acid pool and differences in rate of uptake of amino acids. Probably, fluctuations in pool size account for differences that are found in incorporation of labeled amino acids by different lots of eggs and under different conditions. The values for incorporation of valine-^{14}C by the mature eggs, however, were all significantly less than those obtained with the oocytes. This indicates that variations in the amino acid pool within the two types of suspensions do not account for the differences observed in incorporation. It seems reasonable to assume that oocytes differ from mature eggs also in rate of uptake of amino acids, just as fertilized differ from unfertilized eggs (Mitchison and Cummins, 1966; Tyler *et al.*, 1966). In the case of fertilization, changes in permeability to amino acids do not account for changes in incorporation (Nakano and Monroy, 1958). Very likely, then, the same is true for a comparison between oocytes and mature eggs, but information is not available to enable quantitative assessment to be made of the relative importance of uptake differences between mature eggs and oocytes.

From the present experiments and those cited in the literature, that were done with whole ovaries or *in vivo*, one may conclude that the ripening sea urchin egg synthesizes some, if not most of the materials necessary for growth and for utilization by the fertilized egg. For many other animals, as noted in the Introduction, evidence has accumulated indicating that there is transport of substances into the oocyte from the ovarian cells. That some materials are probably transferred from other tissues to the oocyte also in sea urchins is strongly

suggested by electron micrographs, taken by Dr. Patricia Harris (personal communication), in which ovarian cells, or cellular components, have evidently been engulfed by growing oocytes. Transfer of materials in sea urchins is further suggested by the notable synthetic activity of ovarian cells other than oocytes (Immers, 1961; Ficq, 1964; Gross *et al.*, 1965b; Piatigorsky, unpublished).

In conclusion, then, shed intact oocytes of *Lytechinus pictus* synthesize RNA and protein. RNA synthesis is greatly reduced by dactinomycin but protein synthesis is not. Uridine-^{14}C incorporation into RNA during relatively brief exposures to the isotope is mainly restricted to the nucleolus. Along with the evidence from other sources, one may assume that synthesis, rather than simply accumulation of the RNA, probably occurs in the nucleolus. Protein synthesis takes place primarily in the cytoplasm and nucleolus of the oocyte upon polyribosomes.

SUMMARY

Uridine-^{14}C and valine-^{14}C incorporation into macromolecules by suspensions of eggs of *Lytechinus pictus* containing varying proportions of oocytes were measured by scintillation counting and were localized within the cells by autoradiography. Mature eggs incorporated almost no uridine-^{14}C while oocytes incorporated appreciable amounts. Valine-^{14}C incorporation by suspensions of mature eggs in 9 experiments, ranged from 1.91 $\mu\mu$moles to 3.39 $\mu\mu$moles, and averaged 2.37 $\mu\mu$moles per 10^4 eggs in 15 minutes. The corresponding values obtained in 10 experiments with suspensions containing various amounts of oocytes ranged from 6.12 $\mu\mu$moles to 97.8 $\mu\mu$moles and averaged 37.9 $\mu\mu$moles, namely, approximately 16 times that of the mature eggs. Dactinomycin significantly inhibited uridine-^{14}C incorporation but did not suppress valine-^{14}C incorporation by the mature eggs or the oocytes. In four of the experiments (one with a mature egg suspension) dactinomycin stimulated valine-^{14}C incorporation by three- to fourfold but in 10 others (two with mature egg suspensions) there was no significant difference from the control.

Additional evidence that the incorporation of valine-^{14}C represents synthesis rather than exchange or absorptive processes was provided by a demonstration that it takes place on polyribosomes.

Autoradiographs confirmed that the mature eggs did not incorporate uridine-^{14}C and incorporated only slight quantities of valine-^{14}C, even in the mixed suspensions with oocytes. The oocytes incorporated

uridine-^{14}C primarily in the nucleolus, and this was prevented by dactinomycin. Valine-^{14}C was incorporated, by the oocytes, principally in the cytoplasm and nucleolus.

In sea urchins then, the oocytes appear to undergo a considerable amount of intrinsic protein and RNA synthesis. This is in contrast to the situation in many other organisms in which the oocyte apparently obtains much material from other tissues.

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PART III

LONG-TERM IN VIVO LABELING OF RNA OF SEA URCHIN EGGS DURING OOGENESIS

INTRODUCTION

In studies with sea urchins it is often of considerable value for the investigator to have available unfertilized eggs in which one or more specific substances have been radioactively labeled. The low metabolic activity of the unfertilized egg and its relative impermeability to many substances of biological interest hamper attempts at direct labeling of the shed eggs. For instance, uptake of phosphate (Whiteley, 1949; Litchfield and Whiteley, 1959; Whiteley and Chambers, 1961), nucleosides (Piatigorsky and Whiteley, 1965, see Section 1 of Part I of this thesis; Mitchison and Cummins, 1966; Siekevitz, Maggio and Catalano, 1966) and amino acids (Mitchison and Cummins, 1966; Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis) is suppressed in unfertilized eggs, as is also their utilization until after fertilization (see Monroy, 1965a; Tyler and Tyler, 1966b, for reviews).

It has, however, been shown (Tyler, 1949; Tyler and Tyler, 1966b) that sea urchins, after having been induced to spawn by potassium chloride-injection, can produce additional batches of ripe eggs in the laboratory. The yield after ten days to two weeks may often approach the quantity originally obtained. It is, then, possible to label eggs during oogenesis as has been done in various other animals particularly among the mammals, birds, amphibians, and insects (see Discussion for references) but with the specific advantages that sea urchin eggs provide.

This method was first applied in experiments (Tyler and Hathaway, 1958) to label, with S^{35}, the gelatinous coat (fertilizin) of the egg. Another in vivo procedure without preliminary shedding of the eggs consisted of a four hour incubation of the injected female for labeling of protein (Nakano and Monroy, 1957, 1958; Immers, 1959, 1961; Erb and Maurer, 1962) and of polysaccharides (Immers, 1961). In more recent studies, Gross, Malkin and Hubbard (1965) have again used the longer labeling periods and report effective in vivo labeling of RNA with H^3-uridine or P^{32}-phosphate in a period of one week. Also attempts have been made by Holland and Giese (1965) to label the DNA of unfertilized eggs by long periods of maintenance of the injected animals. Only the small oocytes were found to be radioactive in those experiments; but later experiments by Piko, Tyler and Vinograd (1967) have shown that DNA of ripe eggs can be labeled by the long-term incubation of injected animals in the laboratory.

The present report is for the purpose of demonstrating the effectiveness of the labeling that can be accomplished in sea urchins by the long-term procedure, and of providing some of the parameters for incorporation of C^{14}- and H^3-uridine into the RNA of the ripe unfertilized egg. The results of one experiment of this type have been reported elsewhere (Tyler and Tyler, 1966a, p. 644).

MATERIALS AND METHODS

1) Injection and Care of the Animals

Lytechinus pictus and Strongylocentrotus purpuratus females were induced to spawn most, or all, of their mature eggs by injecting about 0.5 ml of 0.55 M potassium chloride into their perivesceral cavity. The sea urchins were kept continually moist with artificial sea water in order to prevent injury by dehydration of the external epidermis or the gills (Tyler and Tyler, 1966a). This was accomplished either by immersing the sea urchin in sea water every few minutes or by allowing the animal to shed in a moist chamber. Injections were done with a small hypodermic needle (30 gauge, 3/8 inch) carefully inserted through the peristome surrounding the mouth.

One or two days after being shed, the sea urchins were injected with 0.4 to 0.6 ml of C^{14}- or H^{3}-uridine in artificial sea water, at concentrations specified in the individual experiments. The equipment for maintaining the animals was that described by Tyler and Tyler (1966a). The sea urchins were placed, in pairs, in covered, transparent plastic boxes (16 cm x 35 cm) containing about 1500 ml of artificial sea water. They were fed eel grass (Zostera). Gentle rocking was provided to allow for aeration and circulation of the water, and constant illumination provided so as to supply additional oxygen by the photosynthetic activity of the eel grass. The constant illumination is thought also to retain the animals in gamete-ripening condition. The temperature in the room was kept below the critical limits for the species but high

enough to permit reasonably rapid maturation of the gametes; namely, 20°C for Lytechinus pictus and 15°-17°C for Strongylocentrotus purpuratus. Lytechinus pictus proved to be the hardier of the two species. With Strongylocentrotus purpuratus there were fewer long-term survivors and thus only a few experiments are reported for this sea urchin. The water was changed approximately every two weeks and new eel grass was added at that time. Algae would grow along the sides of the boxes and this was allowed to remain.

As has been noted elsewhere (Tyler and Tyler, 1966a) sea urchins often die even under such optimal laboratory conditions present at marine stations. Probably due to the precautions taken in the initial handling of the sea urchins, only few animals died in the present experiments. Sea urchins that survived the first week in the plastic boxes seldom died thereafter even after incubations that lasted longer than one year. It appears, then that the sea urchins can readily adapt to these conditions if they are not damaged during or after their collection.

2) Assay of the labeled material in the shed eggs

Eggs were obtained from the sea urchins by potassium chloride-induced spawning at various times after the labeling injection. The suspensions were screened for oocytes and if more than 1% were present the suspension was discarded. This occurred in very few cases.

For assay the eggs were washed thoroughly, before and after dejellifying at pH 5, the suspension adjusted to 10.0 ml and 8 equal aliquots removed. Six of these were placed on strips of filter paper and

allowed to dry. The other two samples were added to an equal volume of 0.6 M potassium hydroxide, incubated at 37°C for 18 hours and then dried on filter paper strips. Two filter papers (not treated with potassium hydroxide) were assayed directly for radioactivity with a Packard, Tricarb Scintillation Counter. Four strips, including the two containing the eggs subjected to alkaline hydrolysis, were processed for incorporation of C^{14}- or H^{3}-uridine into nucleic acid. Finally, the remaining two filter papers were processed for incorporation of label into protein. The methods for these various measurements have been described elsewhere (Tyler, 1966).

3) Extraction of RNA

RNA was extracted from the unfertilized eggs labeled during oogenesis by a procedure quite similar to that utilized by Gross, Malkin and Hubbard (1965). RNA-labeled unfertilized eggs were homogenized in 0.01 M sodium acetate, pH 5.0, containing 10^{-3} M magnesium chloride, 2% sodium dodecyl sulfate, 0.5% naphthalene disulfonic acid and 0.3% purified bentonite. An equal volume of phenol (Mallinckrodt Chemical Co.), supplemented with 0.1% 8-hydroxyquinoline and saturated with 0.01 M sodium acetate buffer (pH 5.0), was added to the homogenate. The mixture was mechanically shaken at 4°C for 20 minutes, centrifuged and the phenol phase removed. The phenol extraction was repeated three times, the aqueous phase set aside and the interfacial gel reextracted with a 0.01 M solution of Tris-hydrochloric acid at pH 7.4. The resulting aqueous phase was added to the original one and the RNA was precipitated with 66% (v/v) ethanol and 0.1 M sodium chloride overnight at -20°C.

The precipitate was washed in absolute alcohol, dried from ether and redissolved in 0.01 M potassium acetate, pH 5.2 containing 10^{-3} M magnesium chloride and 15 µg/ml of DNase (Worthington, electrophoretically pure). The solution was incubated at 4°C for 30 minutes followed by numerous extractions with buffer-saturated phenol a 4°C until an interface was no longer visible. The aqueous phase was then precipitated as above, washed with absolute alcohol, with ether, air-dried and redissolved in 0.01 M sodium acetate buffer.

The labeled material thus obtained could be made completely soluble in ice-cold 5% trichloroacetic acid by treatment with RNase (50 µg/ml, 37°C for 30 min.) or hydrolysis with potassium hydroxide (0.3M, 37°C for 18 hr.) or hot trichloroacetic acid (5%, 90-100°C for 15 min.). It had a 260 mµ/280 mµ absorption ratio close to 2.

The sedimentation pattern of the labeled RNA was examined by sucrose density-gradient centrifugation under the conditions specified in the section on Results. Sedimentation coefficients have been ascribed as approximate values by analogy with those determined by Slater and Spiegelman (1966a). They compared the rate of sedimentation of RNA extracted from sea urchin eggs with that of 23S and 16S RNA from Bacillus megaterium. The sedimentation coefficients in the present study when calculated by the method of Martin and Ames (1961), are in close agreement with those of Slater and Spiegelman (1966a).

RESULTS

1) Retention of radioactivity by Lytechinus pictus after receiving an injection of H^3-uridine.

To test the retention of radioactively labeled uridine injected into the body cavity of Lytechinus pictus, the following experiment was performed. Two sea urchins, weighing approximately 15 grams apiece, were each injected with 125 µc of H^3-5-uridine in 0.5 ml artificial sea water. They were placed into the same plastic box containing 1500 ml of artificial sea water but lacking eel grass. The sea urchins were kept rocking gently, as usual, and duplicate 0.5 ml samples of the medium were removed and assayed for radioactivity at the indicated times. The sea water was not changed for the three week duration of the experiment. The results are illustrated in Fig. 1.

For the first 12 hours less than 1% of the injected radioactivity appeared in the medium. By 24 hours, however, almost 5% of the label introduced into the animals could be accounted for in the sea water. This value remained approximately constant for 21 days. A slight decrease was observed in the third week. It is evident then that sea urchins retain most of the nucleoside introduced into their body cavity under these conditions. It is of interest to note at this time that Erb and Maurer (1962) injected H^3-leucine and H^3-lysine into the body cavity of the sea urchin Psammechinus miliaris and observed that only 0.1% of the label escaped from the animal after 4 hours in 45 ml of sea water.

FIG. 1. RETENTION OF RADIOACTIVITY BY LYTECHINUS PICTUS AFTER RECIEVING AN INJECTION OF H^3-5-URIDINE. Two females were spawned of their mature eggs by potassium chloride followed by injection with 125 μc of H^3-5-uridine (sp. act. 25,000 c/M) in 0.5 ml artificial sea water. They were placed in the same plastic box (16 cm x 35 cm) containing 1500 ml artificial sea water and incubated as given in Materials and Methods. Duplicate 0.5 ml aliquots were removed at the specified times and assayed for radioactivity by scintillation counting as given in Materials and Methods.

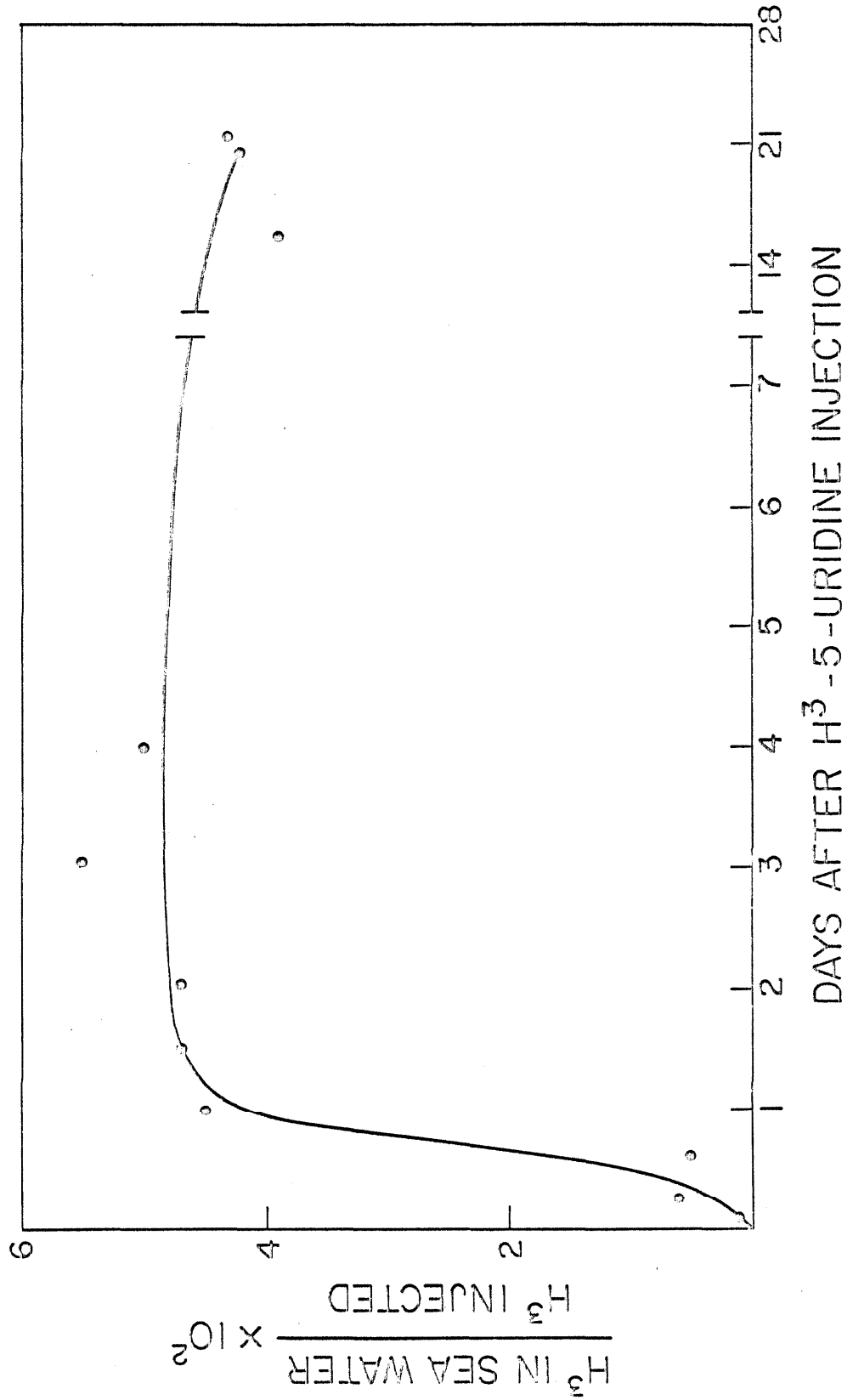
$\frac{H^3 \text{ IN SEA WATER}}{H^3 \text{ INJECTED}} \times 10^2$
6
4
2
1
2
3
4
5
6
7
14
21
28
DAYS AFTER H^3-5-URIDINE INJECTION

Figure 1.

2) Uptake and incorporation of C^{14}- and H^3-uridine by maturing oocytes of Lytechinus pictus and Strongylocentrotus purpuratus.

The data obtained from the radioactivity-measurements of the shed labeled, mature eggs of Lytechinus pictus and Strongylocentrotus purpuratus are given in Table I. The experiments are grouped according to the type of injected isotope (column 2). They are listed within each group in an order corresponding to longer intervals of labeling (column 4). The radioactive measurements were done in duplicate and both values are reported (columns 6, 7, 8 and 9). The data have been adjusted, for comparative purposes, to relate to 10^4 eggs. The actual number of eggs that the animal shed is given in column 5. The data in column 10 list the amount of recovery of the injected label obtained in the shed eggs. The values in column 11 show the ratio of the label incorporated into an acid-insoluble form to the total label accumulated by the maturing eggs. Finally, the sensitivity of the incorporated label to acid and alkaline hydrolysis provides information regarding the types of macromolecules into which the label has become incorporated (columns 12 and 13, respectively).

The values listed in column 10 of the table show that the recovery of the injected radioactivity in the shed eggs of Lytechinus pictus ranged from 0.09% for 24 days labeling (experiment 6) to 32.0% for 40 days labeling (experiment 20). For Strongylocentrotus purpuratus the range was from 0.20% for 16 days labeling (experiment 28) to 10.7% for 13 days labeling (experiment 24).

Since there are many variables that can affect the results of experiments of this type, it was not surprising to find that the percentage of recovery of the label differed considerably in different tests. For

TABLE I

Uptake and Incorporation of C^{14}-and H^{3}-Uridine by Maturing Oocytes of the Sea Urchins Lytechinus pictus and Strongylocentrotus purpuratus During Long-Term in vivo Labeling of Spawned Females. Each Animal Received Only One Injection and Was Maintained Under the Conditions Specified in Materials and Methods.

Expt. No.[b]	Isotope (c/M)	Conc. in μc/ml	Days labeled	No. of eggs shed	CPM per 10^4 eggs				% Recovery of injected radioactivity	Cold TCA / No TCA	Hot TCA / Cold TCA	KOH / Cold TCA
					No TCA[a]	Cold TCA	Hot TCA	KOH				
1	C^{14}-2-uridine (25.2)	10	3	5.0×10^4	23059 25602	3407 3320	1280 1389	-	1.10	0.14	0.40	-
2	C^{14}-2-uridine (25.2)	10	8	8.0×10^5	2006 2037	868 861	-	-	1.47	0.43	-	-
3	C^{14}-2-uridine (25.2)	10	9	1.5×10^5	787 1200	143 190	-	-	0.13	0.17	-	-
4	C^{14}-2-uridine (25.2)	10	15	3.0×10^5	4872 4842	2618 2840	1400 1790	-	1.32	0.56	0.58	-
5	C^{14}-2-uridine (25.2)	10	20	8.0×10^5	1158 1177	300 309	33 33	-	0.84	0.26	0.11	-
6	C^{14}-2-uridine (25.2)	10	24	1.3×10^5	697 675	109 105	22 13	-	0.09	0.16	0.17	-
7	C^{14}-2-uridine (25.2)	10	28	8.2×10^4	2768 3045	416 374	28 35	-	0.21	0.14	0.08	-
8	H^{3}-6-uridine (6550)	100	7	3.8×10^4	22974 22526	7711 7618	1158 1026	-	2.00	0.34	0.14	-
9	H^{3}-6-uridine (6550)	100	9	4.6×10^4	1730 1348	1048 804	213 235	-	0.16	0.60	0.24	-

Table I (continued)

Expt. No.[b]	Isotope (c/M)	Conc. in μc/ml	Days labeled	No. of eggs shed	CPM per 10^4 eggs				% Recovery of injected radioactivity	Cold TCA / No TCA	Hot TCA / Cold TCA	KOH / Cold TCA
					No TCA[a]	Cold TCA	Hot TCA	KOH				
10	H^3-6-uridine (6550)	100	10	8.8×10^4	43918 41827	26818 30245	2490 1827	-	8.57	0.67	0.08	-
11	H^3-6-uridine (6550)	100	13	1.5×10^5	13167 10680	5100 5893	1907 2013	-	4.06	0.46	0.36	-
12	H^3-6-uridine (6550)	150	15	5.0×10^5	20980 22704	14432 14820	10976 8904	-	16.5	0.67	0.68	-
13	H^3-6-uridine (6550)	100	22	8.0×10^4	522 512	307 428	189 286	-	2.35	0.71	0.65	-
14	H^3-6-uridine (6550)	150	28	3.0×10^5	43400 42693	38913 40760	1367 1127	553 427	19.6	0.93	0.03	0.01
15	H^3-5-uridine (25,000)	150	25	7.7×10^5	19340 19223	14304 14065	925 901	483 644	22.5	0.74	0.06	0.04
16	H^3-5-uridine (25,000)	150	31	6.6×10^5	14597 15239	16724 16418	1212 1244	564 664	14.9	1.11	0.07	0.04
17	H^3-5-uridine (25,000)	120	33	2.0×10^5	59190 70310	69120 64940	2030 2370	1360 990	24.5	1.04	0.03	0.02
18	H^3-5-uridine (25,000)	150	34	5.3×10^5	32333 27876	31790 38704	1894 2075	1007 1007	24.4	1.17	0.06	0.03
19	H^3-5-uridine (20,000)	120	36	6.4×10^5	7581 7997	6750 7000	575 744	200 284	9.44	0.88	0.10	0.04
20	H^3-5-uridine (20,000)	120	40	2.3×10^5	74061 73070	57504 57078	3643 3487	1348 1409	32.0	0.78	0.06	0.02

Table I (continued)

Expt. No.[b]	Isotope (c/M)	Conc. in μc/ml	Days labeled	No. of eggs shed	CPM per 10^4 eggs: No TCA[a]	Cold TCA	Hot TCA	KOH	% Recovery of injected radioactivity	Cold TCA / No TCA	Hot TCA / Cold TCA	KOH / Cold TCA
21	H^3-5-uridine (20,000)	120	61	2.0×10^5	32090 31920	29340 25440	2810 2460	-	12.1	0.86	0.10	-
22	H^3-5-uridine (20,000)	125	64	6.4×10^5	19224 19592	18751 14891	1274 1623	938 941	23.6	0.87	0.09	0.06
23	H^3-5-uridine (20,000)	120	89	6.4×10^5	7558 7972	6729 6978	573 741	199 283	9.44	0.88	0.10	0.04
24	H^3-uridine[c] (20,000)	100	13	2.0×10^5	24270 22990	15600 15390	1850 1980	-	10.7	0.66	0.12	-
25	H^3-uridine (20,000)	125	14	4.9×10^6	285 256	196 207	63 62	-	2.40	0.74	0.31	-
26	H^3-uridine (20,000)	125	14	1.7×10^6	440 375	262 338	101 89	-	1.28	0.744	0.32	-
27	H^3-uridine (20,000)	125	14	7.4×10^5	2537 2686	2127 2103	473 500	-	3.51	0.81	0.23	-
28	H^3-uridine (20,000)	100	16	1.5×10^6	61 60	101 33	12 13	-	0.20	1.10	0.19	-
29	H^3-uridine (20,000)	100	16	2.3×10^5	4509 4360	3623 3307	465 518	-	2.29	0.78	0.14	-

[a] TCA: 5% trichloroacetic acid

[b] Experiments 1-23, Lytechinus pictus; Experiments 24-29, Strongylocentrotus purpuratus.

[c] Uniformly labeled (experiments 24-29).

instance, factors that can be expected to influence the final yield of radioactivity in the shed eggs are 1) seasonal differences of the animals during the time various tests were made, 2) variations in the extent of spawning both before and after administering the radioactive material, 3) possible individual differences in the rate and number of maturing eggs and 4) differences in the rate at which the label may be utilized by other tissues of the animal.

Nonetheless, a relationship emerges between the effectiveness of labeling and the time of incubation. This is shown in Fig. 2. The amount of label recovered in the shed eggs gradually increased during the first month, reaching an average maximum of 15 to 20% and remaining approximately at that level during longer incubations. Furthermore, Fig. 3 demonstrates that the ratio of the incorporation of the labeled uridine into an acid insoluble form to that of the total amount taken up by the eggs also reaches a plateau (90-98%) one month after the initial injection. Hence, even though these parameters are strongly influenced by factors, mentioned above, that are difficult to control, it is feasible to predict an order of magnitude of uptake and incorporation of uridine that will result under these conditions. The minimum time necessary to achieve maximum labeling of unfertilized eggs is one month, if only one injection of labeled uridine is given to the animal.

It is possible to reinject an animal several times. Indeed one sea urchin may yield a number of batches of labeled eggs, being reinjected each time, if it is handled with care. This was successfully done in these tests. The percentage of recovered radioactivity, however, did not increase with each batch of regenerated eggs. The amount of label found

FIG. 2. PERCENTAGE RECOVERY OF INJECTED ISOTOPE FOUND IN MATURE EGGS OF STRONGYLOCENTROTUS PURPURATUS AND LYTECHINUS PICTUS LABELED DURING OOGENESIS. Sea urchins were spawned with potassium chloride, injected with labeled uridine and incubated at 20°C as given in Materials and Methods. At the designated time after the injection of radioactive uridine, the mature eggs were shed with potassium chloride and assayed for total radioactivity by scintillation counting. Each point on the figure specifies the percentage of radioactivity, relative to that administered to the animal, that was recovered in the shed eggs.

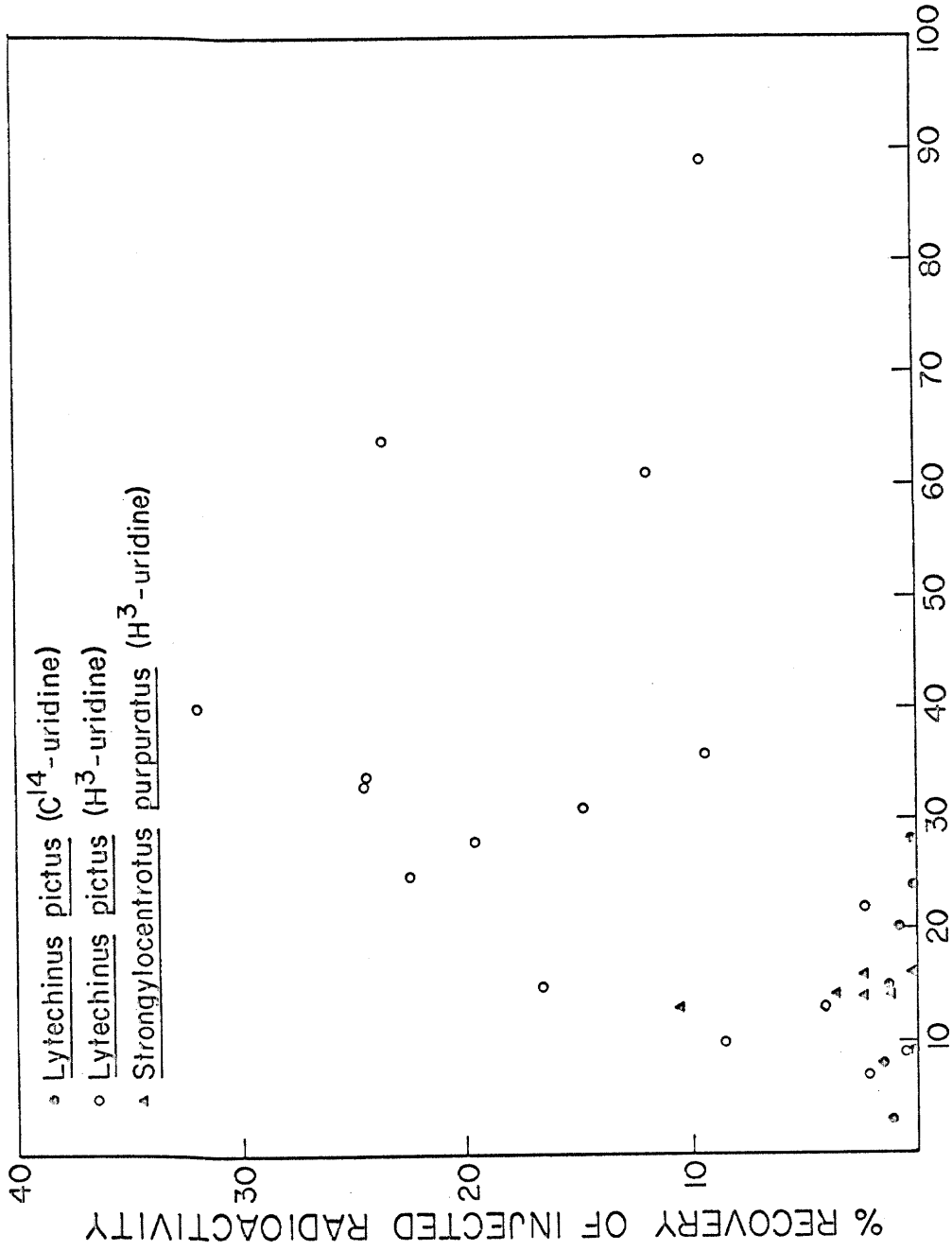
Lytechinus pictus (C^{14}-uridine)
Lytechinus pictus (H^3-uridine)
Strongylocentrotus purpuratus (H^3-uridine)
% RECOVERY OF INJECTED RADIOACTIVITY
DAYS AFTER RADIOACTIVE URIDINE INJECTION
10
20
30
40
50
60
70
80
90
100

Figure 2.

FIG. 3. PERCENTAGE OF RADIOACTIVITY PRECIPITABLE WITH ICE-COLD TRICHLOROACETIC ACID IN THE SHED EGGS OF STRONGYLOCENTROTUS PURPURATUS AND LYTECHINUS PICTUS LABELED DURING OOGENESIS. The percentage of radioactivity precipitable with ice-cold 5% trichloroacetic acid, relative to the total radioactivity accumulated by the shed eggs, has been calculated from the values listed in Table I.

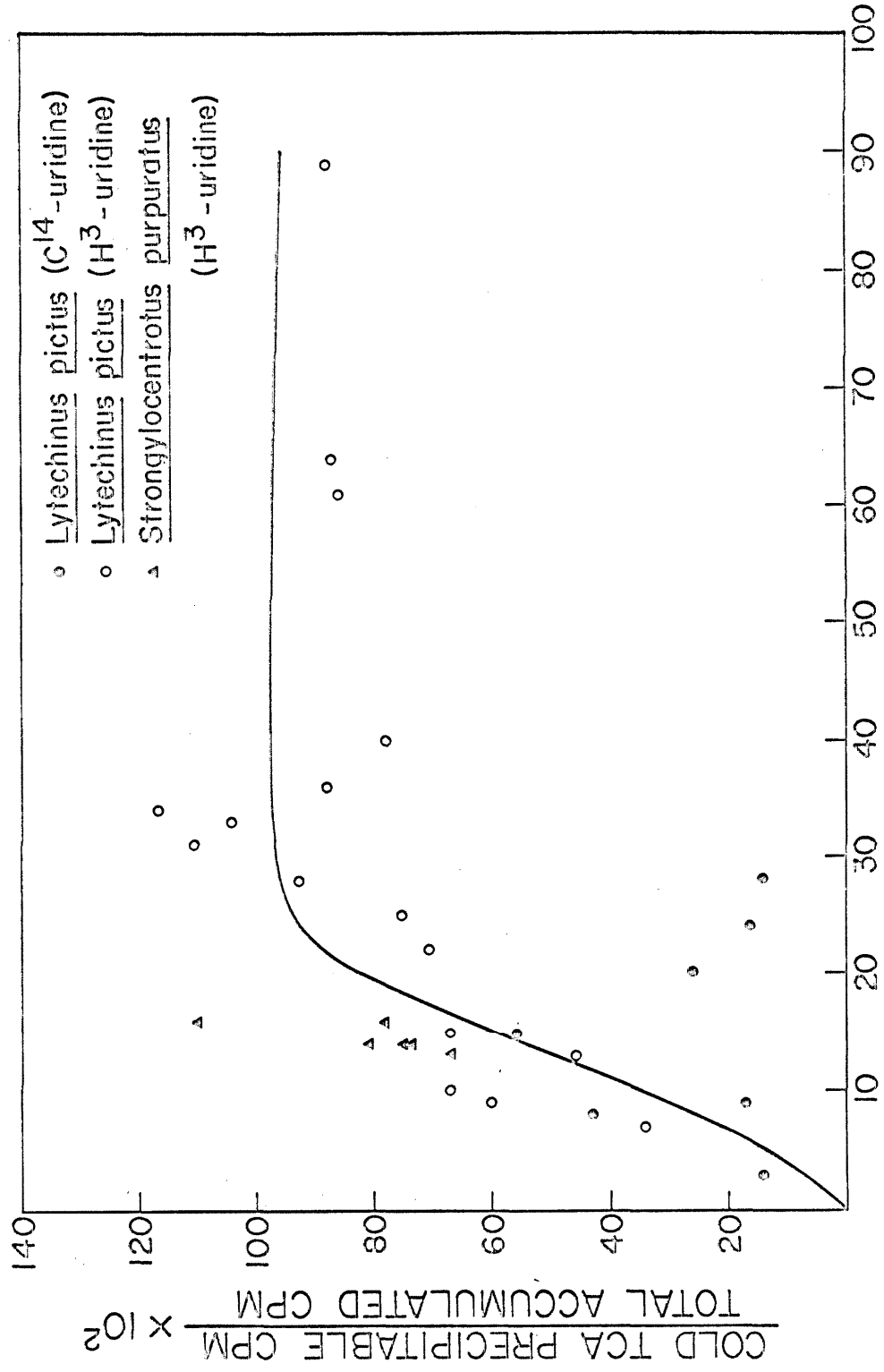
COLD TCA PRECIPITABLE CPM / TOTAL ACCUMULATED CPM × 10²
140
120
100
80
60
40
20
10
20
30
40
50
60
70
80
90
100
DAYS AFTER RADIOACTIVE URIDINE INJECTION
Lytechinus pictus (C14-uridine)
Lytechinus pictus (H3-uridine)
Strongylocentrotus purpuratus (H3-uridine)

Figure 3.

per egg was high, but did not exceed that found in some experiments when only one injection was given. For example, one Lytechinus pictus female was injected with 120 μc of H^3-6-uridine (sp. act. 20,000 c/M) and shed 61 days later. It was reinjected with 150 μc of H^3-5-uridine (sp. act. 25,000 c/M), shed again in 43 days and finally, injected a third time with 150 μc of H^3-5-uridine (sp. act. 25,000 c/M) and incubated for another 55 days. In the final shedding 5.5×10^5 eggs were obtained which had accumulated 5.81×10^4 cpm for 10^4 eggs. The highest value obtained for uptake in the tests in which only one injection of H^3-5-uridine (sp. act. 25,000 c/M) was given (experiment 20) was 7.4×10^4 cpm for 10^4 eggs. A total of 2.3×10^5 eggs were shed. Thus, either the very small oocytes do not mature in appreciable numbers during this time or they are not active in uridine incorporation into RNA. The second possibility is eliminated by the fact that even very small oocytes (15 μ) of sea urchins have been reported to be active in H^3-uridine incorporation into RNA (Ficq, 1964). The former view, on the other hand, is supported by the observation that pre-leptotene primary oocytes which had incorporated, in vivo, H^3-thymidine into DNA failed to develop into growing oocytes or mature eggs in the same annual reproductive season (Holland and Giese, 1965).

The ratios of the radioactivity precipitable with cold 5% trichloroacetic acid, after acid and alkaline hydrolysis of the labeled eggs, to that precipitable before hydrolysis, give an index of the proportion of label that has become incorporated into nucleic acid and RNA, respectively. Acidic hydrolysis (5% trichloroacetic acid, 90-100°C for 15 min.) will make nucleic acid but not protein soluble in cold trichloroacetic acid, while basic hydrolysis (0.3 M potassium hydroxide

at 37°C for 18 hr.) will hydrolyze RNA and some protein but not DNA (Davidson, 1965). The data in column 12 of Table I show that much of the labeled uridine was incorporated into nucleic acid. The precipitable radioactivity, however, was not completely removed by the hot acid hydrolysis, indicating that some label has become incorporated into materials other than nucleic acid. The degree of specificity of incorporation of the label into nucleic acid was different when the injected uridine was labeled in different positions of the molecule. The average amounts of acid hydrolysis of the label incorporated from an injection of C^{14}-2-uridine (experiments 1 - 7), H^3-6-uridine (experiments 8-14), H^3-5-uridine (experiments 15-23) or uniformly labeled H^3-uridine (experiments 24-29) were 73%, 69%, 93% and 78%, respectively. Thus H^3-5-uridine gave the highest specific incorporation into nucleic acid. Alkaline hydrolysis of eggs from animals labeled in vivo with H^3-5-uridine (column 13) left, in every case, slightly less acid precipitable radioactivity than did acidic hydrolysis. This indicates that the label from H^3-5-uridine is indeed incorporated mostly (90-95%) into RNA, but some (1-2%) radioactivity probably goes into other materials.

3) Sedimentation pattern of the RNA labeled during maturation of Lytechinus pictus oocytes.

The radioactive RNA of unfertilized eggs labeled during oogenesis was phenol-extracted and analyzed by sucrose density-gradient centrifugation. Fig. 4 shows the sedimentation pattern obtained by the RNA of

FIG. 4. SEDIMENTATION PATTERN OF PHENOL-EXTRACTED RNA FROM SHED UNFERTILIZED EGGS OF LYTECHINUS PICTUS LABELED DURING OOGENESIS. A female sea urchin was spawned with potassium chloride, injected with 125 μc of H^3-5-uridine (sp. act., 20,000 c/M) and incubated at 20°C for 64 days (see experiment 22 of Table I). The sea urchin was then shed with potassium chloride. The RNA was phenol-extracted from the labeled unfertilized eggs as given under Materials and Methods. The labeled RNA (0.4 ml) was layered on 4.67 ml of a linear 5-20% (w/v) sucrose density-gradient containing 0.1 M NaCl and 0.01 M Na acetate at pH 5.2. The gradient was centrifuged in a SW-39 rotor of a Model L Spinco Ultracentrifuge at 37,000 rpm for 5 hours at 0-4°C. Three drop fractions (approximately 0.20 ml) were collected from the punctured centrifuge tube, diluted with an equal volume of distilled water and absorbancies at 260 mμ measured with a Beckman DU spectrophotometer. Measurements of radioactivity were then made on the same samples by scintillation counting as given in Materials and Methods.

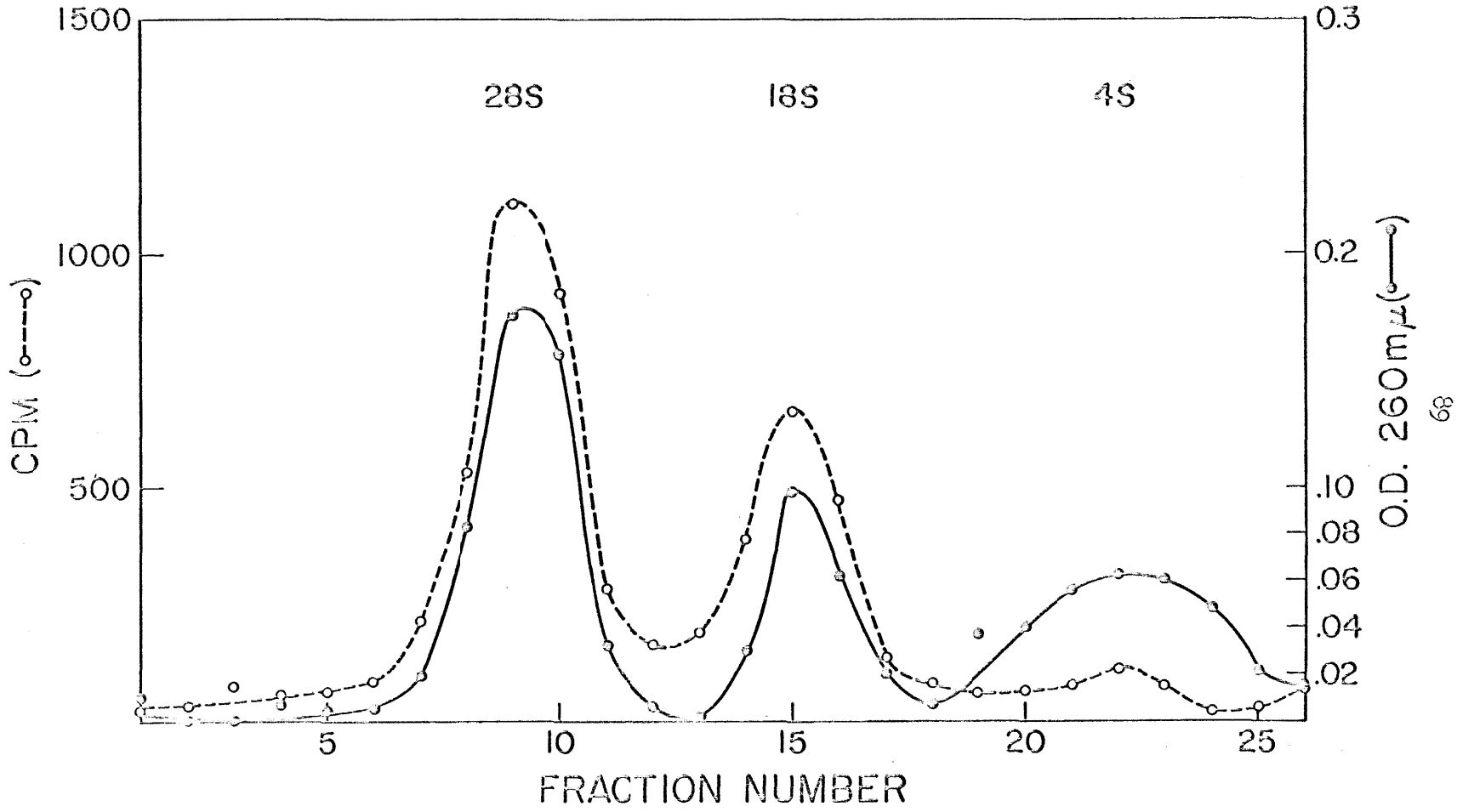
1500
1000
500
CPM (o——o)
28S
18S
4S
0.3
0.2
.10
.08
.06
.04
.02
O.D. 260mμ(o——o)
5
10
15
20
25
FRACTION NUMBER

Figure 4.

eggs collected 64 days (experiment 22) after injection of H^3-5-uridine.

The absorbancy profile shows the three main species of RNA with their characteristic peaks at about 28S, 18S and 4S. The radioactivity pattern shows a similar profile. The low specific activity of the 4S RNA may be more apparent than real since the 260 mμ to 280 mμ absorption ratio is less than two in this region. It is likely, then, that the presence of contaminating material is decreasing the observable specific activity of the 4S RNA.

In addition to the main components, smaller amounts of heterogeneously sedimenting labeled RNA appear in the profile of Fig. 4. These are evident in the regions outside of the 28S, 18S and 4S areas. Assuming the areas under the 28S and 18S regions include heterogeneously sedimenting RNA at the same level as that surrounding these regions, the following percentages of label in the various types of RNA are:

28S = 48%; 18S = 27%; heterogeneous = 21%; 4S = 5%.

In another test of this type utilizing eggs labeled with H^3-5-uridine for 33 days (experiment 17) the distribution of radioactivity was:

28S = 48%; 18S = 29%; heterogeneous = 16%; 4S = 7%.

Thus, most of the labeled RNA is ribosomal. Much less radioactivity is incorporated into transfer RNA and an upper limit of 10 to 20% can be placed on the incorporation of H^3-uridine into heterogeneously sedimenting, presumably messenger, RNA.

DISCUSSION

The results of this study have shown that it is practical and feasible to accomplish long-term labeling of mature, unfertilized eggs of sea urchins. The animals may be kept for many months in relatively small volumes of sea water and may be reutilized to yield several batches of radioactive eggs. Effective labeling of the RNA may be accomplished with only one injection of radioactive nucleoside. About 95% of the injected isotope is retained by the sea urchin. The shortest time for optimum results is one month under the present conditions.

As noted in the Introduction, *in vivo* incorporation of radioactive materials into oocytes has been accomplished in many different types of animals, among them being mammals (Sirlin and Edwards, 1959; Rudkin and Griech, 1962), chickens (Hevesy and Hahn, 1938; Chargaff, 1942; Patterson, 1961), amphibians (Ficq, 1955, 1961, 1966; Brachet and Ficq, 1956; Gall and Callan, 1962; Davidson, Allfrey and Mirsky, 1964; Brown and Littna, 1964a, b; Davidson, Crippa, Kramer and Mirsky, 1966), insects (Sirlin and Jacob, 1960; Favard-Sereno and Durand, 1963a, b; Bier, 1963; Zalokar, 1965) and sea urchins (Gross, Malkin and Hubbard, 1965; Holland and Giese, 1965; Piko, Tyler and Vinograd, 1967). In some organisms, notably chickens and insects (see Tyler, 1955, and Williams, 1965, for reviews), growth of the oocyte is associated with the accumulation of materials synthesized in other cells of the body. This may occur to some extent in all animals. However, at least for amphibians (Izawa, Allfrey and Mirsky, 1963), sea urchins (Piatigorsky, Ozaki and Tyler, 1967, see Part II of this thesis) and marine polychaet worms (Tweedell, 1966)

evidence has been provided that ovarian-free immature oocytes are capable of intense RNA and protein synthesis. It is known also that ovarian, mature sea urchin eggs incorporate little, if any, labeled precursors into RNA (Immers, 1961; Ficq, 1964; Gross, Malkin and Hubbard, 1965). Thus, the labeled RNA of the shed eggs in the present experiments can be assumed to have been synthesized primarily by the oocytes during the course of maturation.

In vivo RNA-labeling experiments (Brown and Littna, 1964b; Davidson, Allfrey and Mirsky, 1964) with the toad Xenopus laevis have shown that growing oocytes synthesize predominantly 28S and 18S ribosomal RNA. Much less 4S RNA was synthesized by oocytes in these tests. The most active time of RNA synthesis was found to occur during the lampbrush phase of oocyte growth (Davidson, Allfrey and Mirsky, 1964). Some non-ribosomal RNA that sediments heterogeneously in a sucrose density-gradient was also shown to be synthesized during oogenesis (Brown and Littna, 1964a; Davidson, Allfrey and Mirsky, 1964). The labeled ribosomal and heterogeneously sedimenting RNA's were conserved throughout oogenesis and during early development of the fertilized egg.

Davidson, Crippa, Kramer and Mirsky (1966) showed that RNA extracted from lampbrush stage oocytes of Xenopus possess considerable capacity to stimulate the in vitro incorporation of labeled amino acids into protein. Hybridization studies indicated that about 1.5% of homologous DNA could be bound with RNA, labeled in vivo, extracted from lampbrush phase oocytes. Furthermore, since unlabeled RNA from later stage, mature oocytes competed effectively with the labeled RNA from lampbrush stage

oocytes for hybridization to homologous DNA, it was concluded that the RNA synthesized throughout oogenesis is conserved and sequestered in the mature oocyte.

Gross, Malkin and Hubbard (1965) investigated in vivo RNA synthesis by oocytes of the sea urchin Arbacia punctulata during their final week of maturation. They showed that in sea urchins, too, growing oocytes synthesize primarily 28S and 18S ribosomal RNA and that the labeled RNA's are preserved in the mature egg. Apart from labeled ribosomal RNA, Gross, Malkin and Hubbard (1965) showed that some 4S RNA becomes labeled during oogenesis. In addition they found small quantities of labeled RNA of higher specific radioactivity than the ribosomal RNA. This labeled RNA sedimented heterogeneously in a sucrose density-gradient and was eluted from a methylated albumin-kieselguhr column at higher ionic strength than was the labeled ribosomal RNA. Furthermore, the phenol-extracted RNA from the labeled unfertilized eggs contained some radioactive materials that hybridized to homologous DNA in the presence of an excess of non-radioactive ribosomal RNA.

The present experiments are generally in accord with those cited above with respect to the high labeling of 28S and 18S RNA and the slight labeling of 4S RNA during oogenesis. These labeled RNA's were conserved in mature unfertilized eggs for prolonged periods of time. The intense labeling of the oocyte nucleolus with labeled RNA-precursors in sea urchins (Ficq, 1964; Piatigorsky, Ozaki and Tyler, 1967, see Part II of this thesis), starfish (Ficq, 1953, 1955; Vincent, 1954), amphibians (Ficq, 1961, 1964; Ozban, Tandler and Sirlin, 1964), polychaet worms (Tweedell, 1966) and

some insects (Zalokar, 1965) is consistent with large quantities of ribosomal RNA being synthesized by the nucleolus (Perry, 1965) of the oocyte.

The present tests show that the incorporation of H^3-uridine into heterogeneously sedimenting RNA does not exceed one-tenth to one-fifth of the total. This value is based on the assumption that labeled messenger RNAs are present in the 28S and 18S regions at the same level as outside these regions. Gross, Malkin and Hubbard (1965) utilized a comparable procedure to estimate a maximum value of label incorporated into heterogeneously sedimenting RNA during the final stages of oogenesis of sea urchins. Their determinations indicated that 10 to 15% of the total label sedimented heterogeneously. These percentages easily account for the 4 to 5% of the total RNA of unfertilized eggs possessing template potential with respect to the in vitro incorporation of labeled amino acids into protein (Slater and Spiegelman, 1966b).

PART IV

STUDIES ON THE STIMULATION OF PROTEIN SYNTHESIS AFTER FERTILIZATION OF EGGS OF THE SEA URCHIN LYTECHINUS PICTUS

INTRODUCTION

Fertilization of sea urchin eggs initiates a rapid increase in the rate of incorporation of labeled precursors into protein (Hultin, 1950, 1952; Hultin & Wessel, 1952, Hoberman, Metz & Graff, 1952; see Gross, 1964; Monroy, 1965a; Tyler and Tyler, 1966b; Monroy & Tyler, 1967 for reviews). Experiments on the extent of protein synthesis before and after fertilization by eggs that have been preloaded, in vivo with radioactively labeled amino acids (Nakano & Monroy, 1958; Nakano, Giudice & Monroy, 1958; Monroy, 1960) and investigations on homogenates of unfertilized and of fertilized eggs (Hultin & Bergstrand, 1960; Hultin, 1961) showed that the increase in amino acid incorporation into protein by fertilized eggs is not due to a greater permeability of fertilized eggs to amino acids, although it is also known that the permeability of sea urchin eggs to amino acids does increase after fertilization (Mitchison & Cummins, 1966; Tyler, Piatigorsky & Ozaki, 1966, see Appendix 1 of this thesis).

Hultin (1961) compared the in vitro incorporating capacity of ribosomes of unfertilized eggs with those of fertilized eggs and suggested that the suppression of protein synthesis in the unfertilized egg is due to inactivity of the ribosomes. Further support for this idea comes from experiments (Maggio, Monroy, Rinaldi and Vittorelli, 1965; Monroy, Maggio and Rinaldi, 1965) that demonstrated that trypsin-treatment of ribosomes of unfertilized eggs increased their capacity to respond, in vitro, to

polyuridylic acid and to RNA extracted from sea urchin eggs and embryos. However, the demonstrations (Tyler, 1962, 1963; Nemer, 1962; Nemer & Bard, 1962; Wilt & Hultin, 1962) that homogenates of unfertilized eggs support polyuridylic acid-dependent C^{14}-phenylalanine incorporation into acid insoluble material as effectively as homogenates of fertilized eggs established that the ribosomes of unfertilized eggs are indeed competent to synthesize protein. In addition, it showed that homogenates of unfertilized eggs contain all the materials necessary for the incorporation of amino acids into protein. The synthesis of messenger RNA, then, was thought to be responsible for the stimulation of protein synthesis after fertilization.

However, experiments with dactinomycin (actinomycin D) (Gross & Cousineau, 1963, 1964; Gross, Malkin & Moyer, 1964) and artificially activated non-nucleate fragments of unfertilized eggs (Tyler, 1963; Brachet, Ficq & Tencer, 1963; Denny & Tyler, 1964; Baltus, Quertier, Ficq & Brachet, 1965; Tyler, 1966) showed that nuclear activity after fertilization is unnecessary for the stimulation of protein synthesis. Furthermore, amino acid incorporation into protein by dactinomycin-treated eggs (Malkin, Gross & Romanoff, 1964) and by parthenogenetically activiated non-nucleate merogones (Burny, Marbaix, Quertier & Brachet, 1965; Denny, 1966) was shown to take place on polyribosomes in these systems as in normally fertilized eggs (Monroy & Tyler, 1963; Stafford, Sofer & Iverson, 1964; Hultin, 1964). It would appear also, from the results of the experiments with dactinomycin, that the activity of cytoplasmic RNA present mainly in the mitochondria of unfertilized eggs (Piko, Tyler & Vinograd,1967)

is not the primary stimulus of the initiation of rapid amino acid incorporation into protein after fertilization. Tests have shown that RNA, extracted from unfertilized eggs, can stimulate, in vitro, the incorporation of labeled amino acids into protein (Maggio, Vittorelli, Rinaldi and Monroy, 1964; Slater and Spieglman, 1966b). Moreover, RNA from unfertilized eggs competes effectively with heterogeneously sedimenting, rapidly labeled RNA of later stages of development for hybridization to homologous DNA (Whiteley, McCarthy & Whiteley, 1966; Glisin, Glisin & Doty, 1966). These findings, in view of the stimulating effect of polyuridylic acid to unfertilized egg-homogenates, support the initial conclusions of Gross and Cousineau (1963), Tyler (1963; 1965a) and Gross (1964); namely, that the stimulation of protein synthesis which occurs upon fertilization of echinoid eggs is the result of the activation of a "masked" messenger RNA in the cytoplasm of the unfertilized egg.

It has also been suggested (Tyler, 1965a; Tyler and Tyler, 1966b; Wilt, 1966) that newly synthesized messenger RNA after fertilization may be stored in an inactive form. Studies of proteins labeled after fertilization, in the presence and absence of dactinomycin, have provided evidence that RNA synthesized after fertilization does not substantially alter the electrophoretic pattern (Spiegel, Ozaki & Tyler, 1965; Terman & Gross, 1965) or the ion-exchange column chromatographic pattern (Ellis, Jr., 1966) of soluble protein synthesized during early development. This new RNA, rather, appears to remain inactive and possibly bound to particles, termed "informosomes" (Spirin, Belitsina, Aitkhozhin, 1964; Spirin and Nemer, 1965) until called into used at a later time.

The present investigation exploits the facts that sea urchins can regenerate new batches of ripe, fertilizable eggs after initial forced shedding (Tyler, 1949; Tyler and Tyler, 1966a) and that, in these, macromolecules can be radioactively labeled during oogenesis (Tyler and Hathaway, 1958) In relatively short-term experiments of this type, Gross, Malkin and Hubbard (1965) examined the types of RNA synthesized by oocytes, in vivo, during their final week of maturation. They found that the oocytes synthesized predominantly 28S and 18S RNA along with some 4S RNA and some heterogeneously sedimenting RNA. The series of experiments presented here has utilized long-term, in vivo, labeling of RNA by oocytes, as described in Part III of this thesis. This allows one to obtain relatively large quantities of labeled, ripe, unfertilized eggs containing highly radioactive RNA. The nature and properties of the "masked" messenger RNA as well as changes occuring upon fertilization which may be responsible for the stimulation of protein synthesis, could thus be advantageously explored.

The starting point for these investigations was the existence of a low level of 260 mμ absorbing material in the polyribosome-area of the sucrose density-gradient of homogenates of unfertilized eggs (Monroy and Tyler, 1963). The significance of this rapidly sedimenting baseline of ultraviolet absorption was studied in the present experiments. This included tests on the sensitivity of the material to treatment with RNase and trypsin. The nature of the RNA's extracted from the fractions obtained at various regions of the gradient of labeled homogenates of unfertilized and fertilized eggs was determined, the formation of poly-

ribosomes upon fertilization quantitated and their amino acid incorporating capacity assayed. In addition electron microscopy was utilized to confirm and extend the results obtained by the radioactive measurements.

MATERIALS AND METHODS

1) Labeling and fertilization of the eggs

Most, if not all, of the mature eggs were spawned out of ripe female sea urchins of the species Lytechinus pictus by the method of potassium chloride-injection (Tyler & Tyler, 1966a). The shed animals were then injected with C^{14}-(10 μc) or H^3-(100-150 μc) uridine in 0.4-0.6 ml of artificial sea water as described in Part III of this thesis. The type of labeled ribonucleoside injected and the time of incubation are specified with the individual experiments.

After one month or more of incubation a batch of labeled eggs was obtained again by injecting potassium chloride into the animal. The egg-suspensions were carefully inspected for oocytes and those contaminated by more than 1% of immature eggs were discarded. The eggs were strained through a mesh of bolting silk to remove debris and washed by centrifugation followed by a brief treatment with artificial sea water at pH 5.2 to remove the gelatinous coat. Eggs were fertilized by addition of one or two drops of a dilute sperm-suspension in 10^{-3} M ethylenediamine tetraacetic acid in artificial sea water at pH 8.0 (Tyler, 1953). At four minutes after fertilization, the elevated fertilization membranes were mechanically removed as described by Spiegel & Tyler (1966). The fertilized eggs were washed free of excess spermatozoa and cultured at

20°C for the specified time. An aliquot was usually reared to the pluteus stage to insure that development of the radioactive eggs proceeded in the typical fashion. The unfertilized and the fertilized eggs were then washed 3 times with about 50 volumes of ice-cold 0.55 M potassium chloride, an aliquot of each suspension removed for counting the number of eggs and the remainder gently packed by centrifugation and placed at 0°C.

2) Preparation of homogenates for sucrose density-gradient centrifugation

The eggs were suspended in homogenization buffer (0.004 M magnesium chloride, 0.025 M potassium chloride, 0.005 M β-mercaptoethanol, 0.05 M tris-hydrochloric acid at pH 7.6). The dilution varied in the different experiments but usually 3 to 4 volumes of buffer were employed. The eggs were homogenized by hand, gently, in a glass homogenization tube with 2 or 3 strokes of a loose-fitting teflon pestle. Complete cytolysis of the eggs was confirmed microscopically.

The homogenate was then processed in one of two ways: 1) it was clarified by addition of sodium deoxycholate (DOC) to a final concentration of 0.5%, centrifuged at 15,000 x g for 15 minutes at about 5°C with the no. 40 rotor of a Beckman model L preparative ultracentrifuge and the supernatant fraction set aside, or 2) it was first centrifuged at 10,000 x g for 10 minutes at 5°C, the supernatant fraction removed and supplemented with DOC to a final concentration of 0.5%. In some experiments the 10,000 x g supernatant fraction of the homogenate was pretreated with RNase (Sigma Chem. Corp., from bovine pancreas, 5x

crystallized) under the conditions specified in the section on Results and then clarified with DOC. The 10,000 x g pellet of the homogenate was washed several times in homogenization buffer, the original volume reestablished, made 0.5% with respect to DOC, centrifuged on the no. 40 rotor of the preparative ultracentrifuge at 10,000 x g for 5 minutes and the supernatant fraction set aside. These preparations were then examined by sucrose density-gradient centrifugation.

3) Extraction of RNA

RNA was phenol-extracted at 4°C by a method similar to that employed by Gross Malkin and Hubbard (1965) with some modifications described in Part III of this thesis. All RNA preparations were incubated with DNase (Worthington, electrophoretically pure) at a concentration of 10-14 µg/ml at 4°C for 30 minutes. The extracted RNA was dissolved in 0.01 M sodium acetate at pH 5.0 and stored at -20°C. Tests on samples of this labeled RNA showed it to be hydrolyzable by RNase (50 µg/ml, 37°C for 30 min.), potassium hydroxide (0.3 M, 37°C for 18 hr.) and hot trichloroacetic acid (5%, 90-100°C for 15 min.). It had a 260 mµ/280 mµ absorption ratio close to 2.

4) Sucrose density-gradient centrifugation and analysis of the density-gradients.

All sucrose solutions (Malinkrodt Chem. Co.) were autoclaved for 3 minutes. For centrifugation of the cell fractions, sucrose solutions of 60%, 30% and 15% (w/v) were made in homogenization buffer, lacking

β-mercaptoethanol. In general, a 0.5 ml cushion of 60% sucrose was placed in a cellulose nitrate centrifuge tube (1/2 in. x 2 in.) and 4.33 ml of a linear 15-30% gradient was formed above the sucrose cushion. Cell extracts (0.25-0.30 ml) were layered on the sucrose gradient and centrifuged at 27,500 rev/min. for 100 minutes, unless otherwise specified, at about 5°C on a Beckman model L preparative ultracentrifuge with an SW 39 rotor.

Examination of the sedimentation pattern of RNA was performed by layering 0.2-0.3 ml of a labeled RNA solution on 4.86 ml of a linear 5-20% (w/v) sucrose gradient made in 0.1 M sodium chloride and 0.01 M sodium acetate at pH 5.0. This was centrifuged for 5 hours at 36,000 rev./min at 5°C in an SW 39 rotor of the ultracentrifuge.

After centrifugation, the bottom of the tubes was pierced and 3 drop fractions (0.15-0.20 ml) were collected and diluted with an equal volume of distilled water. Absorbancies at 260 mμ were determined with a Beckman DU spectrophotometer. The material was then placed on filter paper strips and air-dried.

For assay of the radioactivity in the various fractions, or of the amount of incoporation of label into nucleic acid or protein, the filter papers were processed as described by Tyler (1966). The radioactivity-measurements were made with a Packard TriCarb liquid scintillation counter using a toluene-base scintillation fluid. Counting efficiency was about 50% for C^{14} and approximately 2 to 4% for H^3.

Approximate sedimentation coefficients were given to the major species of RNA by analogy with those determined by Slater and Spiegelman (1966a). They used 23S and 16S Bacillus megaterium RNA as a reference to calculate

the sedimentation coefficients of RNA extracted from sea urchin eggs. Calculations by the method of Martin and Ames (1961) of the sedimentation coefficients in the present experiments agree well with those determined by Slater and Spiegelman (1966a).

5) Preparation of materials from sucrose density-gradients for electron microscopy

The materials from the polyribosome- and monoribosome-regions of a sucrose density-gradient of DOC-treated 10,000 x g supernatant fractions of homogenates of unfertilized and of fertilized eggs were examined by electron microscopy. One drop samples from the specified fractions, treated and untreated with RNase, were placed on electron microscope grids (200 mesh, Ladd Research Industries) that were previously coated with a formvar film made from an 0.4% anhydrous solution in chloroform. After 30 minutes at room temperature in a moist chamber, the drop was removed with a pipette and the materials attached to the grids were treated for 5 minutes with 5% formaldehyde which was buffered in 0.05 M tris-hydrochloric acid at pH 7.4. Formaldehyde was then rinsed from the grids with homogenization buffer followed by distilled water. The grids were dried overnight. The specimens were then subjected to shadowing with platinum-paladium at an angle of 4 to 1. Some preparations were neither treated with formaldehyde nor shadowed but rather stained for 3 minutes with Millipore filtered, 1% aqueous uranyl acetate. The grids were inspected with a Philips EM 200 electron microscope. Electron micrographs of shadowed specimens are presented as negative prints enlarged as stated in the legends to the plates.

RESULTS

1) Sedimentation pattern of phenol-extracted RNA and of fractionated homogenates of unfertilized eggs labeled with uridine during oogenesis.

Experiments reported previously (Gross, Malkin and Hubbard, 1965, see Part III of this thesis) have shown that the growing sea urchin oocyte synthesizes principally ribosomal RNA and some soluble RNA. Thus, homogenates of unfertilized eggs were fractionated initially so that non-ribosomal RNA might be partially isolated before extraction and separation of the various RNA's. A typical experiment is shown in Fig. 1. Labeled fertilized eggs were homogenized. The homogenates were centrifuged at 10,000 rev./min. and the RNA from the supernatant fraction and the washed pellet extracted by the phenol method at 4°C. About two and a half times more RNA was extracted from the supernatant fraction than from the pellet. The RNA from the two preparations were alike in specific radioactivity; i.e., 10,377 cpm per 260 mμ absorbancy unit for the supernatant fraction and 9,239 cpm per 260 mμ absorbancy unit for the pellet. Furthermore, the results given in Fig. 1 show that the RNA from both fractions consisted predominantly of the 28S and 18S species of ribosomal RNA. There was also some 4S soluble RNA.

The profiles of the specific activity of the different species of the labeled RNA's are not significantly different from the sedimentation profiles shown in Fig. 1. It should be noted that the RNA in the experiment was labeled over an extended period of time, namely, for 33 days. There is no a priori reason to assume that heterogeneously sedimenting RNA would be more highly labeled than ribosomal RNA. In fact, Gross,

Fig. 1 SEDIMENTATION PATTERN OF RNA FROM THE SUPERNATANT FRACTION (A) AND THE MITOCHONDRIAL PELLET (B) OF AN HOMOGENATE OF RNA-LABELED UNFERTILIZED EGGS OF LYTECHINUS PICTUS. A spawned female was injected with 150 μc of H^3-5-uridine (sp. act. 25,000 c/M) and shed 33 days later. The labeled unfertilized eggs were washed several times by centrifugation in 0.55 M KCl, homogenized in 3 volumes of 0.01 M Na acetate buffer at pH 5.0, centrifuged at 10,000 x g for 10 minutes and the supernatant fraction set aside at 0°C. The pellet was washed twice by centrifugation with 20 to 30 volumes of homogenization buffer, the supernatant fractions combined and the pellet resuspended in about 40 volumes of buffer. The preparations were phenol-extracted at 4°C and 0.3 ml samples of labeled RNA solution were centrifuged through a linear 5-20% sucrose density-gradient (in 0.01 M Na acetate and 0.1 M Nacl at pH 5.0) at 37,000 rev./min for 5 hr. at 5-10°C, 3 drop (0.15-0.20 ml) fractions collected, the 260 mμ absorption determined and the radioactivity assayed as given under Materials and Methods.

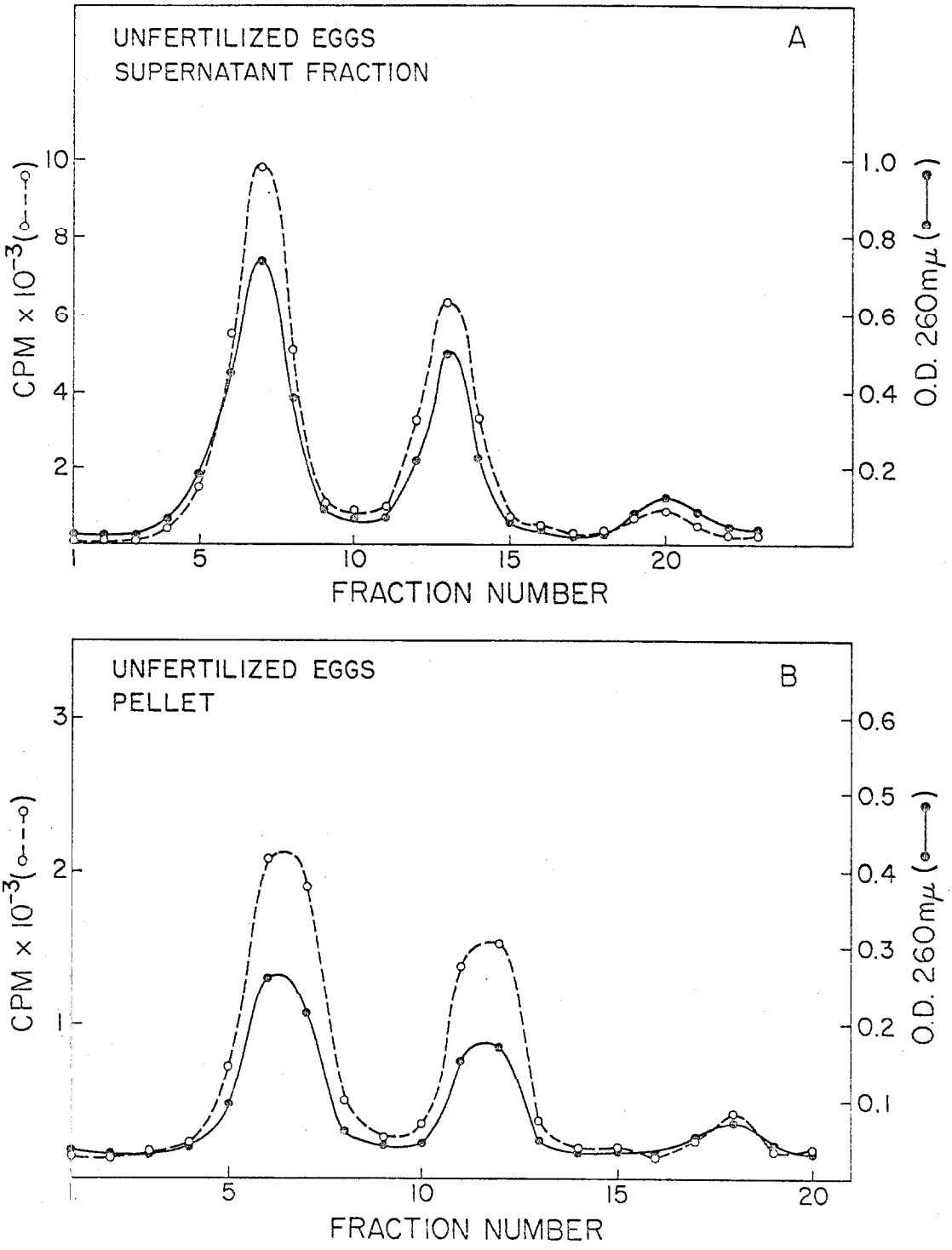
UNFERTILIZED EGGS
SUPERNATANT FRACTION
A
CPM x 10⁻³(o---o)
O.D. 260mμ (●—●)
FRACTION NUMBER
UNFERTILIZED EGGS
PELLET
B
CPM x 10⁻³(o---o)
O.D. 260mμ (●—●)
FRACTION NUMBER

Figure 1.

Malkin and Hubbard (1965) have provided evidence that heterogeneously sedimenting RNA is synthesized by oocytes of sea urchins shortly before they finish their maturation. It is possible then, that the long-term procedure may be less effective in labeling non-ribosomal RNA of unfertilized eggs than the short-term procedure. Non-ribosomal heterogeneously sedimenting RNA may thus be represented by the RNA of lower specific radioactivity present between and on either side of the 28S and 18S RNA.

The radioactive materials in the supernatant fraction of another homogenate of this type was investigated further by sucrose density-gradient centrifugation. The results are given in Figs. 2 and 3. Fig. 2A shows the sedimentation profile of the radioactive materials in the DOC-treated supernatant fraction after a relatively brief centrifugation. A base line of radioactivity sediments faster than the single ribosomes, which were located by their pronounced peak of radioactivity and 260 mμ absorption. Some labeled material remains above the monoribosomes. Fig. 2B shows that the rapidly-sedimenting radioactivity is precipitable with ice-cold trichloroacetic acid. Much of the radioactivity at the top of the gradient, on the other hand, is soluble in cold trichloroacetic acid. However, most of the labeled material in the gradient is made soluble with hot trichloroacetic acid. It appears, therefore, that the radioactively labeled material throughout the gradient is principally nucleic acid.

The slowly sedimenting radioactivity was explored in greater detail by extending the time of centrifugation of the supernatant fraction. The results of two tests are given in Fig. 3. Fig. 3A shows an experiment

Fig. 2. SEDIMENTATION PATTERN OBTAINED BY SHORT-TERM CENTRIFUGATION OF THE LOW-SPEED SUPERNATANT FRACTION OF AN HOMOGENATE OF RNA-LABELED UNFERTILIZED EGGS OF LYTECHINUS PICTUS. A spawned female was injected with 10 μc of C^{14}-2-uridine (sp. act. 25.2 c/M) and shed 30 days later. The labeled unfertilized eggs were homogenized in 9 volumes of homogenization buffer (0.004 M $MgCl_2$, 0.025 M KCl, 0.005 M β-mercaptoethanol, 0.05 M tris-HCl at pH 7.6), centrifuged at 10,000 x g for 10 minutes and the supernatant fraction removed and made 0.5% with respect to DOC. This preparation (0.225 ml) was layered on a linear 15-30% sucrose density-gradient (in homogenization buffer lacking β-mercaptoethenol) and centrifuged at 27,500 rev./min for 100 minutes at 5-10°C. The gradient was analyzed as given under Materials and Methods. (A) 260 mμ absorption and total radioactivity along the length of the gradient. (B) Radioactivity precipitable by ice-cold 10% trichloroacetic acid before and after treatment with 90-100°C 5% trichloroacetic acid for 15 minutes.

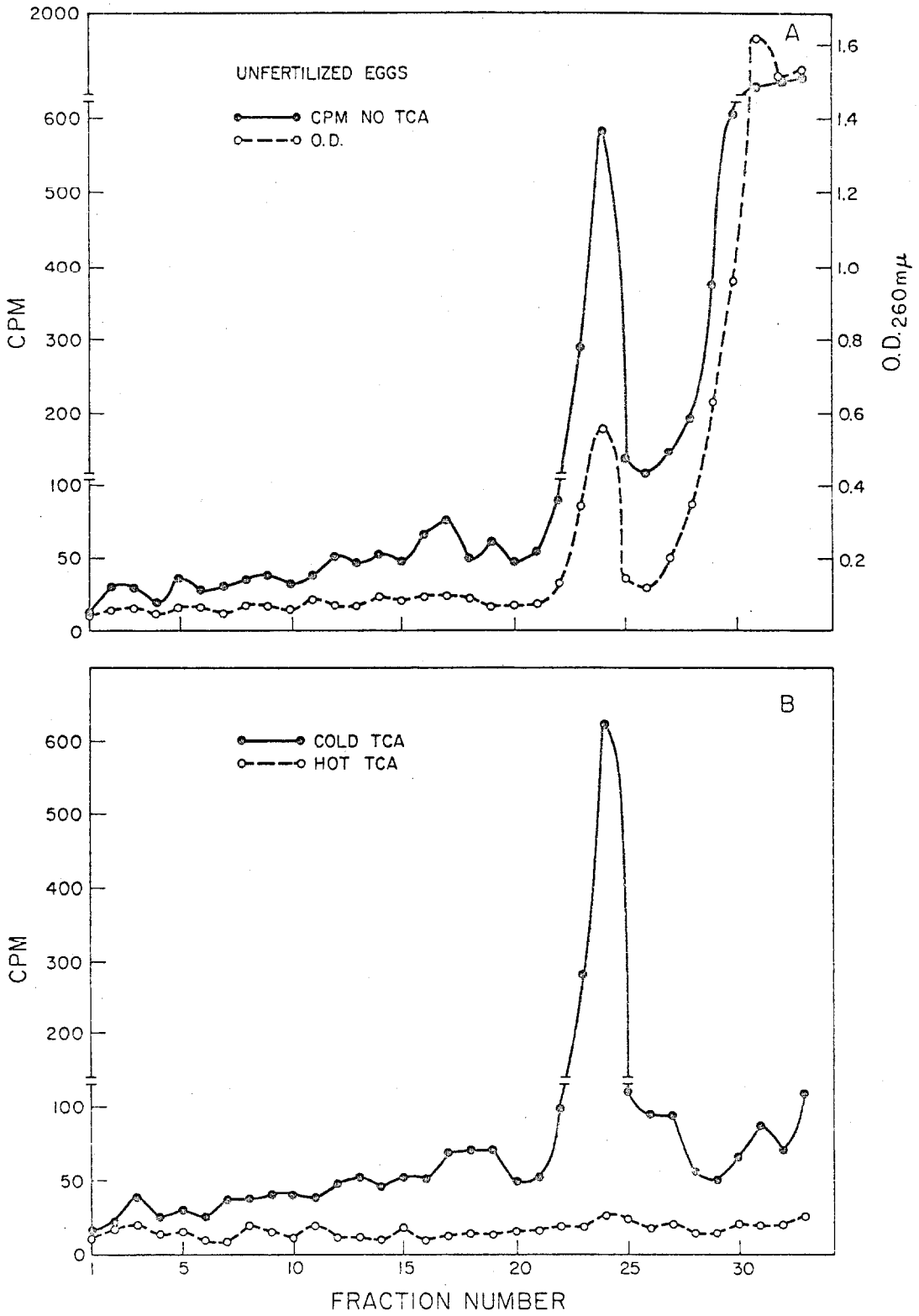
A
UNFERTILIZED EGGS
CPM NO TCA
O.D.
CPM
O.D. 260mμ
2000
600
500
400
300
200
100
50
0
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
B
COLD TCA
HOT TCA
1
5
10
15
20
25
30
FRACTION NUMBER

Figure 2.

Fig. 3. SEDIMENTATION PATTERN OBTAINED BY LONG-TERM CENTRIFUGATION OF THE LOW-SPEED SUPERNATANT FRACTION OF HOMOGENATES OF RNA-LABELED UNFERTILIZED EGGS OF LYTECHINUS PICTUS. Spawned females were injected with C^{14}-2-uridine (sp. act., 25.2 c/M). One (A) was shed 9 days later, another (B) 30 days later (Same eggs as those of Fig. 2.). The labeled eggs were homogenized and post-mitochondrial supernatant fractions prepared as given in the legend to Fig. 2, with the exception that DOC was not added to the low-speed supernatant fraction. Preparations (0.3 ml) were layered on a linear 15-30% sucrose gradient and centrifuged at 25,000 rev./min for 13 hr. at 5-10°C. Three drop fractions (0.15-0.20 ml) were collected after centrifugation. The 260 mμ absorption was determined and the 5% trichloroacetic acid precipitable radioactivity was assayed in each fraction as given under Materials and Methods.

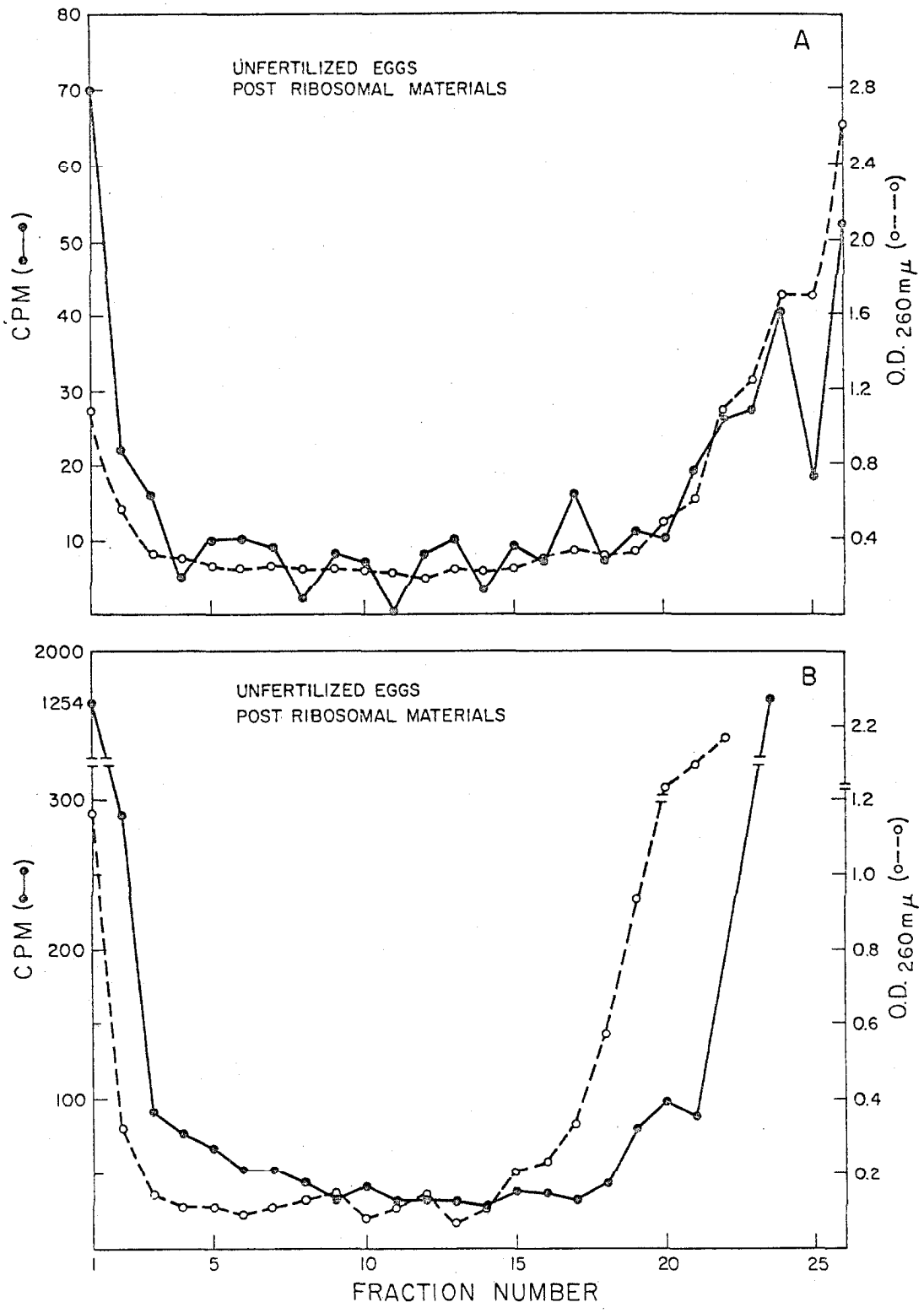
A
UNFERTILIZED EGGS
POST RIBOSOMAL MATERIALS
CPM (●—●)
O.D. 260 mμ (o--o)
80
70
60
50
40
30
20
10
2.8
2.4
2.0
1.6
1.2
0.8
0.4
B
UNFERTILIZED EGGS
POST RIBOSOMAL MATERIALS
2000
1254
300
200
100
2.2
1.2
1.0
0.8
0.6
0.4
0.2
1
5
10
15
20
25
FRACTION NUMBER

Figure 3.

conducted with C^{14}-uridine labeled unfertilized eggs. Fig. 3B shows a comparable experiment performed with the same, C^{14}-uridine labeled supernatant fraction as that of Fig. 2. The shoulder at the bottom of the sucrose gradient marks the position of the ribosomes. The longer centrifugation time has spread radioactivity throughout the length of the sucrose density-gradient. This suggests the presence of labeled RNA of heterogeneous size or of complexes of labeled RNA with other constituents in the supernatant fraction.

The nature of the labeled materials in the homogenates of unfertilized eggs that sediment to the different regions of the sucrose gradient was examined in the following way. Pooled fractions from different regions of the gradient were supplemented with purified, non-radioactive ribosomes from unfertilized eggs, as indicated in Fig. 4A, and the RNA was phenol-extracted followed by sucrose density-gradient centrifugation. The addition of the unlabeled ribosomes helped to coprecipitate the small quantities of RNA from the polyribosome-region. It also provided a marker to facilitate comparisons of the sedimentation of the radioactive RNA with that of ribosomal RNA. Further, it gave an index of the degree of degradation of the RNA during the extraction process. The approximate recovery of radioactivity in the RNA-extracts of regions 1, 2, and 3 of the sucrose density-gradient was 30%, 50% and 13%, respectively. The results of the experiment are given in Fig. 4.

It is clear that the three regions of the gradient each contain labeled ribosomal RNA. In addition, region 3 has a sharp peak of soluble RNA. It is of interest to note that the radioactive peak of 18S RNA is

Fig. 4. SEDIMENTATION PATTERN OF RNA EXTRACTED FROM THE POLYRIBOSOME-(REGION 1), THE MONORIBOSOME-(REGION 2) AND THE POST-MONORIBOSOME-(REGION 3) REGIONS OF A SUCROSE DENSITY-GRADIENT OF THE SUPERNATANT FRACTION OF AN HOMOGENATE OF RNA-LABELED UNFERTILIZED EGGS OF LYTECHINUS PICTUS. The labeled eggs were obtained by the final shedding of a sea urchin injected with 120 μc of H^3-6-uridine (sp. act., 20,000 c/M), shed 61 days later, reinjected with 150 μc of H^3-5-uridine (sp. act., 25,000 c/M), shed again in 43 days and, finally, reinjected with 150 μc of H^3-5-uridine (sp. act., 25,000 c/M) and incubated for another 55 days. A low-speed supernatant fraction of an homogenate was prepared and 5 aliquots (0.25 ml) subjected to sucrose density-gradient centrifugation, as given in the legend to Fig. 2, except a 0.5 ml cushion of 60% sucrose was placed in each gradient. One gradient was analyzed for 260 mμ absorption and radioactivity (A), while only the 260 mμ absorption was determined in the other 4. Each gradient was divided into 3 regions as indicated in (A), and the fractions within each region combined. Purified non-radioactive ribosomes, as indicated in the inserted graph of (A), were added to each region. The 3 regions were phenol-extracted, followed by sucrose density-gradient centrifugation and analysis of the extracted RNA's as given in the legend to Fig. 1.

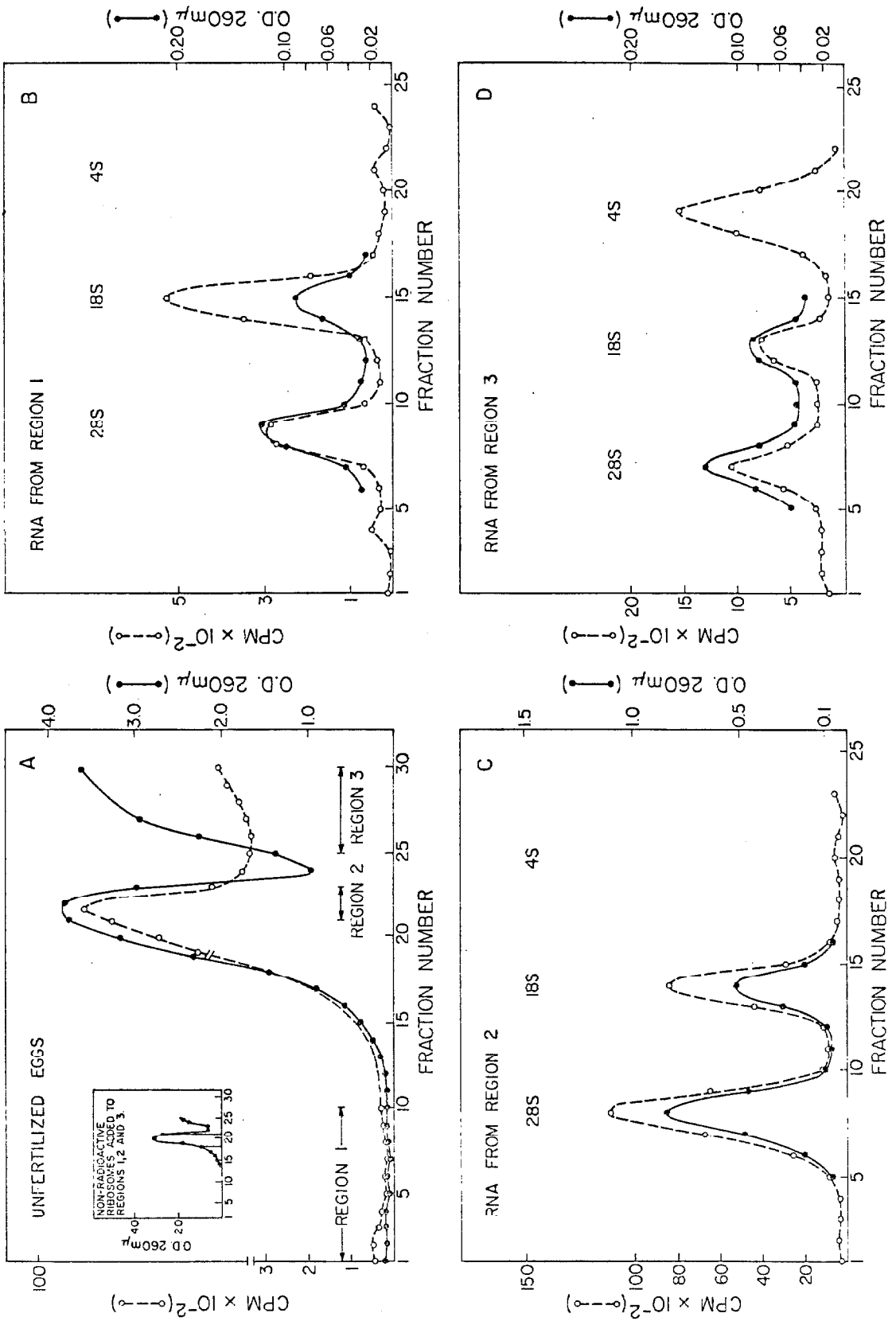
A
UNFERTILIZED EGGS
REGION 1
REGION 2
REGION 3
NON-RADIOACTIVE RIBOSOMES ADDED TO REGIONS 1,2 AND 3.
O.D. 260mμ
CPM × 10⁻²
FRACTION NUMBER
B
RNA FROM REGION 1
28S
18S
4S
C
RNA FROM REGION 2
D
RNA FROM REGION 3

Figure 4.

higher than the 28S peak in region 1. This irregular result was observed again in another experiment of this type, although the difference was smaller than in the present experiment. While the reason for this result is not known, one possibility is that heterogeneously sedimenting RNA with sedimentation coefficients near 18S are present in considerable quantities in the polyribosome-region of the gradient and add to the measurements of 18S ribosomal RNA. Differential extraction of 18S ribosomal RNA is unlikely since the 260 mμ absorption measurements show that more 28S than 18S RNA was recovered from the non-labeled ribosomes which were added to the fractions as a marker.

The identification of ribosomal RNA from the polyribosome-region of the sucrose density-gradient strongly implies that ribosomal aggregates are present in homogenates of unfertilized eggs. That the aggregates are not produced by non-specific agglutination of single ribosomes is indicated by the fact that no appreciable amount of rapidly sedimenting radioactivity appears in the gradient after addition of radioactive ribosomes to an unlabeled homogenate.

Analyses of the data by the method given in Part III (p. 90) give the following distributions of label among the various types of RNA.

Region 1: 28S = 26%; 18S = 46%; heterogeneous = 24%; 4S = 0.6%

Region 2: 28S = 48%; 18S = 27%; heterogeneous = 23%; 4S = 2.2%

Region 3: 28S = 17%; 18S = 10%; heterogeneous = 33%; 4S = 41%

The pellet obtained by centrifuging the homogenate of unfertilized eggs at 10,000 rev./min. for 10 minutes was also examined by sucrose

density-gradient centrifugation. Fig. 5 shows that some rapidly sedimenting radioactive materials are liberated from the low-speed pellet by DOC-treatment, as are also an appreciable number of ribosomes. The peak of radioactivity in the first few fractions of the gradient is caused by an accumulation of labeled material on the 60% sucrose cushion.

These results can be compared with those obtained by density-gradient centrifugation of the supernatant fraction. The data shown in Table I indicate that there is 2.5 times (experiment 1) to 3.3 times (experiment 2) more rapidly sedimenting radioactivity in the supernatant fraction of the homogenate than in the low-speed pellet assuming that relatively little degradation of this material has occurred during extraction of the pellet. Possibly, however, resuspending the pellet in DOC causes the breakdown of some ribosomal aggregates. This would mean, then, that the ratio of aggregates from the supernatant fraction to those from the pellet is actually smaller than 2.5 to 3.3.

2) Formation of additional ribosomal aggregates after fertilization

An experiment was performed to follow the changes after fertilization in the sedimentation profile of radioactive materials in the supernatant fractions of homogenates of eggs. The results are shown in Fig. 6. The ordinate of Fig. 6A is drawn on a linear scale to illustrate the relatively small numbers of ribosomal aggregates that are present in the supernatant fraction of unfertilized or of fertilized eggs. Fib. 6B, on the other hand, shows the same results with the lower portion of the ordinate represented on an expanded scale. This clearly demonstrates the marked

Fig. 5. SEDIMENTATION PATTERN OF THE SUPERNATANT FRACTION AND OF THE MITOCHONDRIAL PELLET OF AN HOMOGENATE OF RNA-LABELED UNFERTILIZED EGGS OF LYTECHINUS PICTUS. A spawned female was injected with 150 μc of H^3-5-uridine (sp. act., 25,000 c/M) and shed 15 days later. The labeled eggs were homogenized in 2 vol. homogenization buffer (see Fig. 2), centrifuged at 10,000 x g for 10 minutes, the supernatant fraction removed, the pellet washed twice by centrifugation in homogenization buffer and the first supernatant fraction combined with the original one. The pellet was dissolved in 10 volumes of homogenization buffer containing 0.5% DOC, centrifuged at 10,000 x g for 5 minutes and the resulting supernatant fraction was subsequently adjusted to the same volume as the original supernatant fraction. The preparations (0.3 ml) were subjected to sucrose density-gradient centrifugation as given in the legend to Fig. 2, except that a 60% cushion sucrose was placed at the bottom of the gradient. Radioactivity of each fraction was measured by scintillation counting as given under Materials and Methods.

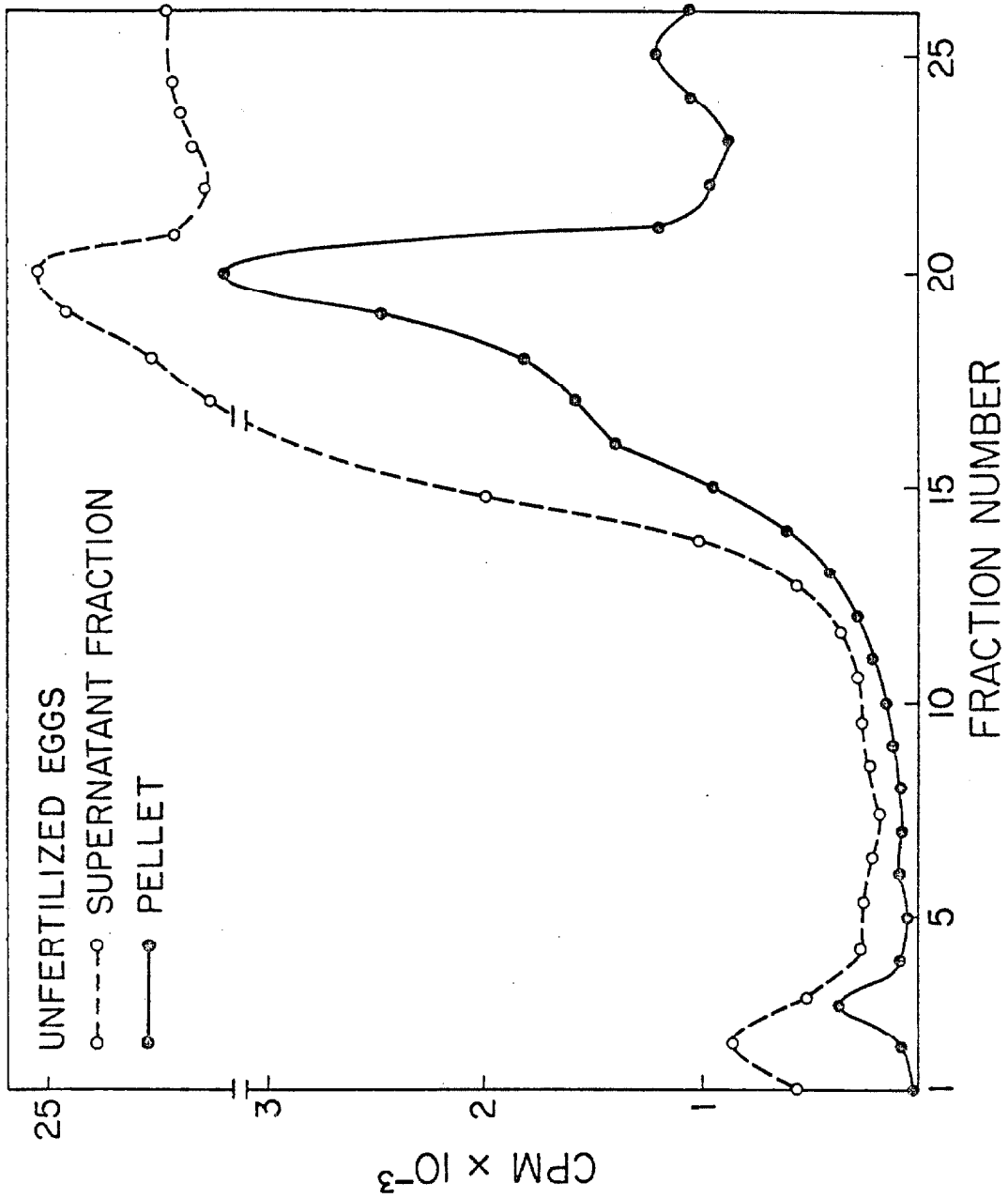
UNFERTILIZED EGGS
SUPERNATANT FRACTION
PELLET
CPM x 10⁻³
FRACTION NUMBER
25
3
2
1
1
5
10
15
20
25

Figure 5.

TABLE I

Radioactivity in the Polyribosome-, Monoribosome- and Post-monoribosome-region of Sucrose Density-Gradients of Equivalent Amounts of Supernatant Fractions[a] and Mitochondrial Pellets[a] of Homogenates of RNA-labeled Unfertilized Eggs of Lytechinus pictus.

Expt. No.	CPM in polyribosome-region[b]			CPM in monoribosome-region[b]			CPM in post-monoribosome-region[b]		
	Supernatant	Pellet	S/P	Supernatant	Pellet	S/P	Supernatant	Pellet	S/P
1[c]	5117	2070	2.5	67778	6455	10.5	43990	2219	19.8
2[d]	3647	1097	3.3	88702	13633	6.5	23191	3347	6.9

[a] RNA-labeled unfertilized eggs were homogenized and fractionated by sucrose density-gradient centrifugation as given in the legend to Fig. 5. Three drop fractions (0.15-0.20 ml) were collected. Radioactivity was measured in each fraction by scintillation counting as given under Materials and Methods.

[b] Polyribosome-region = bottom third of sucrose gradient; monoribosome-region = 8 fractions centering around the 260 mμ absorption peak; post-monoribosome-region = all fractions above monoribosome-region.

[c] Spawned females were injected with 100 μc of H^3-6-uridine (sp. act., 6,550 c/M) and shed 12 days later.

[d] Spawned females were injected with 150 μc of H^3-5-uridine (sp. act., 2,500 c/M) and shed 76 days later. Same experiment as shown in Fig. 5.

Fig. 6. SEDIMENTATION PATTERN OF THE LOW-SPEED SUPERNATANT FRACTIONS OF HOMOGENATES OF RNA-LABELED UNFERTILIZED AND FERTILIZED EGGS OF LYTECHINUS PICTUS. A spawned female was injected with 120 μc of H^3-5-uridine (sp. act., 25,000 c/M) and shed 33 days later. The suspension obtained was divided into 2 equal parts, one of which was fertilized, mechanically demembranated, and further divided into 2 equal samples. After 20 minutes and 40 minutes of development at 20°C, respectively, equal samples were washed 3 times by centrifugation with ice-cold 0.55 M KCl, as were also the unfertilized eggs, post-mitochondrial supernatant fractions of homogenates prepared as given in the legend to Fig. 2 and subjected to sucrose density-gradient centrifugation as given in the legend to Fig. 5. Radioactivity in the collected fractions was measured by scintillation counting as given under Materials and Methods. The results are presented on a linear scale (A) and an expanded scale (B) in the polyribosome-region of the gradient.

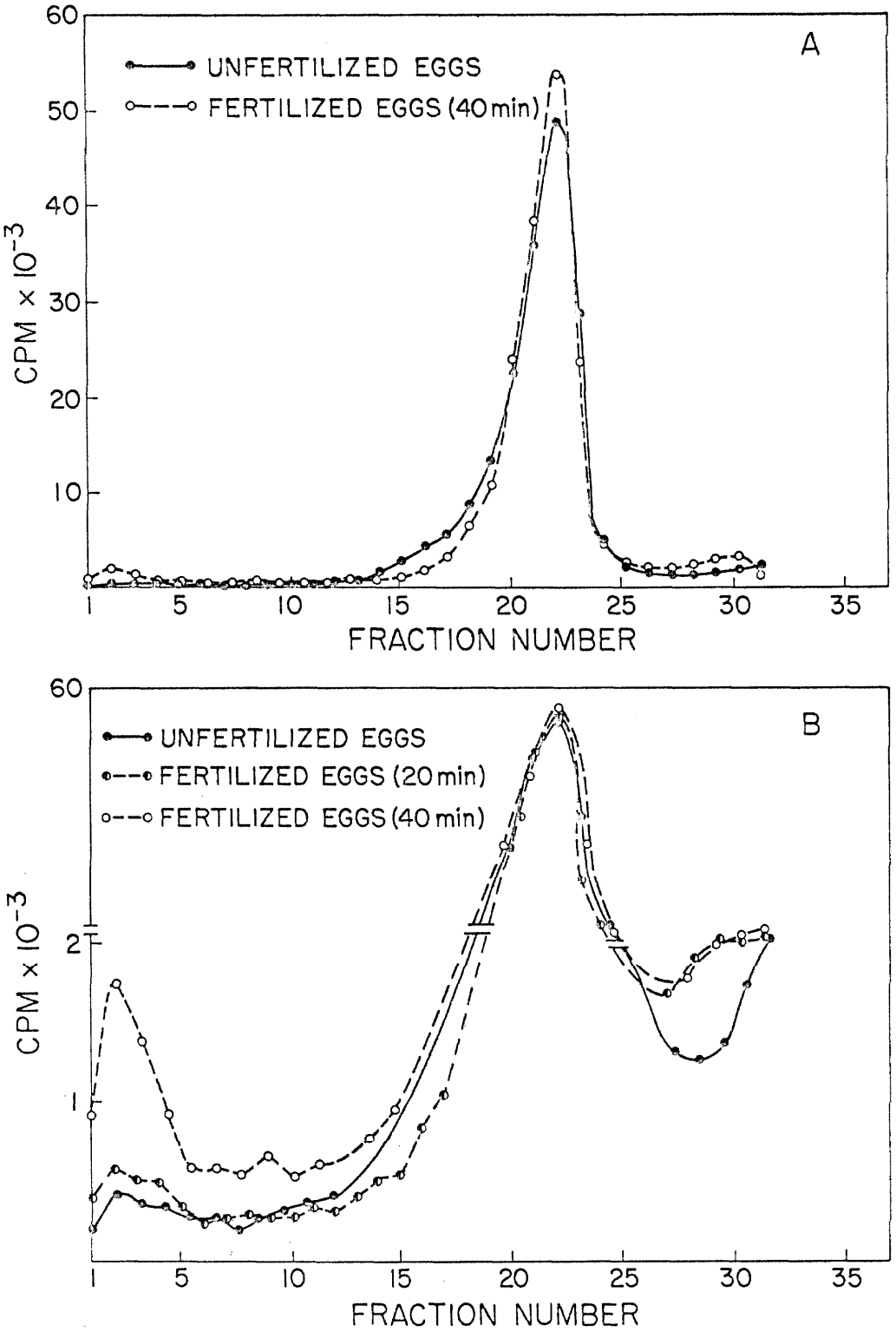
A
UNFERTILIZED EGGS
FERTILIZED EGGS (40 min)
CPM x 10⁻³
FRACTION NUMBER
B
UNFERTILIZED EGGS
FERTILIZED EGGS (20 min)
FERTILIZED EGGS (40 min)
CPM x 10⁻³
FRACTION NUMBER

Figure 6.

difference between homogenates of unfertilized and fertilized eggs with respect to the amount of RNA-labeled material that sediments to the polyribosome-region of the gradient. The results indicate also that this increase pertains to various size classes of ribosomal aggregates.

A similar result was obtained in eight additional experiments of this type. Tabulation of the ratio of radioactivity and of 260 mμ absorption in the polyribosome-region to that in the monoribosome-region of the sucrose gradient is given in Table II. The first five experiments listed in the table were conducted with homogenates that were centrifuged at low speed after DOC-treatment rather than before and are designated as "Homogenates." This preliminary DOC treatment releases much of the radioactive materials that would normally sediment with the low-speed pellet. Experiments 6 to 15, on the other hand, are measurements that have been made on homogenates that have been centrifuged at low speed before addition of DOC and are specified as "Supernatants."

The data given in Table II show that the ratios of cpm's of ribosomal aggregates to those of single ribosomes in homogenates of unfertilized eggs do not exceed 10.7% for the "Homogenates" (experiment 5) and 7.4% for the "Supernatants" (experiment 11). The lowest ratio is 6.5% (experiment 3) and 0.6% (experiment 7) for the "Homogenates" and "Supernatants", respectively. The corresponding ratios for unfertilized eggs calculated on the basis of absorbancy at 260 mμ are consistently higher, expecially in the tests utilizing the "Homogenate" preparations. This is due to the fact that some materials, other than ribosomal aggregates, sediment from "Homogenates" and "Supernatants" to the polyribosome-region of the gradient.

TABLE II

Ratios of Radioactivity and of 260 mμ Absorption in the Polyribosome-region to that in the Monoribosome-region of Sucrose Density-gradients of Homogenates, or Low-speed Supernatant Fractions Thereof[a], of RNA-labeled Unfertilized and Fertilized eggs of Lytechinus pictus.

Expt. No.	Preparation[a]	Minutes after fertilization	Ratios of polyribosome-region to monoribosome-region[b]			
			Ratio of CPM		Ratio of 260 mμ absorption	
			Unfert.	Fert.	Unfert.	Fert.
1	Homogenate	40	--	--	0.316	--
2	Homogenate	40	--	--	0.196	0.284
3	Homogenate	40	0.065	0.179	0.193	0.304
4	Homogenate	40	0.098	0.187	0.346	0.510
5	Homogenate	45	0.107	--	0.226	0.307
6	Supernatant	20	0.019	0.025	0.069	0.089
		40	--	0.050	--	0.116
7	Supernatant	40	0.006	0.034	0.026	0.130
8	Supernatant	45	0.017	0.050	0.016	0.081
9	Supernatant	60	0.018	0.053	0.064	0.107
10	Supernatant	90	--	--	0.038	0.172
11	Supernatant	--	0.074	--	--	--
12	Supernatant	--	0.070	--	0.145	--
13	Supernatant	--	0.016	--	0.037	--
14	Supernatant	--	0.043	--	0.082	--
15	Supernatant	--	0.040	--	--	--

[a]Homogenates and supernatants were prepared, subjected to gradient centrifugation and analyzed as given under Materials and Methods.

[b]Polyribosome-region and monoribosome-region were determined as given in Table 1.

Support for this conclusion comes from the observation, based on many different experiments, that the 260 mμ to 280 mμ absorption ratio in the polyribosome-region is usually on the order of 1.5 to 1.6 in contrast to 1.7 to 1.9 for the monoribosome-region. Furthermore, the ultraviolet absorption spectra, shown in Fig. 7, of the polyribosome- and monoribosome-regions indicate that the former may contain some non-ribosomal material which causes a slight broadening of the 260 mμ absorption peak. This is particularly marked in the spectrum of the polyribosome-region of the supernatant fraction of unfertilized eggs where the number of ribosomal aggregates is small and the relative amount of contaminating material correspondingly larger. Finally it has been observed that ethanol precipitates of the materials in the polyribosome-region are often larger, though less radioactive, than those in the monoribosome- or the post-monoribosome-region. Thus, radioactivity measurements, having been shown in the previous section to be predominantly ribosomal RNA, are more reliable than optical density measurements to quantitate the RNA in the polyribosome-region of the sucrose density-gradient.

Columns 5 and 7 of Table II give the new ratios, for radioactivity and for 260 mμ absorption, of the polyribosome- to monoribosome-regions that are observed soon after fertilization. Comparison of the results obtained with fertilized eggs with those obtained with unfertilized eggs shows that, in every case, the radioactivity and the absorbancy ratios are higher after fertilization. Experiment 6 of the table is the same test that is shown in Fig. 6. It is clear that the increase in radio-

Fig. 7. ULTRAVIOLET ABSORPTION SPECTRA OF THE INDICATED REGIONS OF A SUCROSE GRADIENT OF THE LOW-SPEED SUPERNATANT FRACTION OF HOMOGENATES OF UNFERTILIZED AND FERTILIZED (45 MINUTES) EGGS OF LYTECHINUS PICTUS. Fractions 2-5 (polyribosome-region) and 18-20 (monoribosome-region) of sucrose density-gradients of supernatant fractions of homogenates of unfertilized and of fertilized eggs, as given in the legend to Fig. 6, were combined and the monoribosome-peak diluted 20 times with distilled water. Ultraviolet absorption spectra were determined against a standard of the corresponding regions of a blank sucrose gradient on a Cary Model 15 spectrophotometer. Continuous spectra were recorded although points at 5 mμ intervals have been plotted.

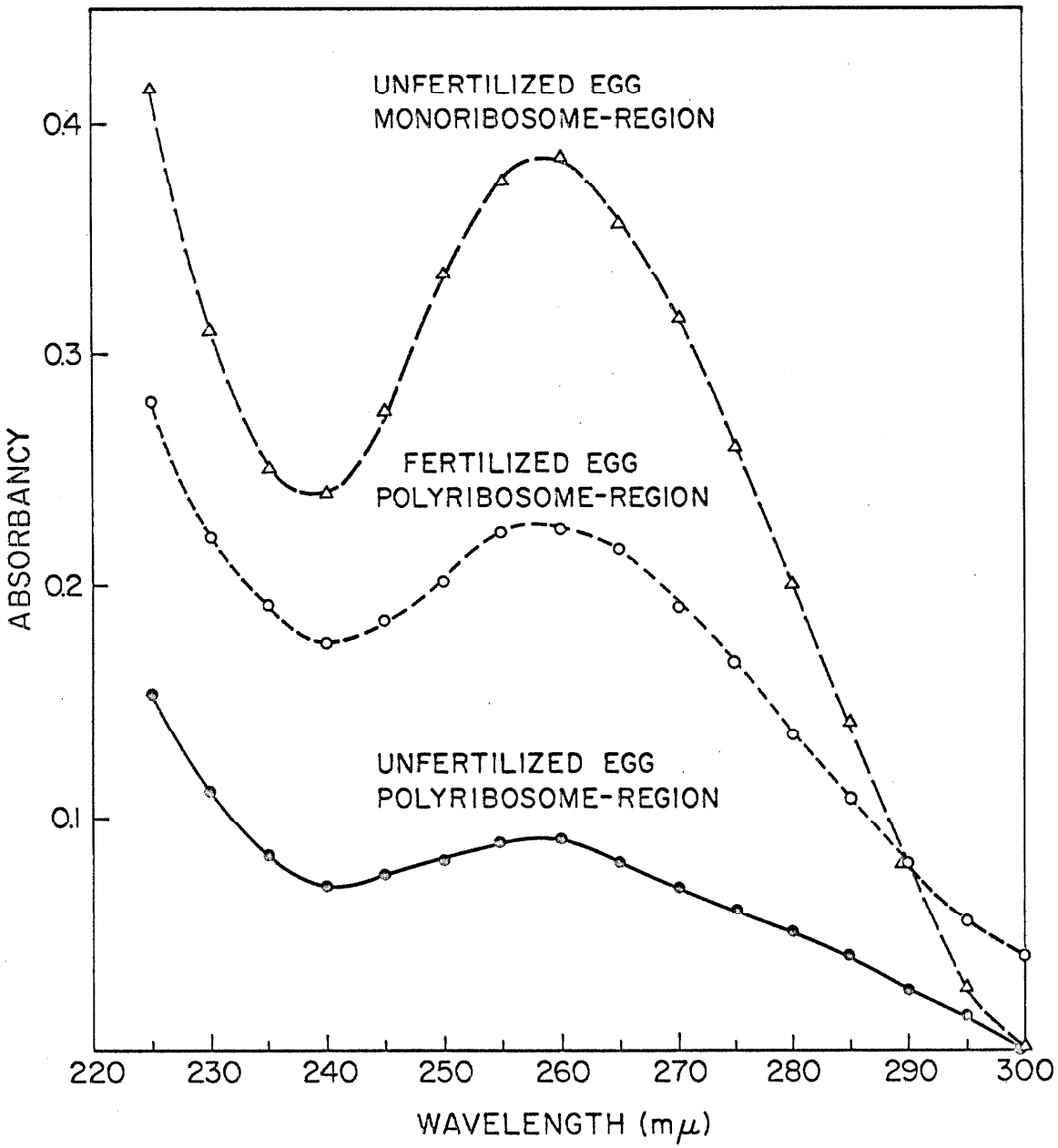
UNFERTILIZED EGG
MONORIBOSOME-REGION
FERTILIZED EGG
POLYRIBOSOME-REGION
UNFERTILIZED EGG
POLYRIBOSOME-REGION
ABSORBANCY
0.4
0.3
0.2
0.1
220
230
240
250
260
270
280
290
300
WAVELENGTH (mμ)

Figure 7.

actively labeled material in the polyribosome-region has already started by 20 minutes after fertilization and continues to increase rapidly thereafter.

The data listed in Table III give the values, obtained from the experiments shown in Table II, on the relative amounts of material sedimenting to the polyribosome-region before and after fertilization. In every instance utilizing both radioactivity and absorbancy measurements, a sharp increase is observed upon fertilization. This increase ranges from 2.6 times (experiment 1) to 5.1 times (experiment 3) for the cpm's and from 1.89 times (experiment 4) to 5.36 times (experiment 1) for the optical density at 260 mμ. The average increase in the rapidly sedimenting material after fertilization observed in these tests is 3.3 and 3.7 for the radioactivity and absorbancy measurements, respectively.

The data presented in Table IV show a similar type of analysis with the exceptions that DOC-treated homogenate was utilized instead of the supernatant fraction thereof, and that, in addition, the radioactivity remaining in the DOC-insoluble pellet has been recorded. This allows one to distinguish between the release of pre-formed rapidly sedimenting particles from the formation of such particles in response to fertilization. The data have been adjusted with respect to the total radioactivity in the homogenates, within each experiment, to allow comparisons of equal numbers of unfertilized eggs with fertilized eggs.

The data shown in columns 5, 6 and 7 of Table IV indicate, that 2.04 times (experiment 1) to 3.55 times (experiment 2) more radioactivity sediments to the polyribosome-region (only fractions

TABLE III

Radioactivity and 260 mμ Absorption of the Polyribosome-region of Sucrose Density-gradients of Low-speed Supernatant Fractions[a] of Homogenates of RNA-labeled Unfertilized and Fertilized Eggs of Lytechinus pictus.

Expt. No.	Minutes after fertilization	CPM in polyribosome-region[b]			260 mμ absorption in polyribosome-region[b]		
		Unfert.	Fert.	Fert./Unfert.	Unfert.	Fert.	Fert./Unfert.
1	40	3033	8034	2.6	--	--	-
2	40	939	2492	2.7	0.214	1.148	5.36
3	40	1215	6240	5.1	0.431	1.590	3.69
4	60	1064	2924	2.7	1.122	2.120	1.89
5	90	--	--	-	0.489	1.911	3.93

[a]Post-mitochondrial supernatant fractions of homogenates of unfertilized and fertilized eggs, labeled with H^3-uridine during oogenesis, were prepared and subjected to sucrose density-gradient centrifugation as given in the legend to Fig. 6. Three drop fractions (0.15-0.20 ml) were collected, the 260 mμ absorption of each fraction measured and the radioactivity determined as given under Materials and Methods.

[b]Polyribosome-region was determined as given in Table 1.

TABLE IV

Demonstration of the Formation of Ribosomal Aggregates by 40 Minutes after Fertilization in RNA-labeled Eggs of Lytechinus pictus.

Expt. No.	CPM in the fractionated homogenate[a]								
	DOC-soluble material			DOC-soluble polyribosome-region			DOC-insoluble pellet		
	Unfert.	Fert.	Fert./Unfert.	Unfert.	Fert.	Fert./Unfert.	Unfert.	Fert.	Fert./Unfert.
1[b]	458060	458260	1.0	11406	23263	2.04	23320	21717	0.93
2	669182	668490	1.0	6352	22570	3.55	32432	23329	0.72
3	101050	101529	1.0	1949	4147	2.13	1728	1113	0.64
4	405040	405430	1.0	1978	4687	2.37	--	--	--

[a] Unfertilized and fertilized eggs, labeled with H^3-uridine during oogenesis, were homogenized in homogenization buffer, and then clarified with 0.5% DOC and centrifuged at 15,000 x g for 15 minutes, as given under Materials and Methods. The supernatant fraction was removed, the DOC-insoluble pellet washed with homogenization buffer by centrifugation and its radioactivity determined by scintillation counting. The supernatant fraction was analyzed by sucrose density-gradient centrifugation, as given in the legend to Fig. 5, and the radioactivity in the first 3 fractions summed as the polyribosome-region.

[b] The RNA was phenol-extracted from the polyribosome-region of the sucrose gradients of the homogenates of both the unfertilized and fertilized eggs. The labeled RNA obtained was examined by sucrose density-gradient centrifugation. The results, illustrated in Fig. 8, show that the increase in radioactivity after fertilization is predominantly an increase in 28S and 18S ribosomal RNA.

1-3 in these experiments) from DOC-treated homogenates of fertilized eggs than those of unfertilized eggs. The values given in columns 8, 9 and 10 of the table show that this increase cannot be accounted for by a corresponding loss of radioactivity from the DOC-insoluble low-speed pellet. The augmented cpm's in the polyribosome-region can be attributed, rather, to an increase in the ratio of ribosomal aggregates to single ribosomes in the DOC-soluble supernatant fraction after fertilization. It is of interest to note, however, that the DOC-insoluble low-speed pellet of the homogenates possesses slightly less radioactivity after fertilization. The nature of the materials that are released from the pellet is not known at the present time.

The labeled RNA of the polyribosome-region in experiment 1 was phenol-extracted and analyzed by sucrose density-gradient centrifugation. This allowed examination of the types of RNA which account for the increase in radioactivity in the polyribosome-region after fertilization. The results are shown in Fig. 8. It is clear that the two-fold increase in the rapidly sedimenting cpm's is quantitatively accounted for by additional labeled 28S and 18S ribosomal RNA.

It can be concluded, therefore, that ribosomal aggregates are present in homogenates of unfertilized eggs. There are more aggregates, however, in corresponding homogenates of fertilized eggs. The increased numbers of ribosomal aggregates present in the post-mitochondrial supernatant fraction of homogenates after fertilization cannot be explained by the release of ribosomal aggregates from the DOC-soluble low-speed pellet. The evidence shows, instead, that additional ribosomal aggregates assemble

Fig. 8. SEDIMENTATION PATTERN OF LABELED RNA FROM THE POLYRIBOSOME-REGION OF SUCROSE GRADIENTS OF THE LOW-SPEED SUPERNATANT FRACTION OF DOC-TREATED HOMOGENATES OF UNFERTILIZED AND FERTILIZED (40 MINUTES) EGGS OF LYTECHINUS PICTUS. A spawned female was injected with 120 μc of H^3-5-uridine (sp. act., 20,000 c/M) and shed 40 days later. The labeled suspension obtained was divided into 2 equal parts. One part was fertilized, mechanically demembranated and cultured at 20°C for 40 minutes. Both parts, unfertilized and fertilized, were then homogenized in 3 volumes of homogenization buffer, the homogenates clarified with 0.5% DOC, centrifuged at 15,000 x g for 15 minutes and 0.275 ml of the supernatant fractions layered on sucrose density-gradients as given in the legend to Fig. 5. Fractions 1-3 of each gradient were combined, the RNA phenol-extracted at 4°C as given under Materials and Methods. The labeled RNA was subjected to sucrose density-gradient centrifugation and the radioactivity analyzed as given in the legend to Fig. 1.

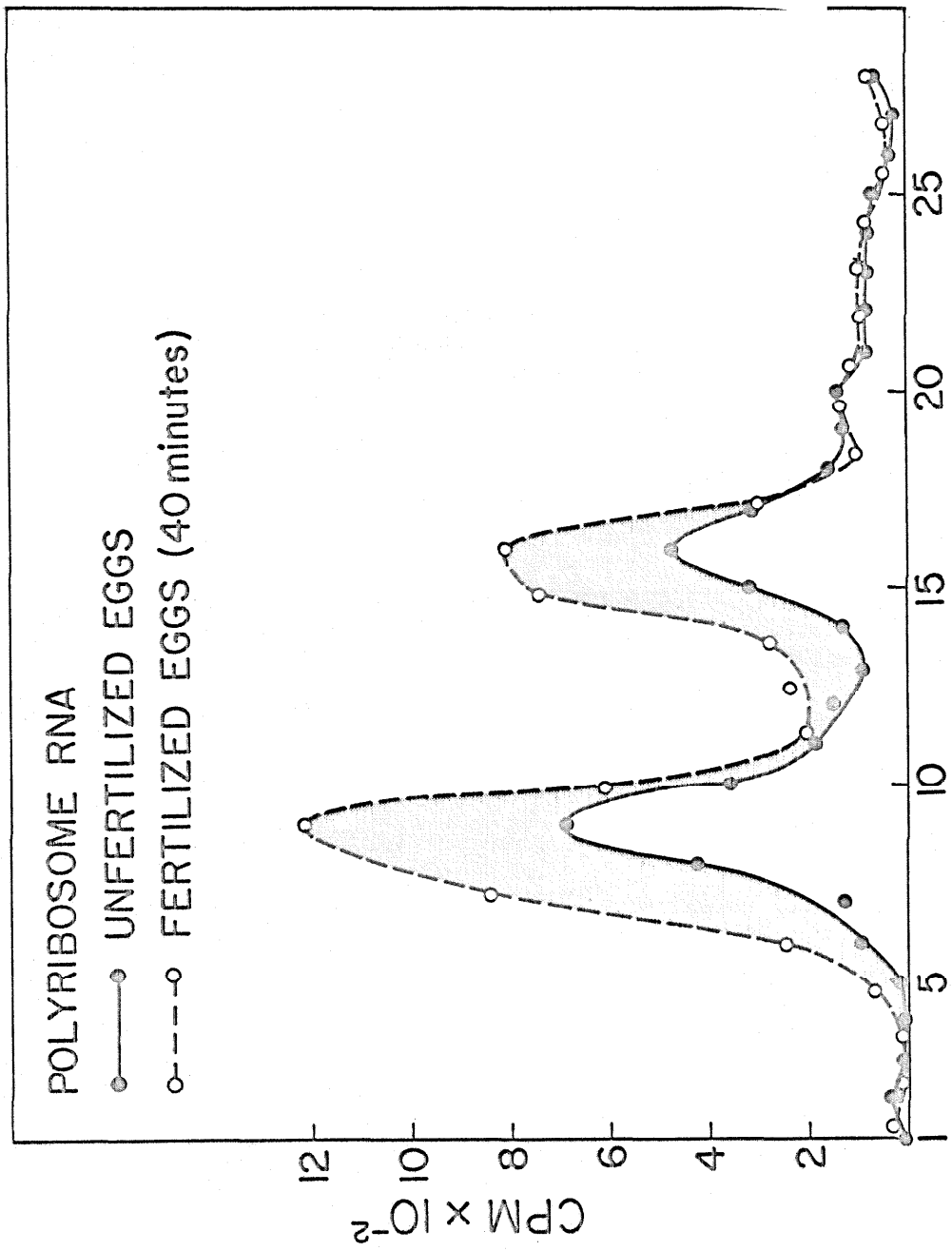
POLYRIBOSOME RNA
UNFERTILIZED EGGS
FERTILIZED EGGS (40 minutes)
CPM x 10⁻²
2
4
6
8
10
12
1
5
10
15
20
25
FRACTION NUMBER

Figure 8.

after fertilization. This is consistent with the earlier observation that relatively few ribosomal aggregates sediment with the mitochondrial pellet.

3) Effect of RNase-treatment of the ribosomal aggregates before and after fertilization

In order to characterize further the ribosomal clusters in homogenates of unfertilized eggs they were subjected to the action of RNase. This is known to disaggregate functional polyribosomes (Warner, Knopf & Rich, 1963; Gierer, 1963; Gilbert, 1963). The results of an experiment of this type are illustrated in Fig. 9 and show that RNase, when added to the supernatant fraction before centrifugation, has no significant effect on the sedimentation profile of the radioactivity or of the 260 mμ absorption. Alkaline hydrolysis of the isolated fractions, however, renders the label soluble in cold trichloroacetic acid and supports the conclusion that the rapidly sedimenting radioactivity is indeed RNA.

Another experiment of this type was performed to compare the effect of RNase on the ribosomal aggregates from homogenates of unfertilized eggs with those of fertilized eggs. The results are shown in Fig. 10. Once again, the sedimentation pattern of the radioactive or of the 260 mμ absorbing materials of unfertilized egg-supernatant fractions was not altered by RNase-treatment (Figs. 10A and B). Alkaline hydrolysis of the fractions of the polyribosome-region, however, eliminated all cpm's precipitable with cold trichloroacetic acid. The shoulder of radioactivity protruding from the heavy edge of the monoribosomes is due to

Fig. 9. SEDIMENTATION PATTERN OF THE LOW-SPEED SUPERNATANT FRACTION, WITH AND WITHOUT RNASE, OF AN HOMOGENATE OF RNA-LABELED UNFERTILIZED EGGS OF LYTECHINUS PICTUS. An equal number of labeled eggs obtained from an animal injected with 150 μc of H^3-6-uridine (sp. act., 6,550 c/M) and one injected with 125 μc of H^3-uridine (uniformly labeled, sp. act., 20,000 c/M), both labeled for 30 days, were combined. Supernatant fractions of homogenates were prepared, as given in the legend to Fig. 2, and then divided into 2 equal parts. One sample received RNase (final concentration of 2 μg/ml) and the other homogenization buffer. Both were incubated at 4°C for 60 minutes, made to 0.5% with respect to DOC and 0.25 ml analyzed by sucrose density-gradient centrifugation as given in the legend to Fig. 5. Three drop fractions (0.15-0.20 ml) were collected, the 260 mμ absorption was determined and the ice-cold 5% trichloroacetic acid precipitable radioactivity was measured as given under Materials and Methods. In addition, each fraction of the RNase-treated preparation was incubated with 0.3 M KOH for 18 hr. at 37°C and the remaining cold trichloroacetic acid precipitable radioactivity determined by scintillation counting.

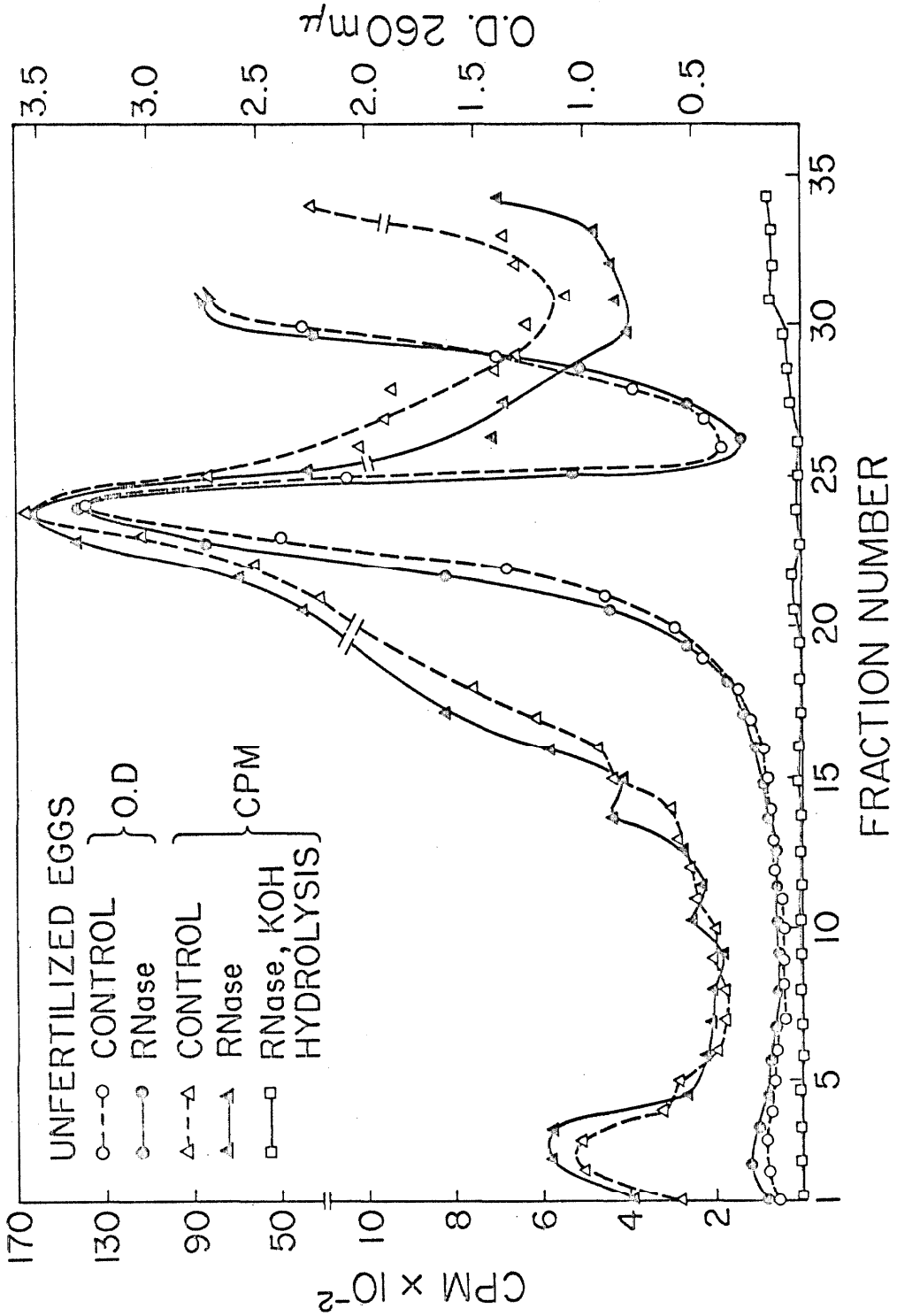
UNFERTILIZED EGGS
CONTROL
RNase
CONTROL
RNase
RNase, KOH HYDROLYSIS
O.D
CPM
CPM x 10⁻²
O.D. 260mμ
FRACTION NUMBER

Figure 9.

Fig. 10. SEDIMENTATION PATTERN OF LOW-SPEED SUPERNATANT FRACTIONS, WITH AND WITHOUT RNASE, OF HOMOGENATES OF RNA-LABELED UNFERTILIZED AND FERTILIZED (60 MINUTES) EGGS OF LYTECHINUS PICTUS. A spawned female was injected with 125 μc of H^3-uridine (uniformly labeled, sp. act., 20,000 c/M) and shed 15 days later. The labeled eggs were divided into 2 equal samples, one of which was fertilized, mechanically demembranated, cultured for 60 minutes at 20°C and washed 3 times with ice-cold 0.55 M KCl in parallel with the unfertilized sample. The unfertilized and fertilized eggs were homogenized, in parallel, in 3 volumes of homogenization buffer, postmitochondrial supernatant fractions prepared, incubated with and without RNase (2.5 μg/ml) and examined by sucrose density-gradient centrifugation as given in the legend to Fig. 2. Three drop (0.15-0.20 ml) fractions were collected, the 260 mμ absorption was determined and the ice-cold 5% trichloroacetic acid radioactivity was assayed in each fraction as given under Materials and Methods.

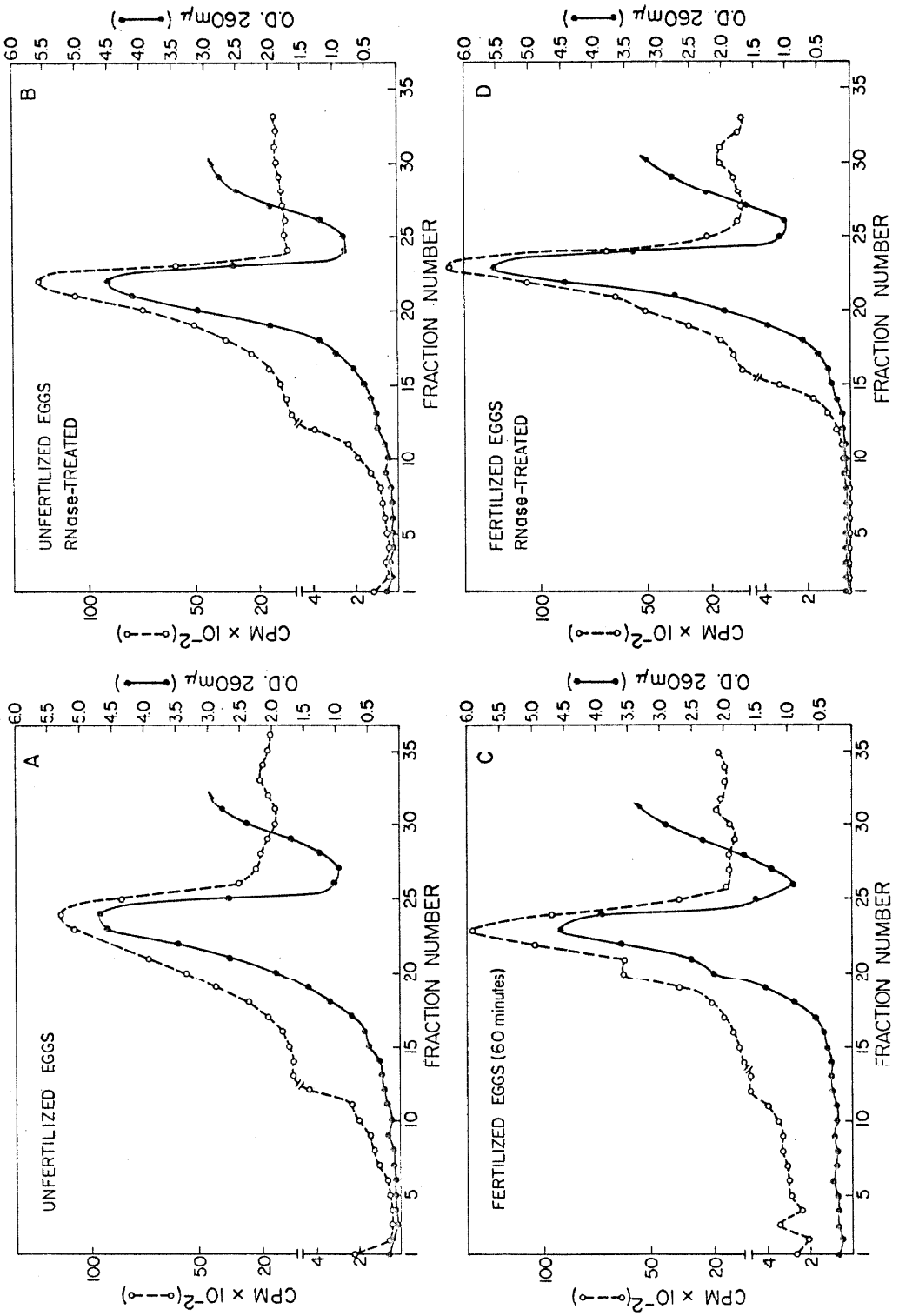
A
UNFERTILIZED EGGS
B
UNFERTILIZED EGGS
RNase-TREATED
C
FERTILIZED EGGS (60 minutes)
D
FERTILIZED EGGS
RNase-TREATED
CPM × 10⁻²(o---o)
O.D. 260mμ (●——●)
FRACTION NUMBER

Figure 10.

the expansion of the scale of the lower portion of the ordinate for the cpm's. This shows more clearly the lack of effect by RNase on the numbers of aggregates in homogenates of unfertilized eggs.

The tests with the fertilized eggs gave a markedly different result. Figs. 10C and D show the usual increase after fertilization in radioactivity and 260 mμ absorption in the polyribosome-region of the gradient. However, RNase-treatment (Fig. 10D) of the supernatant fraction of the fertilized egg-homogenates prevented the sedimentation of radioactive materials to the polyribosome-region of the gradient. Rather, this material could be accounted for by the presence of additional 260 mμ absorption and radioactivity in the nomoribosome-region of the RNase-treated preparation. For instance, the radioactivity sedimenting faster than single ribosomes, before and after RNase-treatment, was 5,606 cpm and 795 cpm, respectively. The values for the monoribosome-region of the sucrose density-gradient were 55,612 cpm without RNase-treatment and 62,371 cpm after RNase-treatment. The sum of the 260 mμ absorption measurements for the polyribosome-region was 3.39 without RNase-treatment and 0.98 after RNase-treatment. The 260 mμ absorption in the monoribosome region was 20.5 before RNase-treatment and 27.6 after RNase-treatment. Thus, the materials in the monoribosome-region of the gradient after treatment with RNase compensate for the corresponding loss of materials from the polyribosome-region.

The reason that RNase failed to dissociate the ribosomal aggregates of unfertilized eggs was investigated further. The possibility that the supernatant fraction of unfertilized egg-homogenates inhibits or indeed

that that of fertilized eggs stimulates RNase activity, was examined. The data given in Table V show the results of a direct test for the inhibitory or stimulatory effect of the low-speed supernatant fractions of unfertilized and fertilized eggs with respect to the activity of added RNase.

A sample of RNA that had been labeled with H^3-uridine was added to homogenization buffer, unfertilized egg-supernatant fraction and fertilized egg-supernatant fraction in each case with and without RNase. The data show that there is little, if any, endogenous RNase activity in the supernatant fractions under these conditions. Furthermore, the added RNase functions approximately to the same extent in the 3 preparations.

Nevertheless, due to the many unknown conditions in homogenates of sea urchin eggs, it was considered of importance to make a direct, quantitative test of the effect of RNase on the ribosomal aggregates of unfertilized eggs. They are, however, present only in small quantity. It is, therefore, difficult to accumulate enough isolated aggregates to enable the performance of experiments on the purified material. In addition, it was considered undesirable to concentrate the aggregates by sedimenting them into a pellet. It was feared that this procedure might produce aggregates not normally present in homogenates of unfertilized eggs. Nonetheless, after sufficiently long-term labeling it was possible to obtain ribosomal aggregates from homogenates of unfertilized eggs that were radioactive enough to perform the experiment. The effect of RNase on the purified aggregates from these RNA-labeled

TABLE V

Effect of RNase[a] on H^3-sRNA[b] in the Presence of Homogenization Buffer or of Low-speed Supernatant Fractions of Homogenates of Unfertilized and Fertilized Eggs (60 minutes) of *Lytechinus pictus*.

Expt. No.	Preparation[c]	CPM of added H^3-sRNA: No TCA[d]	Cold TCA	RNase, cold TCA	% Digestion by supernatant (av. of the duplicate tubes)	% Digestion by RNase (av. of the duplicate tubes)
1	Homo. buffer	760 794	804 765	302 273	-1%	63%
	Unfert. egg supernatant	585 568	500 510	262 261	12.5%	49%
2	Unfert. egg supernatant	653 640	697 631	251 276	-3%	60%
	Fert. egg supernatant	748 738	713 704	303 289	5%	65%

[a] RNase-treatment was 1 µg/ml at 4°C for 60 minutes.

[b] H^3-sRNA (3.38 µg/ml) was purified from HeLa cells labeled with H^3-uridine. The author is indebted to Mr. Loren Hatlen for a gift of this material.

[c] Homogenization buffer: 0.004 M $MgCl_2$, 0.025 M KCl, 0.005 M β-mercaptoethanol, 0.05 M Tris-HCl, pH 7.6 Eggs were homogenized in 6 volumes of homogenization buffer. Post-mitochondrial supernatant fractions of homogenates of unfertilized and fertilized eggs were prepared as given under Materials and Methods.

[d] TCA = 5% trichloroacetic acid.

eggs was compared with its effect on known polyribosomes isolated from fertilized eggs that were labeled with amino acids after insemination. These polyribosomes, naturally, were purified from eggs that were not labeled during oogenesis.

The RNA-labeled ribosomal aggregates of unfertilized eggs and the amino acid-labeled polyribosomes of fertilized eggs were initially isolated by sucrose density-gradient centrifugation. The sedimentation patterns of the purified aggregates, with and without RNase, were then analyzed by density-gradient centrifugation. Non-labeled ribosomes were added to the isolated aggregates before centrifugation to provide a marker indicating the position of single ribosomes in the sucrose gradient. The results are shown in Fig. 11.

The RNase-treatment of the isolated aggregates did not affect their sedimentation, which remained considerably more rapid than single ribosomes. This contrasts to the effect of RNase on the amino acid-labeled polyribosomes. The polyribosomes purified from fertilized eggs sediment with the non-radioactive monoribosomes after treatment with RNase. The ribosomal aggregates present in homogenates of unfertilized eggs, then, are resistent to the action of small quantities of RNase that can dissociate polyribosomes labeled <u>in vivo</u> with amino acids.

Another type of experiment was performed in order to test the effect of RNase on the ribosomal aggregates which were purified from homogenates of unfertilized eggs, but which were not subjected to preliminary treatment that might serve to aggregate ribosomes. RNase was added to the sucrose gradient. The ribosomal aggregates, then, would be exposed to

Fig. 11. SEDIMENTATION PATTERN OF RNA-LABELED RIBOSOMAL AGGREGATES AND OF AMINO ACID-LABELED POLYRIBOSOMES, WITH AND WITHOUT RNASE, ISOLATED FROM HOMOGENATES OF UNFERTILIZED AND FERTILIZED (90 MINUTES) EGGS, RESPECTIVELY, OF LYTECHINUS PICTUS. RNA-labeled unfertilized eggs were obtained from a sea urchin injected with 150 μc of H^3-5-uridine (sp. act. 25,000 c/M), shed 30 days later, reinjected as above and shed 157 days later. Non-radioactive fertilized eggs (cultured for 90 minutes at 20°C) were labeled for 20 minutes at 20°C with a non-competitive mixture (Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis) of 0.75 μc/ml of C^{14}-L-aspartic acid (sp. act., 58 c/M), 1.6 μc/ml of C^{14}-L-arginine (sp. act., 222 c/M) and 1.1 μc/ml of C^{14}-L-valine (sp. act., 208.5 c/M) followed by washing with ice-cold 0.55 M KCl. Low-speed supernatant fractions were prepared as given in the legend to Fig. 2. Three aliquots (0.3 ml) of each preparation were centrifuged at 27,500 rev./min for 100 minutes at 5-10°C through 4.86 ml of a linear 5-20% sucrose gradient made in homogenization buffer, the first 4 drops (0.20-0.25 ml) of each gradient collected, the unfertilized and the fertilized preparations combined, respectively, and each divided into 2 equal parts, one receiving 1 μg/ml of RNase, the other buffer. Both were incubated at 4°C for 45 minutes, diluted with an equal volume of buffer containing purified unlabeled ribosomes and centrifuged for 75 minutes through a sucrose gradient as given in the legend to Fig. 5. Three drop fractions were collected, the 260 mμ absorption determined and the radioactivity assayed as given under Materials and Methods.

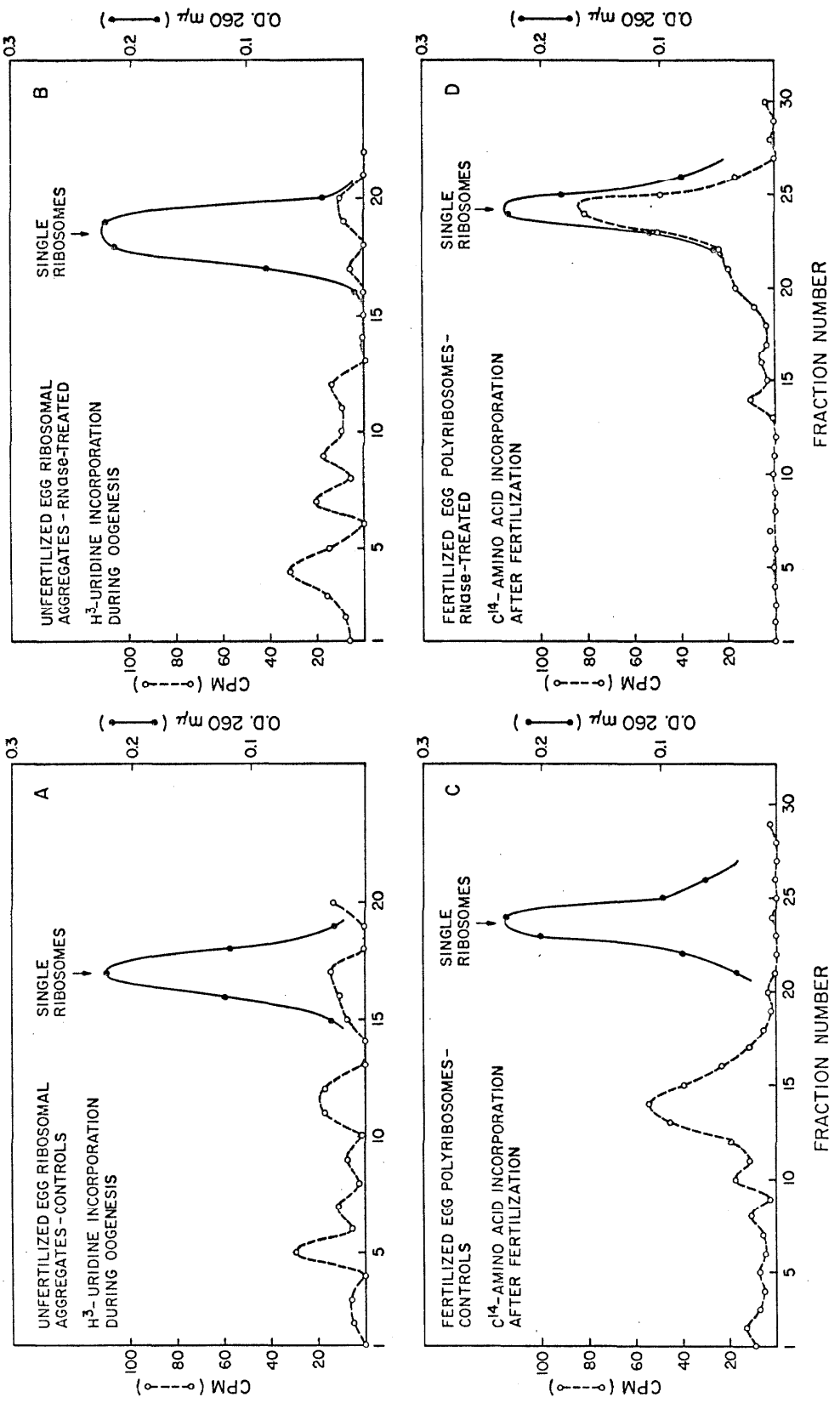
A
UNFERTILIZED EGG RIBOSOMAL AGGREGATES - CONTROLS
H3-URIDINE INCORPORATION DURING OOGENESIS
SINGLE RIBOSOMES
B
UNFERTILIZED EGG RIBOSOMAL AGGREGATES - RNase-TREATED
H3-URIDINE INCORPORATION DURING OOGENESIS
SINGLE RIBOSOMES
C
FERTILIZED EGG POLYRIBOSOMES - CONTROLS
C14-AMINO ACID INCORPORATION AFTER FERTILIZATION
SINGLE RIBOSOMES
D
FERTILIZED EGG POLYRIBOSOMES - RNase-TREATED
C14-AMINO ACID INCORPORATION AFTER FERTILIZATION
SINGLE RIBOSOMES
CPM (o----o)
O.D. 260 mμ (●——●)
FRACTION NUMBER

Figure 11.

the RNase as they were sedimenting through the density-gradient.

The results of an experiment of this type are shown in Fig. 12. The addition of RNase to the sucrose gradient reduced the number of labeled aggregates that sedimented to the polyribosome region from homogenates both of unfertilized (Fig. 12A) and fertilized eggs (Fig. 12B).

This type of experiment was repeated 3 times and similar results were obtained (see Table VI below). These results are contradictory to those reported above and their significance remains obscure. One possible explanation is that the aggregates are reversibly altered in structure during the centrifugation in a manner sufficient to provide the opportunity for RNase to dissociate them during the sedimentation process.

Table VI summarizes the effect of RNase-treatment of homogenates, before and after fertilization, on the numbers of ribosomal aggregates. The results are expressed as the ratio of cpm's and of 260 mμ absorption, in the polyribosome-region of the density-gradient before treatment with RNase to the corresponding values obtained after RNase-treatment. A ratio smaller than unity indicates an increase in the number of aggregates. A ratio greater than one, on the other hand, shows a decrease in aggregate-numbers after the addition of RNase.

The values given in column 2 of the table show that RNase-treatment of the supernatant fraction of unfertilized eggs had little effect on the amount of ribosomal aggregates sedimenting to the polyribosome-region of the gradient in 11 experiments. The ratios ranged from 0.66 in experiment 10 to 1.58 in experiment 5. They were close to unity in the other 9 tests. In experiment 6 DOC was omitted from the supernatant fraction.

Fig. 12. SEDIMENTATION PATTERN OF LOW-SPEED SUPERNATANT FRACTIONS OF HOMOGENATES OF RNA-LABELED UNFERTILIZED AND FERTILIZED (40 MINUTES) EGGS OF LYTECHINUS PICTUS, WITH AND WITHOUT RNASE PRESENT IN THE SOLUTIONS COMPRISING THE SUCROSE GRADIENT. A spawned female was injected with 150 μc of H^3-5-uridine (sp. act., 25,000 c/M) and was shed 31 days later. Post-mitochondrial supernatant fractions of the unfertilized and the fertilized eggs were prepared as given in the legend to Fig. 6 and 0.250 ml samples were analyzed by sucrose density-gradient centrifugation as given in the legend to Fig. 5, with the exception that 2 of the 4 sucrose gradients, as indicated in the graphs, contained 2 μg/ml of RNase. Three drop fractions (0.15-0.20 ml) were collected and the radioactivity of each fraction was measured by scintillation counting as given under Materials and Methods.

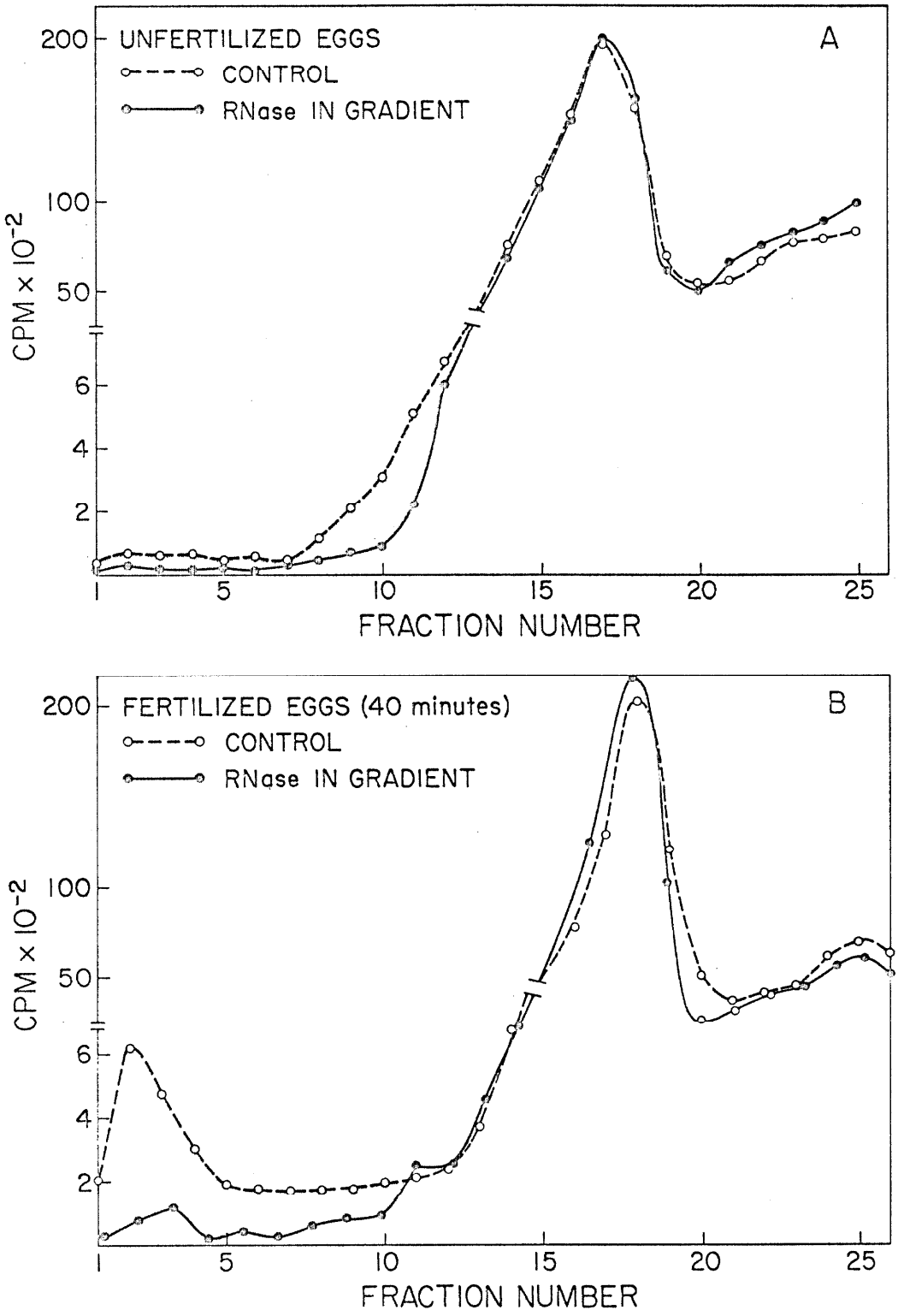
UNFERTILIZED EGGS
A
CONTROL
RNase IN GRADIENT
CPM x 10-2
200
100
50
6
4
2
1
5
10
15
20
25
FRACTION NUMBER
FERTILIZED EGGS (40 minutes)
B
CONTROL
RNase IN GRADIENT
CPM x 10-2
200
100
50
6
4
2
1
5
10
15
20
25
FRACTION NUMBER

Figure 12.

TABLE VI

Effect of RNase[a] on the Numbers of RNA-labeled Ribosomal Aggregates Sedimenting to the Polyribosome-region of a Sucrose Gradient from the Low-speed Supernatant Fraction[b] of Homogenates of Unfertilized and Fertilized Eggs of Lytechinus pictus.

	Ratios of material sedimenting to the polyribosome-region[c] before to that after treatment with RNase			
	CPM		260 mμ absorption	
Expt. No.	U/UR[d]	F/FR[d]	U/UR	F/FR
1	1.04			
2	0.94			
3	1.25		0.86	
4	0.98		1.02	
5	1.58		1.13	
6	1.05		1.00	
7	1.02 0.83[e]			
8	0.88	26.8	0.86	3.67
9	1.02	3.7	0.89	1.90
10	0.66	2.76	0.60	1.62
11	1.05 1.55[f]		0.97 1.05[f]	
12[f]	2.79	3.00	1.07	4.13
13[f]	3.03	4.36	1.03	44.2

[a] RNase-treatment was 1-2 μg/ml at 4°C for 30-60 minutes.

[b] Postmitochondrial supernatant fractions prepared, with and without RNase, and analyzed by sucrose density-gradient centrifugation as given in the legend to Fig. 6.

[c] Polyribosome-region determined as given in Table I.

[d] U and F = unfertilized and fertilized egg controls; UR and FR = RNase treated.

[e] DOC omitted from the supernatant fraction.

[f] RNase (2 μg/ml) was present in the solution comprising the sucrose density-gradient and was, therefore, not added to the supernatant fraction.

However, this did not cause a significant difference in the numbers of ribosomal aggregates sedimenting to the polyribosome region or on their resistance to RNase.

In experiments 11b, 12 and 13 RNase was added to the sucrose gradient rather than to the supernatant fraction. Under these conditions, the ratio of cpm's in the polyribosome-region, with and without RNase in the gradient, was greater than 1. It ranged from 1.55 (experiment 11) to 3.03 (experiment 13). It should be noted that a ratio of 1.55 is comparable with a ratio of 1.58 (experiment 5) and is not appreciably different from a ratio of 1.25 (experiment 3) which were obtained when RNase was added to the supernatant fraction. It is, then, only experiments 12 and 13 that remain contradictory. This has been briefly discussed above.

The data listed in column 4 of Table VI are the corresponding ratios of 260 mμ absorption in the polyribosome-region before and after RNase-treatment. These values, too, are close to unity. However, the difference occuring in the cpm's when RNase is added to the gradient is not reflected in the 260 mμ absorption profile. It is likely that this is due principally to the very low absorption by the few ribosomal aggregates present in homogenates of unfertilized eggs, which allows small experimental fluctuations to obscure the results.

The values of the ratio of ribosomal aggregates for the fertilized eggs, before and after RNase-treatment, are listed in columns 3 and 5 of Table VI. The radioactivity-ratios range from 2.76 (experiment 10) to 26.8 (experiment 8) and the 260 mμ absorption-ratios from 1.62

(experiment 10) to 44.2 (experiment 13). The very high ratios occurred in only one of the five experiments and presumably are atypical. A value of 3 to 4 may thus be considered more representative of the RNase effect. Consequently, RNase seldom destroys all the rapidly sedimenting aggregates in the low-speed supernatant fractions of homogenates of fertilized eggs but consistently and significantly dimishes their number.

4) Effect of RNase-, trypsin- and trypsin followed by RNase-treatment of the ribosomal aggregates of unfertilized eggs.

One possible reason that the ribosomal aggregates found in homogenates of unfertilized eggs are resistant to low concentrations of RNase is that proteinaceous material envelopes, or binds to the aggregates. This would be in accord with the trypsin-labile inhibitor of protein synthesis thought to be attached to ribosomes of unfertilized eggs (Maggio, Monroy, Rinaldi and Vittorelli, 1965; Monroy, Maggio and Rinaldi, 1965). Tests were therefore made on the ability of trypsin, and trypsin followed by RNase, to dissociate the ribosomal aggregates. The results are summarized in Table VII.

Experiments 1 to 4, were performed by the addition of the enzyme(s) to post-mitochondrial supernatant fractions of homogenates of labeled unfertilized eggs. Experiment 5, on the other hand, was conducted with isolated ribosomal aggregates after they were initially purified by sucrose density-gradient centrifugation.

Comparisons of the results shown in column 2 with those in column 3 show that RNase-treatment of supernatant fractions of homogenates of

TABLE VII

Effect of RNase, Trypsin and Trypsin Followed by RNase on the Numbers of RNA-labeled Ribosomal Aggregates Sedimenting to the Polyribosome-region of Sucrose Gradients of Homogenates, or Supernatant Fractions Thereof, of Unfertilized Eggs of Lytechinus Pictus.

Expt. No.[b]	CPM in Polyribosome-region[a]			Trypsin followed by RNase
	Controls	RNase[c] (μg/ml)	Trypsin[d] (μg/ml)	
1	104	123 (1.25)	194 (1.0)	92
2	542	523 (0.5)	219 (5.0)	155
3	269	137 (1.0)	45 (10.0)	114
4		319 (1.0)		137
5	112	145 (0.72)	20 (10.0)	2
	CPM in Monoribosome-region[a]			
5	136	130	299	322

[a]Polyribosome and monoribosome regions of sucrose density-gradient determined as given in Table 1.

[b]Expt. 1-4: Low-speed supernatant fractions of homogenates of unfertilized eggs were prepared and analyzed by sucrose density-gradient centrifugation as given in the legend to Fig. 6. Expt. 5: Homogenates of unfertilized eggs prepared and subjected to sucrose density-gradient centrifugation as given in Table 4. The first 0.3 ml of the gradient was collected and aliquots were treated enzymatically followed by sucrose density-gradient analysis as in experiments 1-4.

[c]Expt. 1-2, 4-5: RNase-treatment for 60 min. at 4°C. Expt. 3, RNase-treated for 30 min. at 4°C.

[d]Trypsin was self-digested for about 1 hr. at 25°C before use. An equal quantity by weight of Soybean Trypsin Inhibitor was added before RNase-treatment. Expt. 1-4: Trypsin-treatment for 30 min at 25°C. Expt. 5, trypsin-treatment for 15 min. at 25°C.

unfertilized eggs (experiments 1-4) or of purified aggregates (experiment 5) did not significantly decrease the amount of ribosomal aggregates. Only experiment 3 showed somewhat fewer aggregates after RNase-treatment. These results are consistent with those of the experiments presented above.

The values listed in column 4 of the table indicate, however, that trypsin-treatment reduced the numbers of ribosomal aggregates in three (experiments 2, 3 and 5) of four tests. In the experiment in which trypsin failed to dissociate the ribosomal aggregates (experiment 1), it should be noted that only 1 μg/ml of trypsin was used. This is five times (experiment 2) to ten times (experiments 3 and 5) less trypsin than employed in the other tests.

Column 5 of Table VII lists the values of radioactivity representing the amount of ribosomal aggregates remaining after treatment with trypsin followed by RNase. The conditions of the enzyme-treatments were the same as those given for the individual treatments for each experiment. It is clear that the combined effects of trypsin and RNase reduced the numbers of ribosomal aggregates relative to the controls. Moreover, experiments 1, 2 and 5 show that the action of trypsin and RNase more effectively dissociated the aggregates than did trypsin alone.

The data of experiment 5, which was performed with purified labeled ribosomal aggregates, follow the changes in the sedimentation of radioactivity after treatment of the aggregates with RNase. The radioactivity abolished from the polyribosome-region after treatment with trypsin, or trypsin followed by RNase, was quantitatively recovered in the monoribosome-region of the gradient. The sum of the radioactivity-measurements

made from the polyribosome- plus the monoribosome-region, in experiment 5, was 248 cpm, 275 cpm, 319 cpm and 323 cpm for the controls, RNase-, trypsin- and trypsin followed by RNase-treatments, respectively. Evidently, then, the ribosomal aggregates are dissociated to single ribosomes by trypsin-treatment.

It is known that trypsin preparations often possess RNase activity (see Glowacki, 1966). Thus, the trypsin solutions employed in the present experiments were examined with respect to such activity.

One set of tests was made on the ribosomal aggregates of fertilized eggs along with those of unfertilized eggs. It was found that incubation of DOC-treated homogenates of RNA-labeled unfertilized and fertilized eggs with self-digested (room temp. for 30-60 min.) trypsin (10 µg/ml, 4°C for 30 min.) reduced the number of ribosomal aggregates by about three-fourths in the case with the unfertilized eggs and by approximately seven-eighths with the fertilized eggs. The radioactivity in the polyribosome-region of the gradient was lowered by the trypsin-treatment from 13,592 cpm to 3,547 cpm for the unfertilized eggs and from 28,849 cpm to 4,339 cpm for the fertilized eggs. The difference in 260 mµ absorption in the polyribosome-region was also lower after trypsin-treatment both of the unfertilized and the fertilized egg-preparations in this test as well as in another which utilized unlabeled homogenates.

In another set of tests, H^3-RNA, obtained by repeated phenol-extraction of 4 hour pulse-labeled embryos of Lytechinus pictus, was made soluble in 5% trichloroacetic acid after an hour exposure to a trypsin solution (1 mg/ml) at room temperature. Trypsin reduced the

number of precipitable cpm's of H^3-RNA by 73% and 84%, respectively. The latter solution had been partially purified by Sephadex G-25 chromatography. Pronase (1 mg/ml), on the other hand, only reduced the precipitable radioactivity by 15% in parallel tests. The evidence shows then that the trypsin solutions possessed considerable RNase activity.

An attempt to obviate this difficulty was made by pretreating the ribosomal aggregates of unfertilized eggs with pronase which showed much less RNase activity than trypsin. In two tests, addition of self-digested (37°C for 60 min.) pronase (experiment 1, 5 µg/ml, 4°C for 15 min.; experiment 2, 50 µg/ml, 4°C for 30 min.) to homogenates of unfertilized eggs did not dissociate the ribosomal aggregates. Furthermore, incubation with RNase (experiment 1, 5 µg/ml; experiment 2, 2 µg/ml) for 15 to 30 minutes at 4°C after pronase-treatment did not reduce the numbers of ribosomal aggregates. A direct test with labeled RNA showed that pronase did not interfere with RNase activity under these conditions.

The experiments on the effect of proteolytic enzymes on the ribosomal aggregates indicate that trypsin-sensitive material has a significant role in the resistance of the aggregates to RNase. Since the trypsin solutions have been found to possess RNase activity, it is not possible to decide at present whether trypsin alone can dissociate the aggregates or whether the combined action of trypsin and RNase is the effective treatment. In any case, the recovery of the disaggregated material in the monoribosome-region of the sucrose gradient shows that trypsin-treatment did not disintegrate individual ribosomes and thus suggests that the

RNase-resistance of the ribosomal aggregates depends upon the integrity of trypsin-labile materials binding the ribosomes together. That trypsin but not pronase readily dissociates the aggregates indicates that the material combined with the ribosomal complex is enriched in lysine and arginine residues.

5) In vivo incorporation of C^{14}-amino acids into protein by ribosomal aggregates of unfertilized and fertilized eggs

In order to test further the properties of the ribosomal aggregates present in homogenates of unfertilized and of fertilized eggs, experiments were made on their in vivo amino acid incorporating activity. Since unfertilized eggs incorporate very little labeled amino acid into protein, the following procedure was utilized.

A suspension of mature, unfertilized eggs was treated with a non-competitive (Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis), unlabeled mixture of threonine, lysine and glutamic acid. This serves to partially deplete the endogenous pool of other neutral, basic and acidic amino acids, respectively, by displacing them from the intact cells into the sea water as the mixture is taken up by the eggs (see Appendix 2 of this thesis). After 30 minutes incubation, the eggs were washed from the exogenous C^{12}-amino acid mixture. An aliquot was fertilized and cultured for the specified time. Both suspensions, unfertilized and fertilized, were then exposed to a mixture of C^{14}-valine, C^{14}-arginine and C^{14}-aspartic acid for 20 minutes. These three amino acids do not compete for entrance into the cells and, therefore, give a

very high degree of labeling of the cells (Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis). Due to the pretreatment of the eggs with the C^{12}-amino acids, the specific activity of the labeled amino acids within the cells is higher than can be achieved without the preliminary step. This results in increased incorporation of the radioactive amino acids into protein presumably without affecting the rate of protein synthesis.

The low-speed supernatant fractions of homogenates of unfertilized and of fertilized eggs treated in this manner were analyzed by sucrose density-gradient centrifugation. The results are shown in Fig. 13. The supernatant fractions of unfertilized eggs (Fig. 13A) and of fertilized eggs (Fig. 13C) have labeled, acid precipitable material sedimenting to the polyribosome-region of the gradient. After treatment of the supernatant fractions with RNase (Figs. 13B and D), however, the labeled material does not sediment past the single ribosomes. Rather, a sharp peak of radioactivity coincides with the 260 mμ absorption peak of the monoribosomes. The evidence indicates, then, that unfertilized and fertilized eggs incorporate amino acids into protein upon RNase-sensitive ribosomal aggregates.

The specific radioactivity, based on the cpm's per 260 mμ absorbancy unit, of the particles labeled with amino acids is given in Fig. 14. Specific activity calculations of the fractions comprising the monoribosome-region are low and similar in the preparations both from the unfertilized and the fertilized eggs. The specific activity of the polyribosome-region, on the contrary, is considerably higher than that

Fig. 13. SEDIMENTATION PATTERN OF THE LOW-SPEED SUPERNATANT FRACTION, WITH AND WITHOUT RNASE, OF HOMOGENATES OF AMINO ACID-LABELED UNFERTILIZED AND FERTILIZED (90 MINUTES) EGGS OF LYTECHINUS PICTUS. A 5% suspension of unlabeled unfertilized eggs was pretreated with a non-competitive mixture (Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis) of C^{12}-L-threonine, C^{12}-L-lysine and C^{12}-L-glutamic acid, each at a concentration of 0.033 M in artificial sea water, washed thoroughly by centrifugation, divided into 2 equal parts and one sample fertilized, mechanically demembranated and cultured for 70 minutes at 20°C. Ten percent suspensions of each preparation were incubated with a non-competitive mixture of 4.2 μc/ml of C^{14}-L-valine (sp. act. 208.5 c/M), 6.3 μc/ml of C^{14}-L-arginine (sp. act., 222 c/M) and 3 μc/ml of C^{14}-L-aspartic acid (sp. act., 58 c/M) for 20 minutes. The reaction was stopped by washing the eggs 3 times by centrifugation with ice-cold 0.55 M KCl. Post-mitochondrial supernatant fractions of homogenates prepared, treated with RNase (1 μg/ml) and analyzed by sucrose density-gradient centrifugation as given in the legend to Fig. 9. Three drop fractions (0.15 - 0.20 ml) were collected and analyzed for 260 mμ absorption and radioactive protein as given under Materials and Methods.

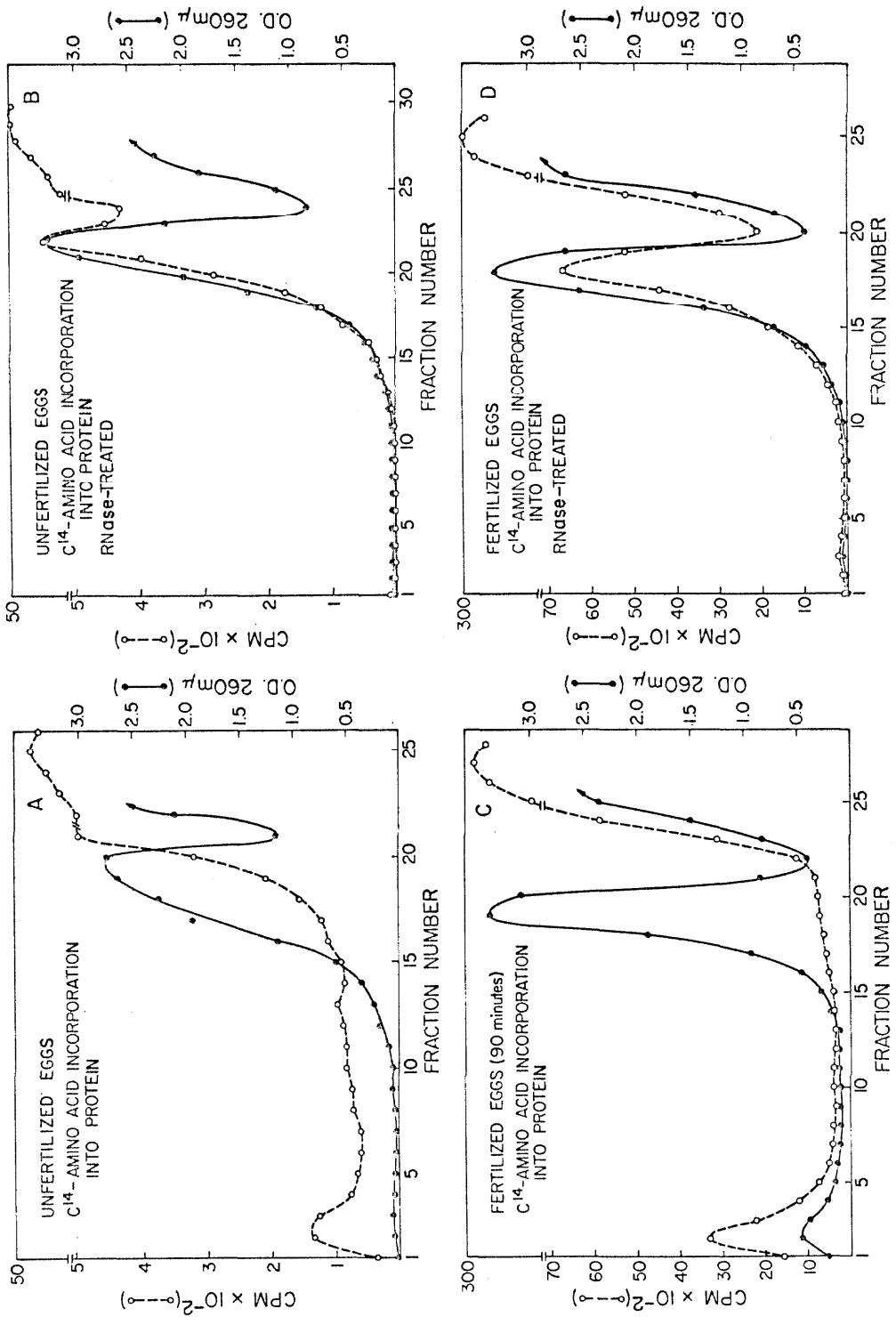
A
UNFERTILIZED EGGS
C14-AMINO ACID INCORPORATION INTO PROTEIN
B
UNFERTILIZED EGGS
C14-AMINO ACID INCORPORATION INTO PROTEIN
RNase-TREATED
C
FERTILIZED EGGS (90 minutes)
C14-AMINO ACID INCORPORATION INTO PROTEIN
D
FERTILIZED EGGS
C14-AMINO ACID INCORPORATION INTO PROTEIN
RNase-TREATED
CPM x 10-2 (o---o)
O.D. 260mμ (●——●)
FRACTION NUMBER

Figure 13.

Fig. 14. SPECIFIC RADIOACTIVITY OF LABELED PROTEIN IN EACH FRACTION OF THE CONTROL SUCROSE GRADIENTS SHOWN IN FIG. 13. The cpm's of each fraction were divided by the 260 mμ absorption.

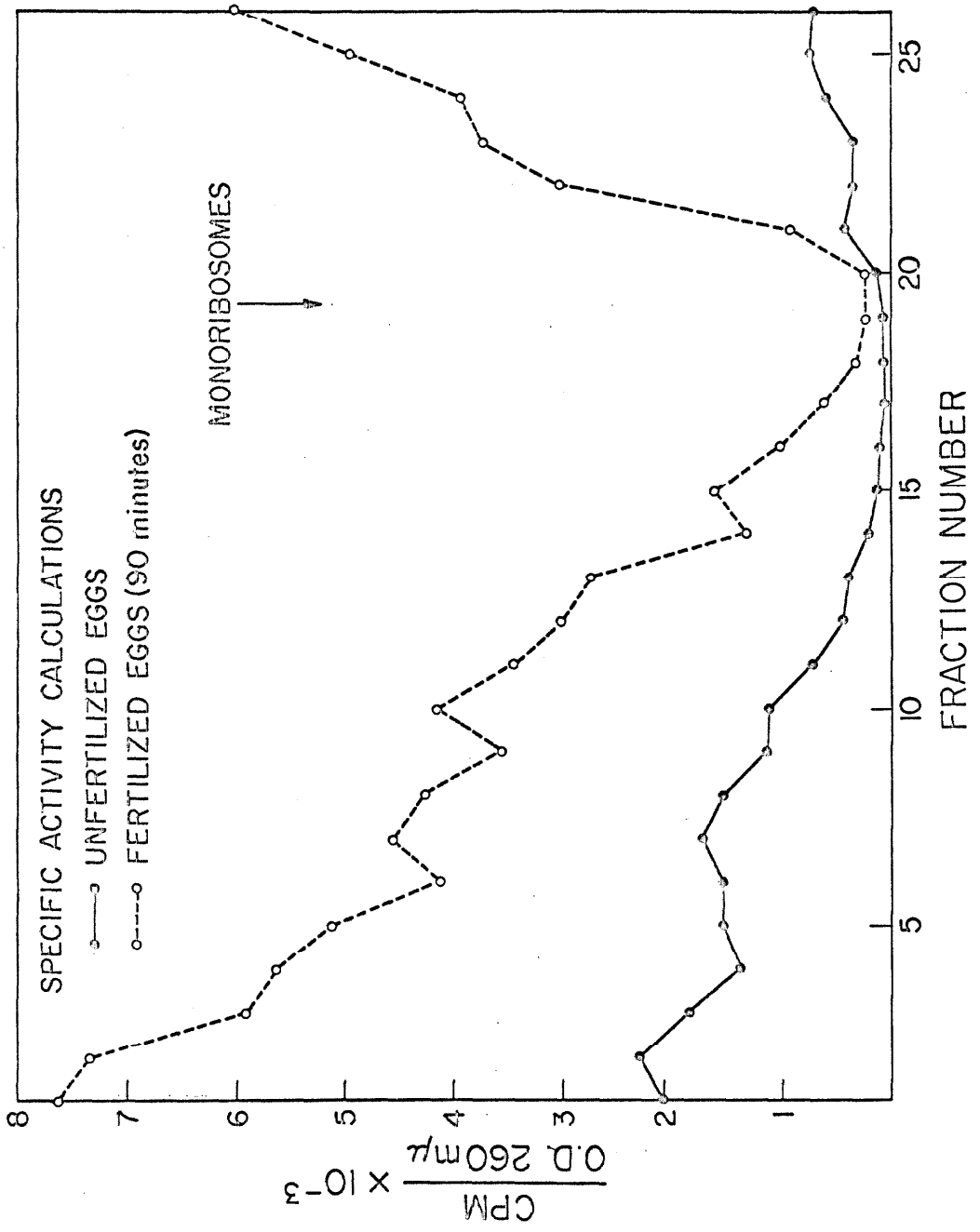
SPECIFIC ACTIVITY CALCULATIONS
UNFERTILIZED EGGS
FERTILIZED EGGS (90 minutes)
MONORIBOSOMES
CPM / O.D. 260mμ × 10⁻³
FRACTION NUMBER
1
2
3
4
5
6
7
8
5
10
15
20
25

Figure 14.

of the monoribosome-region in the two samples. Furthermore, the rapidly sedimenting labeled materials of homogenates of the fertilized eggs possess a specific activity 4 to 6 times greater than that of the corresponding materials of homogenates of the unfertilized eggs.

Measurements of relative rates of amino acid incorporation into protein by whole cells, however, reflect differences in uptake and in pool-size as well as those in incorporation into protein. Moreover, fertilized eggs are known to accumulate amino acids faster than unfertilized eggs (Berg, 1965; Mitchison & Cummins, 1966; Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis).

In order to obtain further information in addition to that provided by the cited workers concerning changes in incorporation with respect to uptake, an experiment was performed with the same labeled amino acid mixture employed in the experiment illustrated in Fig. 13. The results are given in Fig. 15. The 2 upper graphs (Fig. 15A) show that uptake increases only 2 to 3 fold during which time incorporation is enhanced 50 to 60 fold. Since it is likely that the free amino acid pool of unfertilized and of fertilized eggs is approximately the same (Kavanau, 1953, 1954; see Monroy and Maggio, 1966) the percentage of incorporation of the labeled amino acids into protein (Fig. 15B) is 10 to 15 times higher by the unfertilized than by the fertilized eggs after 40 minutes of development. These results are similar to those obtained by Berg (1965) on the incorporation of C^{14}-valine by eggs of Lytechinus anemesus.

Thus, even after adjustment for changes in uptake of amino acids that occur after fertilization, the ribosomal aggregates of fertilized

Fig. 15. RATE OF UPTAKE, INCORPORATION AND PERCENTAGE INCORPORATION OF C^{14}-AMINO ACIDS INTO PROTEIN BEFORE AND AFTER FERTILIZATION OF EGGS OF LYTECHINUS PICTUS. About 10^4 non-radioactive unfertilized and fertilized eggs, at the specified times after fertilization, were exposed, in duplicate tubes to a non-competitive mixture (Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis) of 2.1 μc/ml of C^{14}-L-valine (sp. act., 208.5 c/M), 3.2 μc/ml of C^{14}-L-arginine (sp. act., 222 c/m) and 1.5 μc/ml of C^{14}-L-aspartic acid (sp. act., 58 c/M) for 10 minutes in a total volume of 0.20 ml at 20°C . The reaction was quenched by addition of an ice-cold solution containing an excess of the corresponding C^{12}-amino acids followed by thorough washing by centrifugation with chilled artificial sea water. Uptake and incorporation of the labeled amino acids into protein were measured on the same eggs as described elsewhere (Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis).

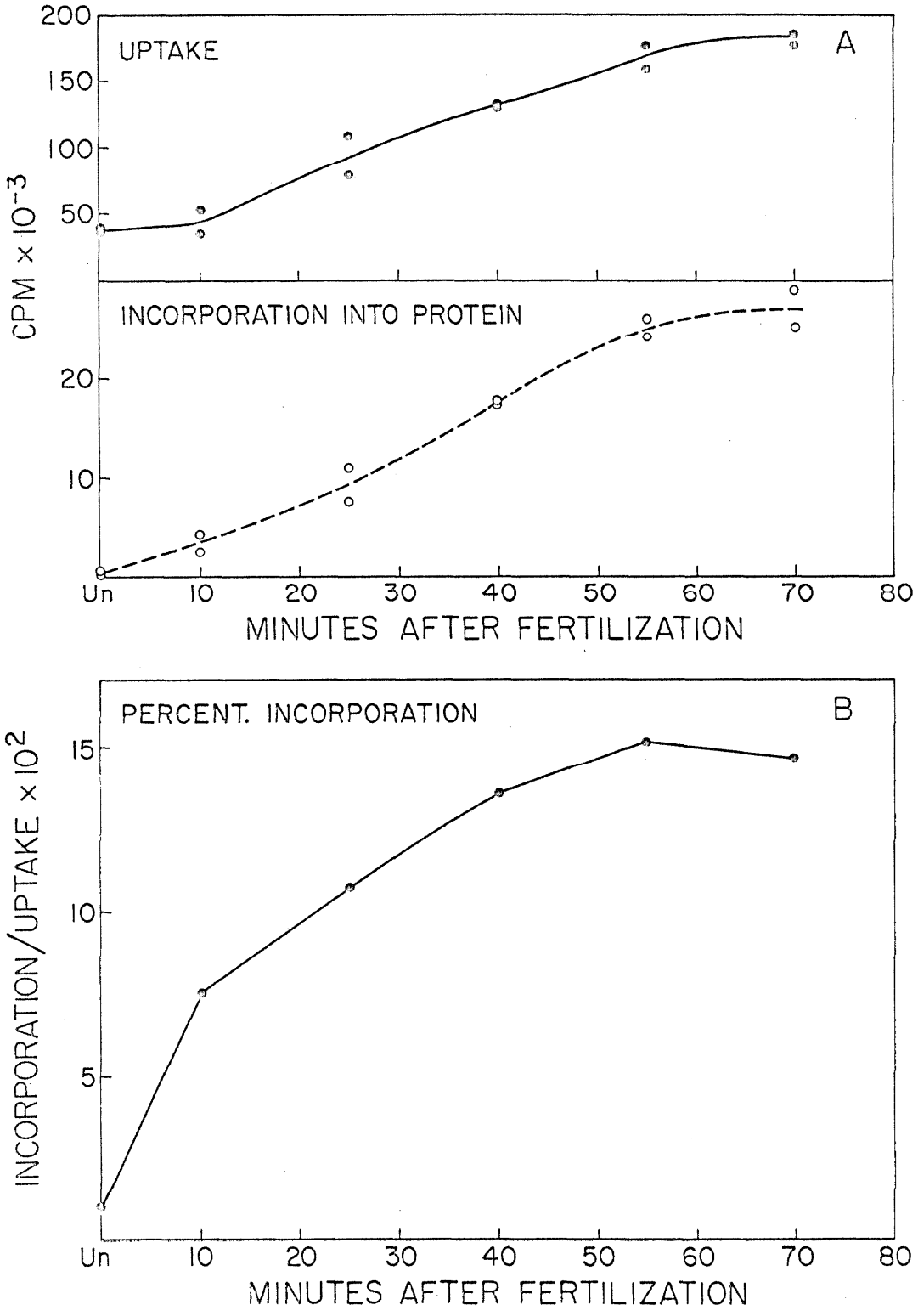
UPTAKE
A
INCORPORATION INTO PROTEIN
CPM x 10⁻³
200
150
100
50
20
10
Un
10
20
30
40
50
60
70
80
MINUTES AFTER FERTILIZATION
PERCENT. INCORPORATION
B
INCORPORATION/UPTAKE x 10²
15
10
5
Un
10
20
30
40
50
60
70
80
MINUTES AFTER FERTILIZATION

Figure 15.

eggs show a 2 to 3 fold higher specific activity of amino acid incorporation into protein than those of unfertilized eggs. This is true whether the calculations are based upon 260 mμ absorption, as shown in Fig. 14, or upon amounts of labeled RNA present in the polyribosome-region before and after fertilization.

6) Examination by electron microscopy of the ribosomal aggregates of unfertilized and fertilized eggs

The materials in the different regions of the sucrose gradients of low-speed supernatant fractions of homogenates of unfertilized and of fertilized eggs were examined by electron microscopy. Electron microscopy was utilized, also, to observe the effect of RNase-treatment of the materials that accumulate on the sucrose cushion. These results were compared, in the case of the unfertilized eggs, with those obtained by the addition of RNase to the supernatant fraction before centrifugation.

Plates IA and B are electron micrographs of the materials from fraction 7 of the polyribosome-region of the sucrose gradient. The larger, somewhat irregular structures in the electron micrographs are evidently aggregates of particles which are 200-300 Å in diameter, although the resolution between subunits is somewhat obscured. This may be caused by the forces of surface tension during the drying of the preparation (Rich, Warner & Goodman, 1963). The aggregates of homogenates of unfertilized and of fertilized eggs are heterogeneous in size and generally similar in appearance. The larger aggregates contain approximately

Plate I. ELECTRON MICROGRAPHS OF MATERIALS IN THE POLYRIBOSOME-REGION OF SUCROSE GRADIENTS OF THE LOW-SPEED SUPERNATANT FRACTION OF HOMOGENATES OF UNFERTILIZED AND OF FERTILIZED (40 MINUTES) EGGS OF LYTECHINUS PICTUS. Post-mitochondrial supernatant fractions were prepared and subjected to sucrose density-gradient centrifugation as given in the legend to Fig. 5. A drop from fraction 7 of the polyribosome-region of each gradient was prepared for electron microscopy and micrographs were taken, as given under Materials and Methods. X 50,000. (A) Unfertilized eggs. (B) Fertilized eggs.

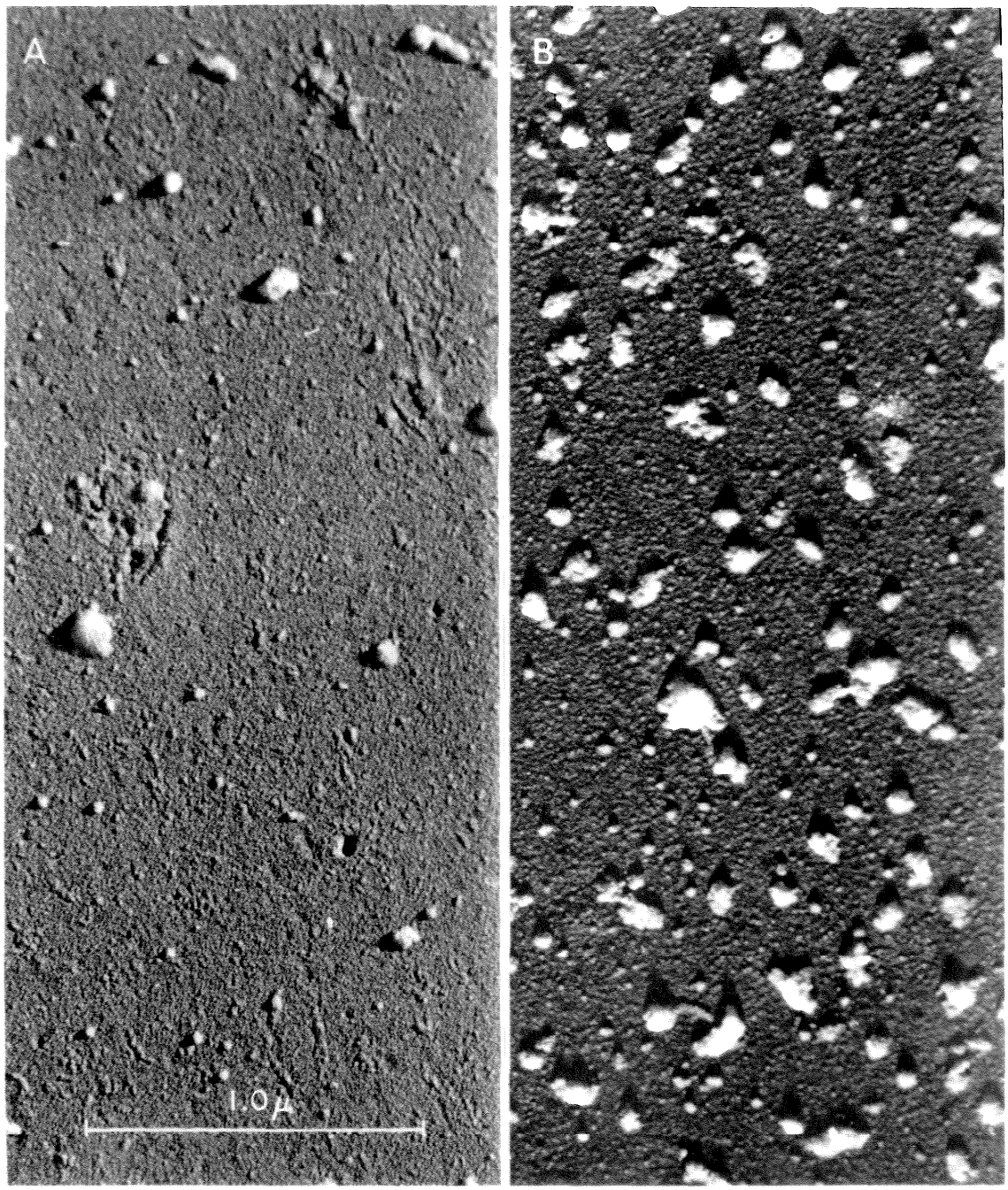

Plate I.

15 to 20 ribosomes. Many more aggregates are present on the grids prepared from fertilized eggs than from unfertilized eggs.

Plate IIA shows that the materials sedimenting to the sucrose cushion from the low-speed supernatant fraction of unfertilized eggs are also aggregates of discrete particles similar to those found in fraction 6. They are, perhaps, slightly larger on the average. Plate IIB, on the other hand, shows the materials from the monoribosome-region of the gradient. The electron micrograph from this region shows that the number of particles per grid is considerably greater than that obtained from the polyribosome-region. Nevertheless, the materials from the monoribosome-region are mostly single particles of a size corresponding to one of the units comprising the aggregates. Ribosomal aggregation, then, does not occur during preparation of the grids for electron microscopy.

Plate III reveals cleavage of the aggregates, prepared from fertilized eggs, after the addition of RNase to the isolated fraction. Plate IIIA shows the clumped particles before RNase-treatment and Plate IIIB shows the material from the same sample after RNase-treatment. The aggregates are almost completely dispersed and the single particles are indistinguishable from those that were obtained from the monoribosome-region (Plate IIB).

In contrast, RNase-treatment of the aggregates of the supernatant fraction of homogenates of unfertilized eggs is strikingly without effect, as already indicated by the experiments reported above. Plate IVA shows that aggregates still accumulate on the sucrose cushion after RNase-

Plate II. ELECTRON MICROGRAPHS OF MATERIALS FROM THE POLYRIBOSOME- AND THE MONORIBOSOME-REGION OF A SUCROSE GRADIENT OF THE LOW-SPEED SUPERNATANT FRACTION OF AN HOMOGENATE OF UNFERTILIZED EGGS OF LYTECHINUS PICTUS. Post-mitochondrial supernatant fractions were prepared and subjected to sucrose density-gradient centrifugation as given in the legend to Plate I. (A) Fraction 2, the materials accumulating on the 60% sucrose cushion. (B) Fraction 20, materials from the monoribosome-peak, diluted 50 times with homogenization buffer.

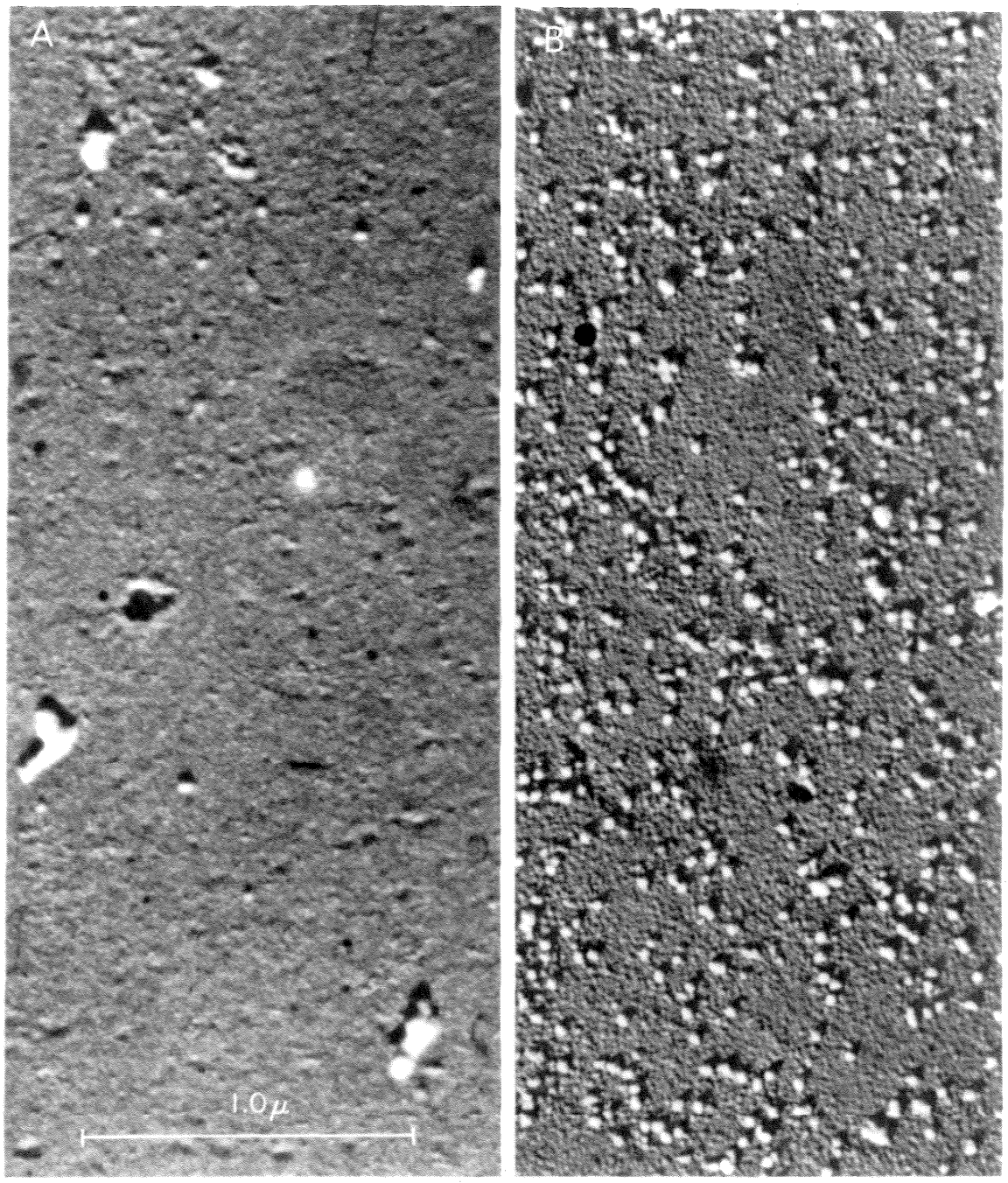

Plate II.

Plate III. ELECTRON MICROGRAPHS OF MATERIALS BEFORE AND AFTER RNASE-TREATMENT, FROM THE POLYRIBOSOME-REGION OF A SUCROSE GRADIENT OF THE LOW-SPEED SUPERNATANT FRACTION OF AN HOMOGENATE OF FERTILIZED (40 MINUTES) EGGS OF LYTECHINUS PICTUS. Post-mitochondrial supernatant fractions were prepared and subjected to sucrose density-gradient centrifugation as given in the legend to Plate I. (A) Fraction 2, the materials accumulating on the 60% sucrose cushion. (B) Fraction 2, treated with RNase (1.4 μg/ml at room temperature) during the 30 minutes that the drop remained on the grid.

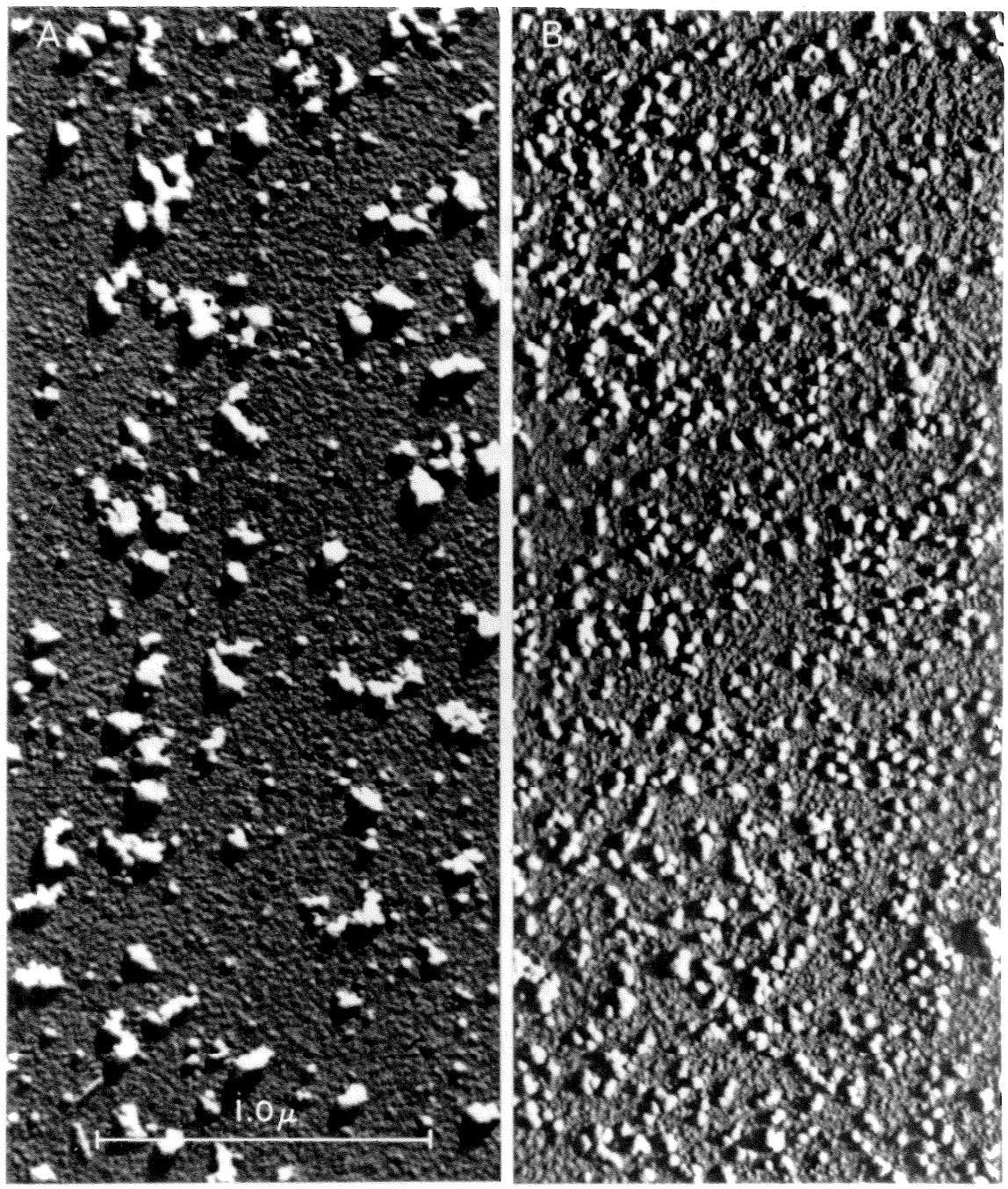

Plate III.

Plate IV. ELECTRON MICROGRAPHS DEMONSTRATING THE EFFECT OF RNASE ON THE RIBOSOMAL AGGREGATES SEDIMENTING TO THE POLYRIBOSOME-REGION OF A SUCROSE GRADIENT OF THE LOW-SPEED SUPERNATANT FRACTION OF AN HOMOGENATE OF UNFERTILIZED EGGS OF LYTECHINUS PICTUS. Post-mitochondrial supernatant fractions were prepared and subjected to sucrose density-gradient centrifugation as given in the legend to Plate I, with the following exceptions. (A) Fraction 2, the materials accumulating on the 60% sucrose cushion, after the supernatant fraction was treated with RNase (1 µg/ml at 4°C for 45 minutes) before density-gradient centrifugation. (B) Fraction 2, treated with RNase (1.4 µg/ml at room temperature) during the 30 minutes that the drop remained on the grid.

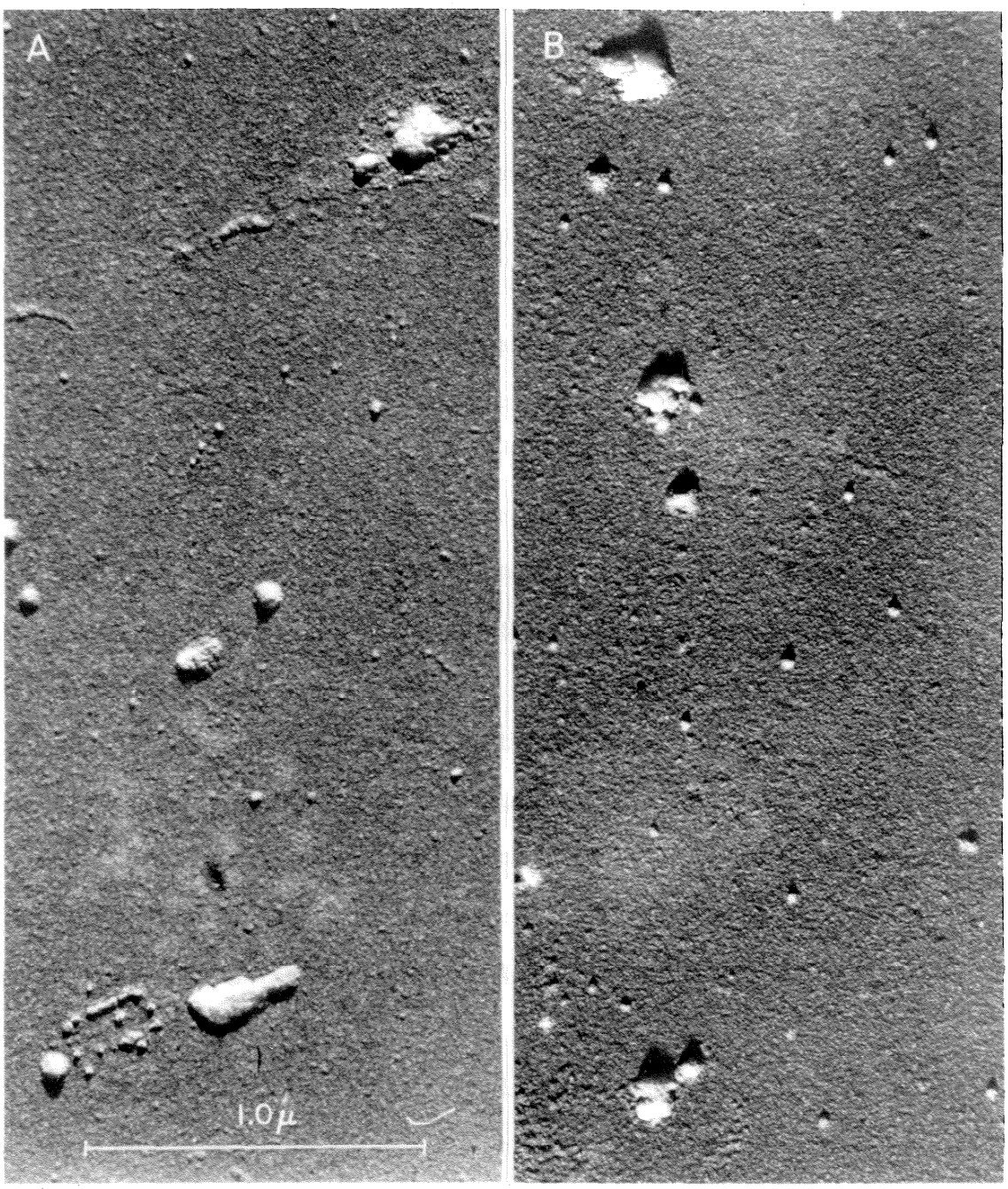

Plate IV.

treatment of the supernatant fraction preceding the centrifugation. Also, as is illustrated in Plate IVB, most of the aggregates are not dispersed by the addition of RNase to the isolated fraction of the sucrose gradient. It may be of significance, however, that more single particles are visible in the micrographs after RNase-treatment of the isolated fraction than after similar treatment of the supernatant fraction. These could represent the dissociation of ribosomal aggregates active in protein synthesis present in unfertilized eggs.

Plates V and VI are selected electron micrographs of ribosomal aggregates of unfertilized eggs. A fine strand, stainable by uranyl acetate (Plate VIE), apparently connects individual particles. It is impossible to evaluate the significance of these strands only on the basis of their appearance in the electron microscope. They vary in thickness from approximately 40-60 Å (Plates VB, VIA, B, C, D, F) to about 150 Å (Plate VA), and are, on the average, 0.3-0.6 μ long assuming continuity through the ribosomes. They are, then, broader than the 10-20 Å generally considered (Rich, Warner & Goodman, 1963) characteristic of messenger RNA. Nonetheless, this does not exclude their containing messenger RNA and the following reasons support that interpretation.

First, if strands of considerable thickness, such as for instance, the ones shown in Plate V, were remnants of uncoiled ribosomes they might be expected to be seen extending from particles of a smaller diameter than intact ribosomes. But, in fact, the diameter of these thick strand-associated particles measures the same, or at times even slightly larger

Plate V. ELECTRON MICROGRAPHS OF MATERIALS FROM THE POLYRIBOSOME-REGION OF A SUCROSE GRADIENT OF THE LOW-SPEED SUPERNATANT FRACTION OF AN HOMOGENATE OF UNFERTILIZED EGGS OF LYTECHINUS PICTUS. Post-mitochondrial supernatant fractions were prepared and subjected to sucrose density-gradient centrifugation as given in the legend to Plate I. Materials from fraction 2, namely those materials accumulating on the 60% sucrose cushion, were prepared for electron microscopy as given under Materials and Methods. (A) X 123,000. (B) X 83,500.

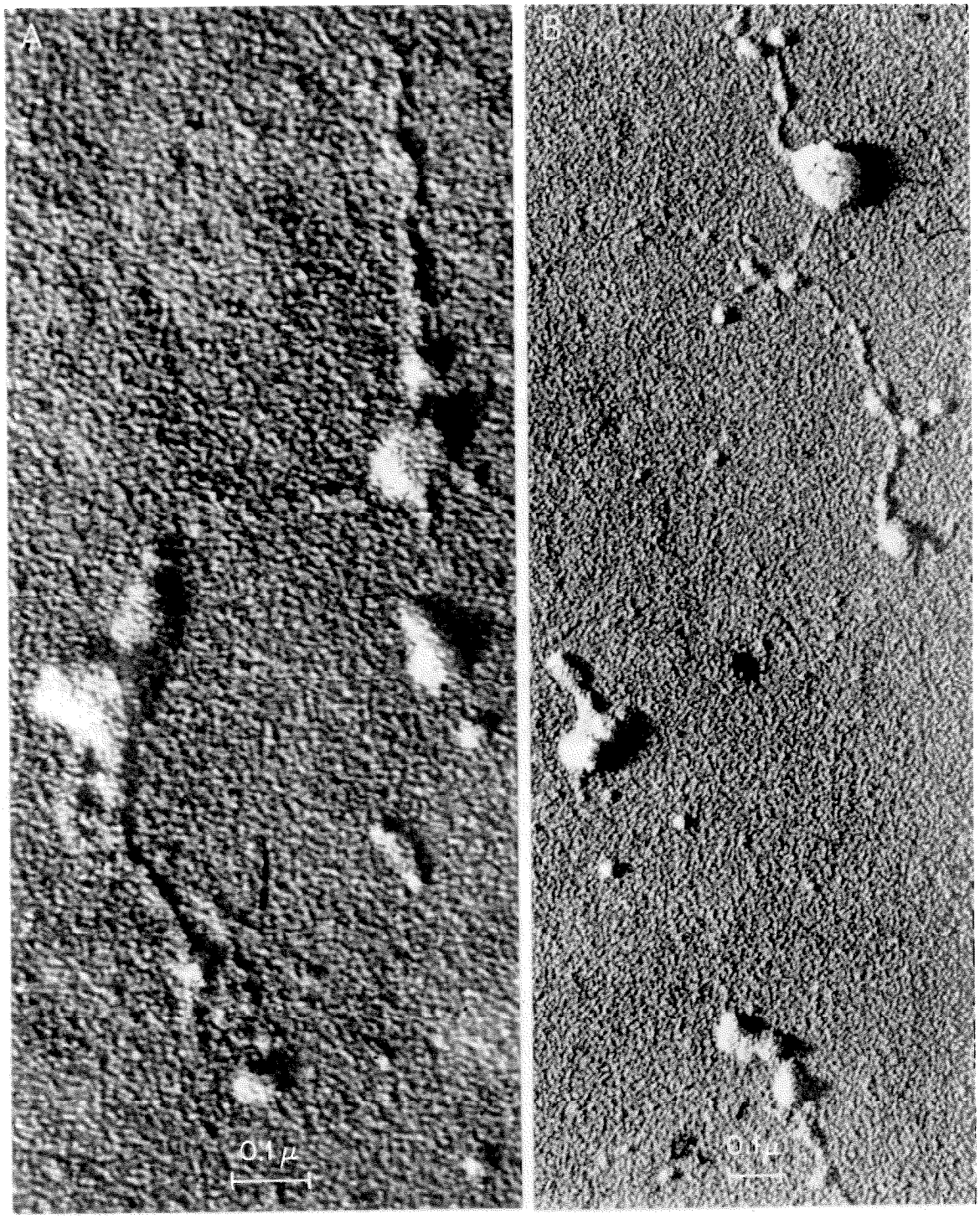

Plate V.

Plate VI. ELECTRON MICROGRAPHS OF MATERIALS FROM THE POLYRIBOSOME-REGION OF A SUCROSE GRADIENT OF THE LOW-SPEED SUPERNATANT FRACTION OF AN HOMOGENATE OF UNFERTILIZED EGGS OF LYTECHINUS PICTUS. Post-mitochondrial supernatant fractions were prepared and subjected to sucrose density-gradient centrifugation as given in the legend to Plate I. Materials from fraction 2, namely those materials accumulating on the 60% sucrose cushion, were prepared for electron microscopy as given under Materials and Methods. (A-D, F) shadowed (angle 4:1) with Pt-Pd X 83,500, (E) stained with uranyl acetate as given under Materials and Methods X 50,000.

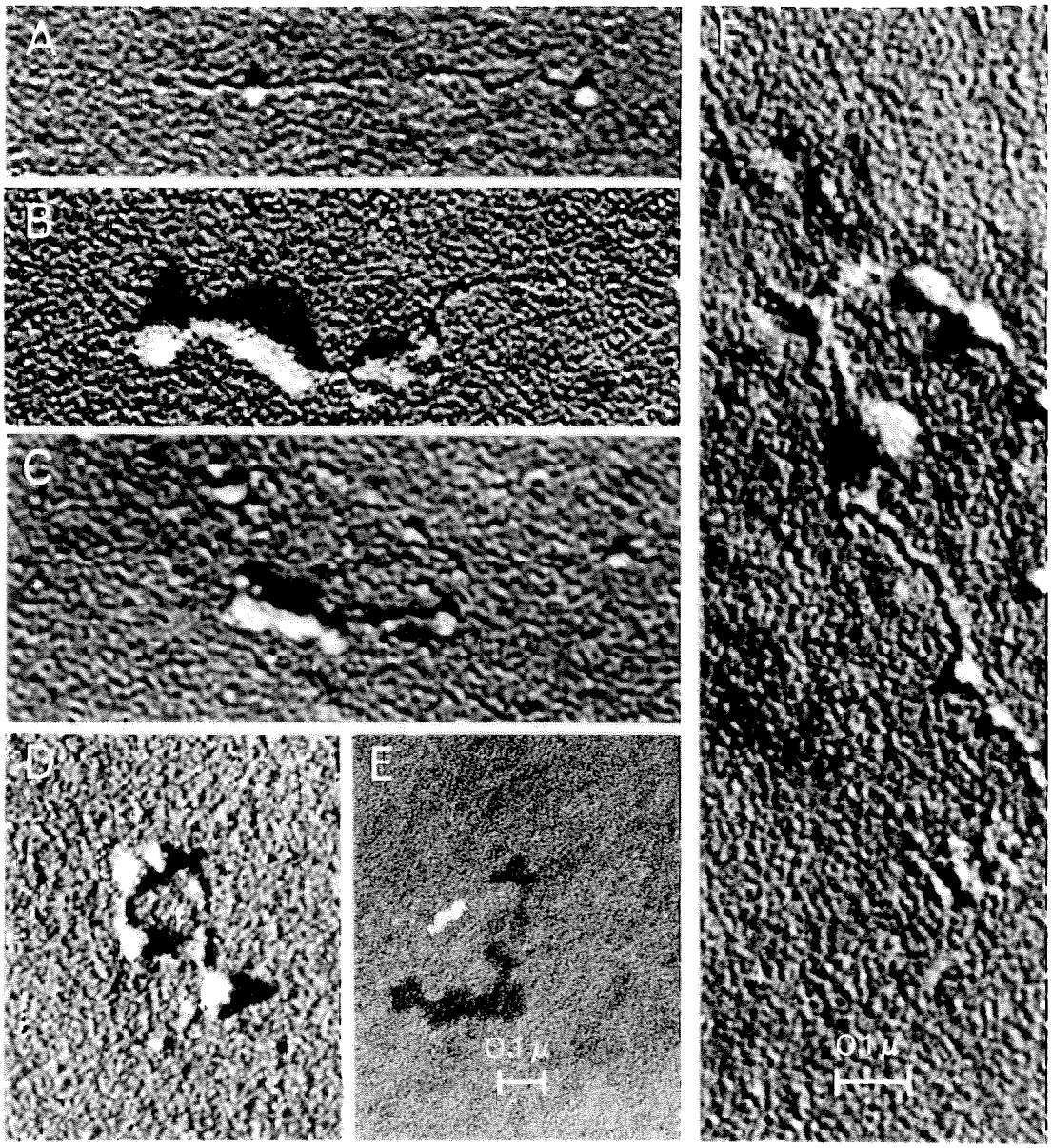

Plate VI.

than that of single ribosomes or of particles associated with somewhat thinner strands. Second, the apparent width of a molecular strand of RNA might be increased by a coiling of the RNA upon itself as it settles on the grid. With this in view, it should be noted that the thickness of the strands in these micrographs vary considerably along their length. Another factor that might increase the thickness of messenger RNA in these shadowed preparations would be its accumulation of platinum and paladium, as would also its accumulation of contaminating material suspected to be present in the polyribosome-region of the sucrose gradient. An additional argument favoring the interpretation of these strands as messenger RNA is that several ribosomes appear to be attached to individual strands. Furthermore, the length of the strands in these micrographs range from 3000 Å to 6000 Å. This would allow the occupancy of 12 to 24 ribosomes of an average diameter of 250 Å, which is the average size of the ribosomal aggregates observed in this region of the sucrose gradient (see Plates II and III).

The electron micrographs suggest, then, that the strand-associated-particle-aggregates isolated from homogenates of unfertilized eggs may be polyribosomes. They serve to provide additional evidence that unfertilized eggs contain a population of polyribosomes.

DISCUSSION

The present results indicate that the mature unfertilized sea urchin egg incorporates amino acids into protein upon polyribosomes. After fertilization, the rate of incorporation increases sharply. This increase is associated with the formation of new polyribosomes. In addition, the data suggest that inactive polyribosomes may be activated immediately after fertilization.

1) Protein synthesis by unfertilized eggs

The active polyribosomes of unfertilized eggs are readily dissociated by RNase-treatment. Electron micrographs of the ribosomal aggregates suggest that these structures consist of ribosomes linked together by a thread, possibly messenger RNA. Since much evidence has been accumulated that the cytoplasm of unfertilized eggs contains a substantial store of inactive messenger RNA (see Introduction), the demonstration of protein synthesis by unfertilized eggs means that inactive and active template may coexist in a similar environment. This adds indirect support to the hypothesis (Tyler, 1963, 1965a; Tyler and Tyler, 1966b; Spirin & Nemer, 1965; Wilt, 1966) that the new messenger RNA synthesized after fertilization (Nemer, 1963; Wilt, 1963, 1964; Glisin & Glisin, 1964; Gross, Malkin and Moyer, 1964; Gross, Kraemer & Malkin, 1965; Baltus, Quertier, Ficq & Brachet, 1965; Comb, Katz, Branda and Pinzino, 1965; Spirin & Nemer, 1965; Nemer and Infante, 1965; Siekevitz, Maggio & Catalano, 1966) may be inactive in protein synthesis until a time specified by the needs of the cell. With respect to protein synthesis, then,

the unfertilized egg may differ from the fertilized egg principally in the ratio of inactive to active messenger RNA.

Experiments to be reported elsewhere (Tyler and Piatigorsky, in preparation) exclude the possibility that bacterial contamination of the medium is responsible for the protein synthesis exhibited by suspensions of unfertilized eggs. Moreover, they show that the incorporation of labeled amino acid into protein by unfertilized eggs is inhibited by puromycin, as it is also in fertilized eggs. The reservation that the protein synthesis is a reflection either of artificial activation of a small number of eggs in the suspension or of the presence of a few oocytes, is impossible to eliminate rigorously. However, the suspensions were carefully examined and no eggs with fertilization membranes were seen nor were there any oocytes.

2) The possible activation of arrested polyribosomes after fertilization

Although the assembly of new polyribosomes occurs after fertilization, their relatively slow rate of formation does not fully account for the immediate and large increase in the rate of protein synthesis. For instance, the present evidence indicates that the rate of incorporation of labeled amino acids into protein, relative to uptake, increases five to ten fold by twenty minutes after fertilization. During this time, however, the numbers of ribosomal aggregates have not yet doubled. By forty minutes after fertilization amino acid incorporation is ten to fifteen times its initial value while the numbers of ribosomal aggregates augment only three to five fold. Thus, the specific activity of the

incorporation of labeled amino acids into protein is two to three times lower for the ribosomal aggregates of unfertilized eggs than for those of fertilized eggs.

Among the various possible interpretations of these results, a reasonable one is that polyribosomes are suppressed in unfertilized eggs and are activated after fertilization. The evidence shows that the arrested polyribosomes are resistant to the action of RNase before their activation. The tests on the sensitivity of the aggregates to proteolytic enzymes indicate that the RNase-resistance may be due to the attachment of a trypsin-labile protective sheath.

RNase-resistant ribosomal aggregates inactive in protein synthesis, however, may be considered as arrested polyribosomes only after the unequivocal demonstration that they possess messenger RNA. If only the RNase-sensitive aggregates are defined as polyribosomes, it is clear that the homogenates of unfertilized eggs would contain many fewer polyribosomes than estimated above. The relative increase in their numbers after fertilization, therefore, would be a correspondingly larger value. Since the RNase-resistant aggregates are the predominant species of ribosomal aggregates present in homogenates of unfertilized eggs, the increase in RNase-sensitive aggregates after fertilization would readily account for the elevated protein synthesis.

It would seem that non-specific clusters of ribosomes could easily arise in egg-homogenates. Two observations argue against this possibility. First, labeled single ribosomes added to homogenates of unfertilized eggs do not aggregate. Second, RNase-treatment dissociates the ribosomal

aggregates in homogenates of fertilized eggs below the level of those present in the corresponding homogenates of unfertilized eggs. For the following reasons, however, neither of these facts conclusively eliminates the possibility that the ribosomal aggregates of unfertilized eggs are experimentally induced artifacts.

Homogenates of unfertilized eggs contain a large excess of single ribosomes. The aggregates then represent only a small proportion of the total number of ribosomes present. This means that labeled ribosomes added to a non-labeled homogenate would have only a small probability of becoming entangled in a non-specific ribosomal aggregate.

With respect to the second point, it is known that constituents of unfertilized and fertilized sea urchin eggs differ in a number of ways. For example, the solubility of egg-proteins in potassium chloride (Mirsky, 1936) decreases after fertilization. Also, electrophoretic mobilities of proteins precipitated at different concentrations of ammonium sulfate change following fertilization (Monroy, 1950; Monroy and Monroy-Oddo, 1951). Moreover, the viscosity of urea solutions of proteins of unfertilized eggs differs from that of fertilized eggs (Ceas, Impellizzeri and Monroy, 1955). Furthermore, the sensitivity of certain proteins to trypsin-treatment increases after fertilization (Giardina and Monroy, 1955; D'Amelio, 1955). Finally, the solubility properties of a glycoprotein component changes after fertilization (Monroy and Vittorelli, 1960).

The cited investigators have suggested that these changes in property are due to intrinsic alterations, or rearrangements, of the preexisting

components of the unfertilized egg. Thus, an homogenate of unfertilized eggs provides a significantly different environment for ribosomes than one from fertilized eggs. One must interpret with caution, then, differences observed in homogenates of fertilized and unfertilized eggs.

Nevertheless, the highly suppressed protein synthesis in unfertilized eggs and the abundance of evidence favoring the idea that "masked" messenger RNA exists in unfertilized eggs provide additional arguments in favor of the proposition that the RNase-resistant ribosomal aggregates are one site of translational control of messenger RNA activity.

3) Examples of RNase-resistant ribosomal aggregates inactive in protein synthesis

There have been several reports on the occurence of RNase-resistant ribosomal aggregates lacking synthetic capacity. The first of these was by Humphreys, Penman & Bell (1964). They showed that the feather primordia of chicken embryos contain a class of ribosomal aggregates consisting of four ribosomes, resistant to RNase-treatment, relatively inactive, *in vivo*, in amino acid incorporation and stable during prolonged exposures to dactinomycin. These properties are reversed when the cells become active in the synthesis of keratin, which occurs on the thirteenth day of development. Later, Bell, Humphreys, Slayter & Hall (1965) showed that the synthetically inactive aggregates appear in the electron microscope as closed quartets of ribosomes. On the thirteenth day, however, when the cells are induced to synthesize keratin the quartets incorporate radioactively labeled amino acids into

protein and appear electron-microscopically as linear arrays, four ribosomes in length. Yatvin (1966a, b) confirmed these observations and, in addition, provided evidence that the developing hypophysis of the chicken embryo plays a key role in the activation and elongation of these ribosomal aggregates.

The experiments on the developing feather of the chicken embryo provide several analogies to those in the present study on the stimulation of protein synthesis upon fertilization. RNase-resistant, inactive ribosomal aggregates are found in homogenates of both systems. In contrast to chicken feather primordia, unfertilized eggs possess synthetically inactive ribosomal aggregates of various sizes. This, of course, may be easily reconciled by the fact that chicken feather primordia synthesize principally one protein, keratin, while fertilized sea urchin-eggs synthesize an assortment of proteins (Spiegel, Ozaki and Tyler, 1965; Termen and Gross, 1965; Ellis, Jr., 1966). A more important difference between the two systems is that the ribosomal aggregates of unfertilized and fertilized eggs appear morphologically comparable in the electron microscope. This contrasts to the structural modifications of the aggregates evident in the case of feather induction. The analogy, then, is not complete and although the two phenomena may be related in principle they differ in detail.

Kaulenas and Fairbairn (1966) have performed experiments on eggs and embryos of the nematode worm Ascaris lumbricoides. They showed that ribosomal preparations of these eggs contained a large proportion of ribosomal aggregates relative to single ribosomes. After a short exposure

(30 or 60 min.) of the developing eggs to H^3-uridine, radioactive material became associated with the ribosomal aggregates. Furthermore, radioactivity also appeared in materials of homogenates that sedimented slower than single ribosomes in a sucrose density-gradient. Both the rapidly sedimenting 260 mμ absorbing material and much of the labeled material were resistant to mild RNase-treatment (1 μg/ml; room temperature for 20 min.). Some label from the light polyribosome-region of the density-gradient was transferred to the monoribosome-region after the RNase-treatment. Treatment of the ribosomal preparations with 5 μg/ml of RNase transferred most of the 260 mμ absorbing material from the polyribosome- to the monoribosome-region and all of the label from the polyribosome-region to the top of the gradient. Treatment with 10 μg/ml abolished all the 260 mμ absorption from the polyribosome-region of the sucrose gradient. This concentration of RNase also began to degrade the single ribosomes.

As development progressed to the blastula stage, the sedimentation profile of the ribosomal preparations indicated only a slight increase in numbers of ribosomal aggregates. Mild treatment with RNase (1 μg/ml), however, now sharply reduced, but did not totally abolish, the 260 mμ absorption from the polyribosome-region. A compensatory increase was observed in the monoribosome-region of the sucrose density-gradient. In addition, the RNase-treatment completely transferred the H^3-uridine labeled material from the polyribosome-region to the surface of the sucrose gradient.

Preliminary treatment of the ribosomal preparations obtained from the newly fertilized eggs with trypsin (100 µg/ml; 37°C for 30 min.) sensitized the ribosomal aggregates such that they were capable of being subsequently dissociated to single ribosomes by 1 µg/ml of RNase. Furthermore, trypsinization also resulted in a five-fold increase in the rate of incorporation of labeled amino acids into protein by the ribosomes when added to a cell-free system. The amino acid incorporating activity stimulated by trypsin could be abolished by a secondary treatment of the ribosomal preparation with small quantities of RNase.

The results obtained by Kaulenas and Fairbairn are particularly germane to those of the present investigation. In both series of experiments a trypsin-sensitive inhibitor is implicated in the suppression of polyribosome activity, the inactivated polyribosomes being resistant to relatively small amounts of RNase. Perhaps the most significant difference between the two studies is that the release of arrested polyribosomes would make only a relatively small contribution to the stimulation of protein synthesis upon fertilization of sea urchin eggs. This is because of the many new polyribosomes that form after fertilization. In the Ascaris embryo, on the other hand, the polyribosome may represent the main site of control of messenger RNA translation.

Another case providing evidence for RNase-resistant inhibited polyribosomes comes from the initial studies of Amos and Moore (1963). Amos and Moore showed that primary cultures of chicken embryo fibroblasts did not synthesize protein unless the medium contained horse serum. The cells, however, could be maintained in culture for at least four days

without protein synthesis. Even after four days the addition of serum to the medium initiated protein synthesis by the cultured cells. In fact, longer deprivations of serium resulted in faster rates of protein synthesis upon subsequent serum addition. The authors inferred from these results that "protein-synthesizing machinery" accumulated when the fibroblasts were starved for serum.

Soeiro and Amos (1966) continued to investigate the effects of serum on the cultured cells. They showed that dactinomycin, when added concurrently with serum to starved cells, did not prevent the initiation of protein synthesis. Furthermore, when dactinomycin was present in the medium during serum starvation the capacity for the reinitiation of protein synthesis was only slightly affected. It appeared, therefore, that messenger RNA decay was not responsible for the suppression of protein synthesis in the absence of serum.

Microsomes obtained from cells that had been starved for serum for twenty-four hours incorporated much less C^{14}-phenylalinine into protein than did microsomes from control cells. Treatment of the microsomes in vitro with serum did not increase their responsiveness to polyuridylic acid. These tests suggested, then, that the microsomes were affected by serum deprivation.

The sedimentation patterns obtained by sucrose density-gradient centrifugation studies indicated that the numbers of ribosomal aggregates remained approximately constant when starved cells were maintained in the presence of dactinomycin for twenty-four hours. However, the ability of the rapidly sedimenting materials of the sucrose gradient to incorporate

labeled amino acids into protein in a cell-free system was about one-third that found by the comparable structures from cells cultured with serum.

Finally, structures possessing low amino acid incorporating activity were resistant to mild treatment with RNase. Trypsin plus RNase, however, dissociated the ribosomal aggregates. Soeiro and Amos proposed that the serum factor required for protein synthesis may act directly on the polyribosome or, alternatively, may intervene between the polyribosome and a cytoplasmic regulator of polyribosome activity.

The results of Soeiro and Amos are similar to those obtained on experiments with unfertilized and fertilized sea urchin eggs with one important exception, namely, only microsomes from fibroblasts cultured with serum were responsive to polyuridylic acid. This is in contrast to the situation in sea urchin eggs where microsomes from both unfertilized and fertilized eggs are equally capable of being stimulated to incorporate phenylalanine into protein by polyuridylic acid (see Introduction). The experiments on cultured fibroblast cells provide an additional example of suppressed polyribosomes associated with the property of RNase-resistance. The investigations of Soeiro and Amos also support the possibility that a trypsin-labile inhibitor may be the responsible agent.

4) Other evidence indicating translational control of polyribosomal activity.

There have been several other reports, including investigations on sea urchin eggs, providing evidence that translation of messenger RNA may be suppressed on the polyribosome. These studies, however, briefly outlined below, indicate that the inhibition of polyribosomal activity need not be associated with a resistance to RNase.

Candelas and Iverson (1966) have obtained ribosomal preparations of unfertilized and fertilized eggs from the polyribosome-region of sucrose density-gradients. They showed that the material from fertilized eggs was twenty to thirty times more active, per mg protein, in the incorporation of C^{14}-leucine into protein than the corresponding material from unfertilized eggs when added to a post-microsomal supernatant fraction of fertilized eggs. Candelas and Iverson suggested that polyribosomes of unfertilized sea urchin eggs may be activated after fertilization.

Experiments by Spirin and Nemer (1965) and by Wilt (1966) showed that upon incorporation of H^3-uridine and C^{14}-leucine into RNA and protein, respectively, by early cleavage embryos the newly synthesized RNA was associated with small polyribosomes. Nascent protein, however, was being synthesized primarily upon larger, more rapidly sedimenting polyribosomes. Nemer and Infante (1965) interpreted H^3-RNA associated with the small relatively inactive polyribosomes as messenger RNA on the basis of its sedimentation properties in a sucrose density gradient and its high affinity for homologous DNA.

Spirin and Nemer (1965) showed that RNase-treatment of post-mitochondrial supernatant fractions completely digested the labeled RNA associated with the small polyribosomes and transferred this radioactivity to the top of the sucrose gradient. The labeled protein, on the other hand, sedimented to the monoribosome-region of the density-gradient after treatment with RNase.

These experiments thus provide another example of suppressed polyribosomes. The experiments on the relatively inactive, light polyribosomes, however, contrast in at least one significant way with those in the present tests upon the stimulation of protein synthesis after fertilization. The small polyribosomes of Spirin and Nemer (1965) are readily dispersed by RNase. The light polyribosomes are not, then, the same as the inactive aggregates of ribosomes in homogenates of unfertilized eggs. They may, however, represent a mechanism of translational control of protein synthesis which operates along lines similar to that in unfertilized eggs but which may more easily cope with the rapidly changing exigencies of development.

Evidence for control of polyribosomal activity has not been limited to studies on embryos or embryonic cells. For example, Dietz, Reid and Simpson (1965) have studied growing yeast. They showed that the 260 mμ absorption profile of ribosomes in a sucrose density-gradient did not change during the transition from early log-phase to mid log-phase growth of the yeast. The ribosomal aggregates from early log-phase yeast were considerably more active <u>in vitro</u> in the incorporation of labeled amino acids into protein than those from mid log-phase yeast. It appears then

that polyribosome activity may be suppressed during the early log-phase of growing yeast.

Translational control of the rate of protein synthesis in normal, starved and regenerating rat liver has been studied with fruitful results by Hoagland and his collaborators. Hoagland, Scornik and Pfefferkorn (1964) presented evidence that normal adult rat liver contained a heat-labile inhibitor of microsomal synthetic activity when tested in vitro. This inhibitor was readily released from normal liver microsomes by sonication, but not from regenerating liver microsomes. Its suppressive action could be antagonized by guanosine triphosphate. These investigators suggested that the rate of protein synthesis in adult tissues may be regulated by a microsomal inhibitor which, in turn, may be controlled by guanosine triphosphate.

Sox and Hoagland (1966) compared the protein-synthetic capacity of polyribosomes isolated from livers of starved rats with those obtained from livers of fed rats. They showed that polyribosomes from the former are sluggish in the in vitro incorporation of labeled amino acids into protein relative to the polyribosomes from livers of fed rats. Tests with polyuridylic acid indicated that the defect is due not to the individual ribosomes but rather to the polyribosome.

These experiments compare favorably with those on sea urchin eggs. Ribosomes of unfertilized sea urchin eggs also respond to polyruidylic acid, but the present evidence indicates that the polyribosomes may be suppressed in synthetic activity.

5) Evidence for translational control of protein synthesis during mitosis

Autoradiographic studies on chinese hamster connective tissue cells (Taylor, 1960) HeLa and Osgood leukemic cells (Feinendegen, Bond, Shreeve and Painter, 1960), Ehrlich ascites tumor cells (Baserga, 1962) and HeLa cells (Terasima and Tolmach, 1963) indicated that inhibition of RNA synthesis during metaphase of mitosis is a general characteristic of animal cells. Other autoradiographic investigations on hamster fibroblast and HeLa cells (Prescott and Bender, 1962) and hamster epithelial and human amnion cells (Konrad, 1963) confirmed the suppression of RNA synthesis and extended the observations to show that protein synthesis was also inhibited during metaphase.

Two facts indicate that metaphase-inhibition of protein synthesis in animal cells occurs at the level of translation rather than transcription. First, human amnion cells, which Konrad (1963) showed decrease in the rate of protein synthesis during metaphase, continue to incorporate labeled amino acids actively into protein thirty hours after enucleation (Goldstein, Micou and Crocker, 1960). Second, and more compelling, is that HeLa cells, shown by Prescott and Bender (1962) to decrease at metaphase in the rate of protein synthesis, have messenger RNA with an average half-life of three to four hours (Penman, Scherrer, Becker, and Darnell, 1963) Messenger RNA in these cells, then, does not decay rapidly enough to account for the inhibition of protein synthesis during metaphase.

Scharff and Robbins (1965) have further investigated the translational arrest of protein synthesis during mitosis of HeLa cells by using a com-

bination of electron microscopy and density-gradient centrifugation techniques. They showed that in vivo incorporation of labeled amino acids into protein decreased in proportion corresponding to a loss of polyribosomes, both during normal and colchicine induced metaphase. The active polyribosomes present during interphase as well as those remaining during metaphase were converted to single ribosomes by small amounts of RNase.

The observation of the inhibition of protein synthesis during metaphase of intact HeLa cells was extended to the cell-free system by Salb and Marcus (1965). In addition they showed that isolated ribosomes from metaphase cells were less responsive in vitro to polyuridylic acid than ribosomes from interphase cells. Trypsin-treatment of the metaphase ribosomes, however, restored most (85%) of the ability of the ribosomes to support C^{14}-phenylalanine incorporation into protein in combination with polyuridylic acid. The optimum concentration of trypsin was 20 µg/ml. At higher concentrations of trypsin both interphase and metaphase ribosomes lost protein-synthesizing capacity at equal rates.

The experiments by Salb and Marcus are similar to those by Monroy, Maggio and Rinaldi (1965) (see below) on trypsin activation of ribosomes from unfertilized eggs of sea urchins. Both groups of investigations provide evidence for trypsin-sensitive regulators of protein synthesis acting on ribosomes. The two studies also serve to relate mitotic mammalian cells to unfertilized sea urchin eggs and suggest that the stimulation of protein synthesis occurring after fertilization may be one rather striking example of general mechanisms operating in cells of diverse types.

Experiments (Sofer, George and Iverson, 1966) on fertilized sea urchin eggs have indicated that polyribosomes, during metaphase of the first cleavage, are less active than during interphase in vivo and in cell-free systems, in the incorporation of radioactively labeled amino acid into protein. On the other hand, Gross and Fry (1966) have reported that incorporation of labeled amino acids into protein does not decrease at metaphase during the first two cleavages of sea urchin eggs. And, finally, Timourian (1966) has found that amino acid incorporation is occassionally but not always depressed during metaphase of fertilized sea urchin eggs. Perhaps these divergent results indicate that the interruption of protein synthesis during metaphase in sea urchin development is brief and difficult to correlate precisely with the experimental procedures. In any case, the results of Sofer, George and Iverson (1966) provide additional evidence that polyribosomes may be inhibited during metaphase. This contrasts with the report of the disaggregation of polyribosomes during metaphase in HeLa cells (Scharff and Robbins, 1965).

The results of these experiments on metaphase arrest of protein synthesis in mammalian cells and in sea urchin eggs provide, then, further examples of translational control of protein synthesis.

6) Evidence for translational inhibition of protein synthesis upon maturation of reticulocytes

Erythropoiesis may be stimulated in rabbits by the experimental production of severe anemia or by the administration of large doses of

erythropoietin (see Borsook, 1964). This results in the production of non-nucleate reticulocytes that are larger than normal, that have more hemoglobin per cell and that give rise to erythrocytes that die prematurely. Borsook and his colleagues have interpreted these findings as the consequence of additional stem cell proliferation coupled with skipping of mitosis by polychromatic cells. Thus, the orthochromatic erythroblast stage is bypassed and reticulocytes are formed more quickly than usual.

The studies cited above showed also that the in vitro protein synthesizing-capacity of intact reticulocytes is significantly higher by cells isolated from anemic than from normal rabbits, even though RNA synthesis does not occur in reticulocytes. Studies on reticulocytes fractionated (Glowacki and Millette in Borsook, 1964; Glowacki and Millette, 1965) by bovine serum albumin-gradient centrifugation showed that denser, more mature reticulocytes were less active in the incorporation of labeled amino acids into protein than younger reticulocytes. These studies indicated, then, that the rate of protein synthesis decreases during reticulocyte maturation. Furthermore, this decrease cannot be dependent upon the cessation of RNA synthesis.

Investigations into the nature of the protein-synthesizing capacity by reticulocytes showed that they incoporate amino acids into protein upon polyribosomes (Warner, Rich and Hall, 1962; Marks, Burka and Schlessinger, 1962; Warner, Knopf and Rich, 1963; Gierer, 1963; Burka and Marks, 1964). Other investigations into the properties of maturing reticulocytes demonstrated that the older the reticulocyte the fewer the polyribosomes (Marks, Rifkind and Danon, 1963; Rifkind, Luzzatto and Marks, 1964;

Rifkind, Danon and Marks, 1964; Mathias, Williamson, Huxley and Page, 1964; Danon, Zehavi-Willner and Berman, 1965; Glowacki and Millette, 1965). However, the point of special relevance to the present investigation is that more mature reticulocytes possess polyribosomes of lower specific activity with respect to amino acid incorporation than those of younger reticulocytes (Glowacki and Millette, 1965). Electron microscopic autoradiography (Miller and Maunsbach, 1966) also showed that inactive polyribosomes are present in rabbit reticulocytes. These studies, then, are in accord with the possibility that polyribosomes become suppressed during reticulocyte maturation. They serve to add still another bit of evidence that polyribosomes may be a site of translational control of protein synthesis.

7) Examples of RNase-resistant polyribosomes active in protein synthesis

There have been a number of reports on RNase-resistant polyribosomes that are active in protein synthesis. Rabinowitz, Zak, Beller, Rampersad & Wodl (1964) have shown that polyribosomes from fourteen to eighteen day chicken embryo heart muscle are active in protein synthesis but partially resistant to RNase. They are, however, largely disrupted by trypsin or chymotrypsin, and the action of chymotrypsin and RNase are additive. In another study, Manner, Gould and Slayter (1965) demonstrated that polyribosomes from lymph nodes of immunized rats synthesize gamma globulin but they are not destroyed by RNase. These also are disassociated by trypsin or chymotrypsin. Finally, Kretsinger, Manner, Gould & Rich (1964) observed that large polyribosomes, synthesizing collagen,

from nine day old chicken embryos are partially resistant to RNase. They believe that an interaction of the nascent polypeptide chains on the individual ribosomes is the primary factor which keeps these polyribosomes intact even after their messenger RNA strand has been cleaved.

It is unlikely that the nascent polypeptides present on the monomers comprising the ribosomal aggregates of the unfertilized egg, in the present investigation, are interacting in a way to hold adjacent ribosomes together after RNase-treatment. The strongest evidence against this possibility is that the active ribosomal aggregates of unfertilized eggs, namely those that contain growing polypeptide chains, are disassociated by the action of RNase.

These observations on protease-sensitivity of polyribosomes are similar to those of the present investigation with respect to the dispersive effect of trypsin. The experiments by Rabinowitz, Zak, Beller, Rampersad and Wool (1964) are of particular interest on account of the combined effect of chymotrypsin and RNase. The interpretation of experiments on the effect of protease activity on ribosomes or polyribosomes, however, is complicated by several factors.

Kaji, Suzuka and Kaji (1966) have recently studied the effect of proteolytic enzymes on the properties of ribosomes of Escherichia coli. Trypsin-treatment caused the ribosomes to lose progressively 1) the ability to make polyphenylalanine in response to polyuridylic acid, 2) to bind specific transfer RNA's and 3) to form polyribosomes with a polyuridylic acid messenger. More extensive treatment with trypsin dissociated the 70S particles to 50S and 30S subunits. The sedimentation

of the subunits after trypsin-treatment was not altered.

Trypsin, then, can affect individual ribosomes without causing their disintegration. Thus, it is not clear in what way(s) trypsin dissociates either active polyribosomes or inactive ribosomal aggregates in the experiments cited above. The fact that both active and inactive ribosomal aggregates may be RNase-resistant, however, does not discard the possibility that polyribosome suppression may be associated with resistance to RNase. It would seem of central importance for the understanding of RNase-resistant ribosomal aggregates to experimentally alter RNase-sensitive polyribosomes to their resistant inactive counterparts.

8) Formation of additional, active polyribosomes after fertilization

The present evidence, then, supported by observations on other systems, indicates that fertilization might activate suppressed polyribosomes. However, it is also established that a significant part of the increase in the rate of amino acid incorporation into protein after fertilization is due to the formation of new polyribosomes. The evidence given in the Introduction indicates that much of the messenger RNA responsible for the rapid assembly of polyribosomes has been synthesized before fertilization. The present study has shown a three to five fold increase in the number of ribosomal aggregates and a ten to fifteen fold enhancement in the percentage incorporation of labeled amino acid into protein by eggs forty minutes after fertilization. This means that approximately 70% of the increase in the rate of protein synthesis is directed by messenger RNA which was not on polyribosomes before fertilization.

Spirin, Belitsina and Aitkhozhin (1964) have provided some indication of the location of what is presumably inactive messenger RNA in developing eggs of the fish Misgurnus fossalis. They found that C^{14}-adenine and C^{14}-uridine or C^{14}-leucine and C^{14}-lysine were incorporated into particles sedimenting at about 40S to 60S in a sucrose density-gradient. These ribonucleoprotein particles sedimented in a preformed cesium chloride gradient to a density of 1.4 to 1.5 indicating an RNA content of 15 to 35%. This differed from ribosomes which sedimented in cesium chloride to a density of 1.5 to 1.6. The rapidly labeled particles were considered, then, not to be ribosomal subunits.

These particles were newly synthesized both before and during gastrulation of the fish embryo. Labeled RNA, however, only became associated with polyribosomes, in vivo, at gastrulation. However, the labeled ribonucleoprotein particles combined with unlabeled ribosomes in vitro to form polyribosomes. Furthermore, the particles stimulated amino acid incorporation into protein six fold when added to a cell-free ribosomal system.

These investigators termed the ribonucleoprotein particles "informosomes". They inferred that "informosomes" are messenger RNA-protein complexes and that they function in vivo by directing protein synthesis in combination with ribosomes. From their evidence it appeared as if "informosomes" retained their protein component when attached to ribosomes. The authors suggested that the protein moiety may stabilize the messenger RNA-ribosome (polyribosome) complex. A more attractive interpretation, however, would be that the protein component of "informosomes"

inactivates the associated messenger RNA. The messenger RNA, then, would have become spontaneously activated in Spirin's in vitro test system.

Spirin and Nemer (1965) have shown that cleaving sea urchin eggs also incorporate nucleosides into RNA-containing particles that sediment between 40S to 60S in a sucrose gradient. They extracted this RNA from the gradient and demonstrated that it readily hybridized to homologous DNA saturated with non-radioactive ribosomal RNA. Spirin and Nemer concluded that the particles were analogous to "informosomes".

Experiments by Smith (1966) have shown that blastula and gastrula stage sand dollar embryos also incorporate labeled nucleosides into particles sedimenting at about 40S. These particles contain newly synthesized RNA that sediments heterogeneously in a sucrose density-gradient. It would be of great interest to learn if this RNA possesses other properties in common with messenger RNA.

The studies initiated by Spirin and his collaborators on the "informosome" provide evidence for one possible location of "masked" messenger RNA. Another possible interpretation of "informosomes" is that they are preparative artifacts. Support for this idea comes from the studies of Girard and Baltimore (1966). They showed that the addition of cytoplasmic extract of HeLa cells increased the rate of sedimentation of isolated RNA from poliovirus and HeLa cells.

Nonetheless, "informosomes" may prove to be an important site of the "masked" messenger RNA of unfertilized sea urchin eggs. The present investigation shows that 40S to 60S material, labeled with radioactive

uridine during oogenesis, is present in homogenates of unfertilized eggs. Due to the relatively small quantities of ribosomal RNA, it was possible to distinguish high percentages of heterogeneously sedimenting RNA extracted from the post-ribosomal region of sucrose density-gradients. This RNA may be messenger that is complexed, in vivo, in a manner so as to be inactive as template before fertilization. It would, then, conform to the criteria of "informosomes."

Slowly sedimenting particles, however, are not the only clues as to possible locations of "masked" messenger RNA. Maggio, Monroy, Rinaldi and Vittorelli (1965) and Monroy, Maggio and Rinaldi (1966) have provided evidence that "masked" messenger RNA may be on the ribosomes. They have treated microsomes of unfertilized eggs with trypsin and demonstrated a subsequent increase in their ability to incorporate amino acids, in vitro, into acid insoluble material after the addition of polyuridylic acid or of purified RNA from blastulae and from unfertilized eggs. Moreover, it is of particular interest that the ribosomes were also stimulated by trypsinization to incorporate amino acids into protein without the addition of RNA. Monroy and his colleagues have postulated, on the basis of these experiments that the "masked" messenger RNA is, in fact, a messenger RNA-ribosome complex coated with a protein inhibitor. Further support for this proposition comes from the report (Maggio, Vittorelli, Rinaldi and Monroy, 1964) that RNA extracted from the ribosomal pellet of unfertilized eggs is more active in template activity of amino acid incorporation than RNA from the corresponding supernatant

fraction. Also, Verhey, Moyer and Iverson (1965) have provided electron-microscopic evidence favoring the idea of a ribosome-protein complex. They demonstrated that microsomes of unfertilized eggs, but not of fertilized eggs, are associated with RNase-resistant, trypsin-labile amorphous material.

Monroy and his colleagues (Monroy, Maggio and Rinaldi, 1965) have suggested that the ribosome-messenger RNA complex of unfertilized eggs is released of inhibition by the transient proteolysis activated immediately after fertilization (Lundblad, 1949).

The experiments on ribosomal inhibition of unfertilized eggs have an interesting similarity to those by Salb and Marcus (1965) on mitotic HeLa cell microsomes (see above). In both cases a trypsin-sensitive inhibitor is suspected to act directly on the ribosome. The present experiments also imply a ribosomal inhibitor, acting at the level of polyribosomes, and capable of being destroyed by trypsin-treatment.

On the other hand, Mano and Nagano (1966) have claimed, in a recent report, that the 12,000 x g pellet of homogenates of unfertilized sea urchin eggs contains more template active RNA than either the microsomal pellet or the post-microsomal supernatant fraction. This depends, however, on the presence of calcium in the homogenization medium. Without calcium the microsomal pellet possesses the greatest <u>in vitro</u> template activity. After fertilization, also, template RNA is associated principally with the microsomal pellet.

Furthermore, they reported that labeled RNA, extracted from ovaries exposed to P^{32}-phosphate, sedimented at approximately 10S. This RNA was

found primarily in the mitochondrial pellet of ovarian-homogenates. Trypsin-treatment of the homogenate, however, transferred the labeled 10S RNA to the microsomal pellet. Finally, the labeled ovarian RNA, when added to ribosomes, spontaneously formed polyribosomes. Thus, Mano and Nagano propose still another location for the "masked" messenger RNA of unfertilized eggs; namely, upon particles that sediment faster than microsomes.

Following the footsteps of Monroy, Mano (1966) has postulated that a burst of proteolysis occurs after fertilization. This serves, he says, to free the "masked" messenger from its particulate inactivator. To support the hypothesis, Mano has measured proteolytic activity in the 12,000 x g pellet of homogenates of unfertilized and fertilized eggs. He found that the mitochondrial pellet, microsomal pellet and post-microsomal supernatant fraction of homogenates of unfertilized eggs possessed protease activity with maxima at pH 4.3 and 6.7. The same fractions prepared from eggs ten minutes after fertilization, however, showed similar activity and, in addition, pronounced protease activity with a maximum at pH 8. This activity largely subsided by one hour after fertilization.

It is impossible, then, to decide at present where the "masked" messenger RNA, responsible for the assembly of additional polyribosomes after fertilization, resides. The experiments of Mano and Nagano (1966) and of Mano (1966) are interesting but inconclusive. One of the several unresolved points is the role played by calcium. Then, the possibility that trypsin is activating and partially cleaving large polyribosomes

cannot be completely excluded. And, finally, it is impossible to convincingly attribute synthetic activity of whole ovaries to the oocytes above. It is well established that the other ovarian cells undergo intense RNA synthesis (Immers, 1961; Ficq, 1964; Gross Malkin and Hubbard, 1965). Nevertheless, the possibility that "masked" messenger RNA is attached to large, trypsin-sensitive particulate matter remains open and very much alive.

The results of Spirin and his collaborators supporting the "informosome" also cannot be excluded as a primary site of "masked" messenger RNA (see Spirin, 1966, for review). In fact the present investigation provides evidence compatible with the "informosome" theory. Only further exploration will establish the validity and importance of this particle.

The present results, however, are generally in accord with those of Monroy and his colleagues. A trypsin-labile inhibitor may attach to messenger RNA-ribosome complexes and prevent synthetic activity upon maturation of the oocyte. Proteolysis after fertilization would digest the inhibitor and thereby activate polyribosomes. This would also allow single ribosomes attached to messenger RNA to unite with other ribosomes not bearing messenger RNA. Additional polyribosomes thus form. Activation of suppressed polyribosomes and assembly of new polyribosomes, then, would be two manifestations of a single mechanism.

SUMMARY AND CONCLUSIONS

1) Part I: Uptake of C^{14}-uridine by unfertilized and fertilized sea urchin eggs.

Section 1 of Part I of this thesis presents the results of an investigation of the permeability of unfertilized and fertilized eggs of the sea urchin Strongylocentrotus purpuratus to radioactively labeled uridine. Unfertilized eggs were suspended in C^{14}-2-uridine and the ratio of C^{14} inside to that outside of the cell was measured while the eggs remained suspended in the radioactive uridine. It was found that very little uridine penetrated the unfertilized egg. After fertilization, however, the label became concentrated in the egg and depleted from the environment.

The amount of labeled uridine accumulated by the eggs, determined by measuring the ratio of label inside to that outside of the eggs, per unit volume, was compared with the value obtained for uridine uptake determined after copiously washing the eggs with sea water. The two types of measurements were in close agreement. No significant quantity of label, then, was lost from the unfertilized or fertilized eggs by washing with sea water.

Uridine uptake after fertilization was studied with respect to its time course, dependence on exogenous uridine concentration and response to 2, 4-dinitrophenol. A lag phase, acceleration phase and constant accumulation pahse was found in the rate of uridine uptake which reached a plateau within the first hour of development. Once the maximal rate of accumulation was attained, fertilized eggs were shown to be very efficient in

concentrating exogenous C^{14}-uridine. As the concentration of uridine was raised beyond 0.5-1.0 μM in the sea water, there was no further increase in the rate of uptake. Dinitrophenol inhibited uridine uptake. At -3°C only a very small amount of uridine penetrated into the eggs, and it did not reach equilibrium with the outside concentration, indicating penetration involves more than diffusion.

Most of the radioactivity taken up by the fertilized egg remained soluble in ice cold 0.2 M perchloric acid. No appreciable free C^{14}-uridine was detected inside the fertilized eggs. The soluble radioactivity was identified on the basis of anion-exchange column chromatography as nucleoside-5'-monophosphate and triphosphate, primarily the latter.

The observations indicate that fertilized eggs accumulate uridine by phosphorylating the nucleoside at the surface of the cell and thereby convert uridine to uridine-5'-monophosphate. Uridine-5'-monophosphate is then rapidly transformed within the cell to uridine-5'-triphosphate and possibly other nucleotides. This process is evidently deficient in unfertilized eggs.

Section 2 of Part I demonstrates that a mixture of C^{14}-uridine-2' and 3'-monophosphate is accumulated by fertilized eggs more effectively than C^{14}-uridine-5'-monophosphate. C^{14}-uridine-5'-diphosphate and triphosphate are not taken up by the eggs. These results provide further and compelling evidence that the activity of uridine kinase on the cell surface is responsible for the uptake of uridine. The strong inhibition of H^3-cytidine uptake by the presence of an excess of non-radioactive uridine or thymidine and the correspondingly weak inhibition by cytosine

or uracil (Mitchison and Cummins, 1966) is also consistent with the interpretation that phosphorylation of the ribose moiety is necessary for the uptake of nucleosides by sea urchin eggs.

Section 2 of Part I shows also that uridine kinase undergoes a large increase in activity rather than a net synthesis after fertilization. This statement stems from the results of the puromycin experiment, namely, that the system for uridine uptake develops after fertilization even when protein synthesis is strongly suppressed.

The activity of several other enzymes (see Discussion to Section 2 of Part I) is known to be stimulated after fertilization. Evidence has been provided that this may be associated with the release of the inactive enzyme from particulate matter after fertilization. By analogy one many suggest that uridine kinase is sequestered within the unfertilized egg and thus incapable of activity at the cell surface until after fertilization.

2) Part II: In vitro RNA and protein synthesis by intact isolated sea urchin oocytes.

Part II of this thesis shows that isolated immature oocytes of the sea urchin Lytechinus pictus can incorporate considerable amounts of C^{14}-valine and C^{14}-uridine into macromolecular materials. This has been shown by Izawa, Allfrey and Mirsky (1963) to be true also for isolated immature oocytes of amphibians and by Tweedell (1966) for ovarian free immature oocytes of marine polychaet worms. A combination of scintillation counting and autoradiographic methods were employed in the present study to quantitate

and localize within the cell the incorporation of the labeled precursors by the oocytes.

The results revealed that suspensions of mature eggs incorporated little or no C^{14}-uridine into macromolecular materials while suspensions of oocytes incorporated appreciable amounts of the labeled uridine into acid precipitable materials. C^{14}-valine, on the other hand, was incorporated by suspensions of mature, unfertilized eggs but very much less than by oocyte-suspensions. C^{14}-valine incorporation by suspensions of mature eggs in nine experiments, ranged from 1.91 μμmoles to 3.39 μμmoles, and averaged 2.37 μμmoles per 10^4 eggs in 15 minutes. The corresponding values obtained in ten experiments with suspensions containing varying proportions of oocytes ranged from 6.12 μμmoles to 97.8 μμmoles and averaged 37.9 μμmoles, namely, approximately 16 times that of the mature eggs.

The average rate of incorporation of C^{14}-valine by eggs one hour after fertilization (Denny and Tyler, 1964; Timourian and Denny, 1964; Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis) may be compared with that by the oocytes. The present investigation indicates that oocytes can incorporate C^{14}-valine into protein during some stages of their growth at a significantly higher rate than fertilized eggs. For example, in one experiment the oocytes incorporated C^{14}-valine at a rate 80 times that of mature eggs. The fastest rate of C^{14}-valine incorporation obtained by fertilized eggs, however, has been only 23 times that of mature eggs (Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis). These comparisons are made apart from consideration of possible variations in the

size of the free amino acid pool and differences in the rate of uptake of valine.

Dactinomycin (actinomycin D) significantly inhibited C^{14}-uridine incorporation but did not suppress C^{14}-valine incorporation by the mature eggs or the oocytes. In four of the experiments (one with a mature egg suspension) dactinomycin stimulated C^{14}-valine incorporation by 3- to 4-fold, but in ten others (two with mature egg suspension) there was no significant difference from the control.

Additional evidence that the incorporation of C^{14}-valine represents synthesis rather than exchange or absorptive processes was provided by a demonstration that it takes place on polyribosomes.

Autoradiographs confirmed that the mature eggs did not incorporate C^{14}-uridine and incorporated only slight quantities of C^{14}-valine, even in the mixed suspensions with oocytes. The oocytes incorporated C^{14}-uridine primarily in the nucleolus and this was prevented by dactinomycin. C^{14}-valine was incorporated, by the oocytes, principally in the cytoplasm and nucleolus. These results are comparable to those obtained by Ficq (1964) who made an autoradiographic investigation of RNA and protein synthesis by sea urchin oocytes when they were still lodged within the excised ovary.

In sea urchins then, the oocytes appear to undergo a considerable amount of intrinsic protein and RNA synthesis. This is in contrast to the situation in many other organisms in which the oocyte apparently obtains much material from other tissues (see references cited in the Introduction to Part II). In addition, the data suggest that oocytes of sea urchins

may go through stages during their growth of very intense protein synthesis, significantly greater than that of fertilized eggs. Furthermore, it is of interest to note that oocytes, like fertilized eggs, synthesize protein upon relatively stable messenger RNA molecules.

3) Part III: In vivo RNA synthesis by ovarian sea urchin oocytes.

Part III of this thesis presents measurements on long-term in vivo uptake and incorporation of radioactively labeled uridine by growing oocytes of the sea urchins Lytechinus pictus and Strongylocentrotus purpuratus. The results demonstrate that labeling the RNA of mature, unfertilized eggs is feasible and effective.

Uptake and incorporation of labeled uridine by oocytes in twenty-nine different sea urchins (23 Lytechinus pictus and 6 Strongylocentrotus purpuratus) gave the following results. Ripe female sea urchins that had been spawned-out by potassium chloride injection and given a single injection of labeled uridine showed the highest percentages (15-20%) of uptake of the label in the mature eggs after one month incubation of the animals. Longer incubations did not result in higher recovery of the injected isotope. The greatest total recovery obtained in the shed eggs in these experiments was 32%.

The ratio of the incorporation of the labeled uridine into an acid insoluble form to that of the total amount taken up by the eggs also reached a maximum (90-95%) after about one month incubation. Most of the label appears in nucleic acid, as opposed to other macromolecules. The specificity of incorporation, however, is dependent upon the isotope

employed. The isotope providing the most specific incorporation into nucleic acid in the present studies was H^3-5-uridine (93% relative to total incorporation) and that least specifically incorporated into nucleic acid was H^3-6-uridine (69% relative to total incorporation). Extraction with cold phenol showed the label to be distributed among the different types of RNA as follows: 70-80% 28S and 18S, 10-20% heterogeneously sedimenting and 5-10% 4S RNA. These results are in close agreement with the findings of Gross, Malkin and Hubbard (1965) for oocytes of the sea urchin Arbacia punctulata.

4) Part IV: Evidence for arrested polyribosomes in unfertilized eggs and the demonstration of the formation of new polyribosomes after fertilization of sea urchin eggs.

Part IV of this thesis presents the studies made on the mature eggs of the sea urchin Lytechinus pictus which were labeled with radioactive uridine during oogenesis, as described in Part III. Homogenates of the radioactively labeled eggs, fertilized and unfertilized, were examined by sucrose density-gradient centrifugation. The labeled RNA was extracted from different regions of the gradient and was, in turn, also analyzed by sucrose density-gradient centrifugation. The results were correlated with the difference in protein synthesizing activity between fertilized and unfertilized eggs. The interpretations made were supported by electron-microscopic observations of the materials in the homogenates which sedimented to the various regions of the sucrose density-gradient.

Sucrose density-gradient centrifugation of the labeled homogenates of unfertilized eggs showed that most of the label sedimented with the single ribosomes. Some labeled material, however, sedimented faster than the single ribosomes. There was also some acid precipitable radioactivity that remained behind the ribosomes in the sucrose gradient.

Phenol-extraction followed by sucrose density-gradient centrifugation showed that the labeled materials in the polyribosome region of the gradient were principally 28S and 18S ribosomal RNA. Electron microscopy of the rapidly sedimenting material indicated that it consisted of aggregates of ribosomes.

Addition of RNase to post-mitochondrial supernatant fractions of homogenates, at concentrations sufficient to disaggregate amino acid labeled polyribosomes of oocytes (Part II of this thesis) and of fertilized eggs (Monroy and Tyler, 1963; Stafford, Sofer and Iverson, 1964), did not dissociate the labeled ribosomal aggregates of unfertilized eggs. Furthermore, RNase-treatment of labeled ribosomal aggregates which were first purified by sucrose density-gradient centrifugation did not affect their subsequent sedimentation through another sucrose gradient. In addition, ribosomal clusters which were isolated by sucrose gradient centrifugation followed by treatment with RNase still appeared as ribosomal aggregates in electron micrographs. The aggregates, then, possess resistance to the action of RNase.

Evidence was obtained that the RNase-resistant ribosomal aggregates may be disrupted by the combined effects of trypsin and RNase. In these

experiments, however, trypsin alone was also capable of dissociating the aggregates. Since the trypsin solutions utilized were found to possess RNase activity, the interpretation of the experiments is subject to some uncertainty. They indicate, though, that trypsin-sensitive material plays a central role in the resistance of the aggregates to RNase.

RNA-labeled homogenates of fertilized eggs showed two significant differences from corresponding homogenates of unfertilized eggs. Experiments consistently revealed three to five times more radioactively labeled and 260 mμ absorbing material sedimenting faster than single ribosomes from post-mitochondrial supernatant fractions of homogenates of fertilized eggs than of unfertilized eggs. Phenol-extraction and sucrose density-gradient centrifugation of the rapidly sedimenting labeled material in homogenates established that the increase after fertilization could be attributed to an increase in 28S and 18S ribosomal RNA. Electron microscopy of the materials that sedimented to the heavy region of the sucrose gradient confirmed that homogenates of fertilized eggs were largely enriched in numbers of ribosomal aggregates as compared with those present in homogenates of unfertilized eggs.

Experiments quantitating the amounts of ribosomal aggregates associated with deoxycholate soluble materials in homogenates of fertilized and unfertilized eggs demonstrated that additional ribosomal aggregates assemble after fertilization. Measurements of the amounts

of ribosomal aggregates sedimenting with the low-speed pellet of homogenates of unfertilized eggs also indicated that new ribosomal aggregates must form to account for the large increase in their numbers in the post-mitochondrial supernatant fraction after fertilization. Thus, the present results cannot be interpreted as the release after fertilization of appreciable numbers of ribosomal aggregates that were previously associated with deoxycholate-soluble material. Rather, a net formation of ribosomal aggregates occurs after fertilization.

The second difference that appeared in homogenates of fertilized eggs was the sensitivity of the ribosomal aggregates to RNase. The addition of RNase to the post-mitochondrial supernatant fraction abolished most, if not all, of the rapidly sedimenting radioactivity. This material could be accounted for by the presence of additional radioactivity and 260 mμ absorbing material in the region of the single ribosomes. Furthermore, electron microscopy confirmed that ribosomal aggregates purified from homogenates of fertilized eggs by sucrose density-gradient centrifugation can be dissociated by RNase-treatment.

Tests on the <u>in vivo</u> protein-synthesizing capacity of mature, unfertilized eggs showed that homogenates possessed labeled protein sedimenting faster than single ribosomes in a sucrose density-gradient. This radioactivity was completely eliminated from the heavy region of the sucrose gradient and was quantitatively recovered in the monoribosome region of the gradient after the addition of RNase to the post-mitochondrial supernatant fraction of the homogenate. The evi-

dence indicates then that two classes of ribosomal aggregates exist in homogenates of unfertilized eggs. First, a predominant species inactive in amino acid incorporation into protein and resistant to mild treatment with RNase and, second, a minor population of ribosomal aggregates that incorporates labeled amino acids into protein and is readily dissociated to single ribosomes by RNase-treatment.

Calculations based upon the 260 mμ absorption or upon consideration of the numbers of labeled aggregates that typically sediment to the heavy region of the sucrose gradient, indicated that the specific radioactivity of rapidly sedimenting protein was considerably higher in the fertilized than in the unfertilized egg. Moreover, this difference was greater than could be attributed to enhanced uptake of the labeled amino acids by fertilized eggs. The evidencs shows, then, that more protein is synthesized per unit ribosomal aggregate of fertilized eggs than of unfertilized eggs.

One attractive interpretation of the ribosomal aggregates present in homogenates of unfertilized eggs is that messenger RNA remains attached to ribosomes forming polyribosomes, as the synthetic activity of the oocytes is reduced upon maturation. These polyribosomes are, however, altered in property in some manner, perhaps coated with a trypsin-labile inhibitor, so as to be RNase-resistant and inactive as template.

Evidence has been provided that is consistent with the hypothesis that polyribosomes suppressed at maturation are reactivated after fertilization (Ozaki, Piatigorsky and Tyler, 1966). Labeled unfertilized eggs were obtained by incubating Lytechinus pictus females with C^{14}-valine for a period of four to six weeks as described for H^3-uridine in Part III of this thesis. The electrophoretic pattern of the labeled soluble proteins was compared with that of the soluble proteins prepared from eggs labeled with C^{14}-valine during the first ninety minutes after fertilization. It was found that the soluble proteins labeled shortly after fertilization have electrophoretic mobilities coinciding with the principal components identified in the extracts of the labeled unfertilized eggs.

In another set of experiments, Ozaki and Tyler (1966) examined anti-sera prepared against homogenates of unfertilized eggs. They showed that ten components could be demonstrated immuno-electrophoretically by testing the anti-sera against C^{14}-valine labeled extracts of blastulae. Three of these components were radioactively labeled. Ozaki and Tyler (1966) suggested, on the basis of these results, that protein synthesis during cleavage may be in part a resumption of the types of proteins that were synthesized during oogenesis.

The present study has shown that the percentage incorporation of labeled amino acids, relative to uptake, increases ten to fifteen-fold while the numbers of ribosomal aggregates augments only three to five times. This indicates that messenger RNA inactivated upon polyribosomes could represent, at most, only about thirty percent of that responsible for the amount of incorporation of labeled amino acids into protein that

occurs by forty minutes after fertilization. The other seventy percent of messenger RNA directing protein synthesis by forty minutes after insemination becomes associated with polyribosomes only after fertilization.

The evidence presented in the Introduction to this thesis, as well as in the Introduction to Part IV, strongly implies that this messenger RNA is "masked" in the unfertilized egg. The bulk of experiments concerning the nature of the "masked" messenger RNA in unfertilized eggs has been already discussed in Part IV. One possibility receiving support in the present investigation is that "masked" messenger RNA is complexed with protein or other material and is represented by the heterogeneously sedimenting radioactivity in the post-ribosomal region of the sucrose-density gradient of homogenates of unfertilized eggs which were labeled with uridine during oogenesis. This would be consistent with the interpretation of similar results obtained by labeling the RNA synthesized during the early development of fish (Spirin, Belitsina and Aitkhozhin, 1964) and sea urchin (Spirin and Nemer, 1965) embryos.

On the other hand, the experiments indicating a trypsin-sensitive inhibitor of protein synthesis on the ribosomes of unfertilized eggs (Monroy, Maggio and Rinaldi, 1965) provide an interesting analogy to the present findings. The present investigation shows that a trypsin-labile material is associated with the stability of the ribosomal aggregates in the presence of RNase. One might propose, then, that both single ribosomes bearing messenger RNA and polyribosomes are inhibited by a trypsin-sensitive material in unfertilized eggs. This causes the polyribosomes to be RNase-resistant and both the polyribosomes and monoribosomes to be

suppressed in protein synthesis. Removal of this inhibitor after fertilization would result in the activation of the suppressed polyribosomes. It would also cause the assembly of additional polyribosomes by the attachment of single ribosomes to ribosome-messenger RNA complexes released after fertilization. Thus polyribosome activation and formation would be two occurrences following the removal of a single inhibitor.

Experiments by a number of investigators correlating the synthesis of RNA and protein, in general, and the synthesis of specific types of proteins with the determination and differentiation of cells during development, have led (Tyler, 1965a; Tyler and Tyler, 1966b; Wilt, 1966) to the postulate that the regulation of messenger RNA activity is of primary importance to the mechanics of embryogenesis. This hypothesis has been based on numerous experiments with the different types of cells and organisms to which many are referred in the articles cited above. In form of summary, a number of experiments have shown that embryonic determination and overt differentiation can be separated with respect to time by a change in sensitivity of the tissue to dactinomycin. Inhibition of RNA synthesis by dactinomycin-treatment before embryonic determination has occurred prevents subsequent differentiation of that tissue. After determination has taken place, on the other hand, differentiation can proceed in the absence of RNA synthesis. If, however, protein synthesis is suppressed by puromycin treatment of a tissue after determination has occurred, the tissue does not visibly differentiate.

Unfertilized eggs, then, may represent an extreme example of a general case; namely, "masked" messenger RNA has determined the oocyte to a fate of

rapid cleavage. The demonstration, in Part IV, that radioactively labeled amino acids are incorporated into protein upon polyribosomes in unfertilized eggs provides additional support, although indirect, that cells in general may possess the means to regulate the activity of given populations of messenger RNA. Assuming the presence of "masked" messenger RNA in unfertilized eggs, one can conclude that inactive and active messenger RNA can coexist in a common environment.

APPENDIX 1

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INFLUENCE OF INDIVIDUAL AMINO ACIDS ON UPTAKE AND INCORPORATION OF VALINE, GLUTAMIC ACID AND ARGININE BY UNFERTILIZED AND FERTILIZED SEA URCHIN EGGS

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INFLUENCE OF INDIVIDUAL AMINO ACIDS ON UPTAKE AND INCORPORATION OF VALINE, GLUTAMIC ACID AND ARGININE BY UNFERTILIZED AND FERTILIZED SEA URCHIN EGGS [1]

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In the course of investigations (*cf.* Tyler, 1965a) into the mechanism of the initiation of protein synthesis by sea urchin eggs, some variable results were obtained in tests with dactinomycin (actinomycin D). This inhibitor of DNA-primed RNA synthesis stimulated incorporation of labeled valine into protein in four experiments with suspensions of eggs that contained many oocytes but failed to do so in several subsequent tests. The nutritional status of the animals, and consequently of the eggs, was considered as one possible source of this variation. Tests were therefore made of the effects of glucose, which experiments by Honig and Rabinovitz (1965) had shown could prevent or relieve dactinomycin-induced inhibition of protein synthesis in sarcoma-37 cells. However, glucose did not enable dactinomycin to enhance incorporation of amino acid into protein by sea urchin eggs. Tests were then made with mixtures of amino acids. Again no stimulation was obtained with dactinomycin on the incorporation of a labeled amino acid. In these tests another phenomenon appeared, namely, a marked inhibition by the amino acid mixture on the incorporation of the labeled amino acid. The experiments on the oocytes, including the erratic dactinomycin effect, will be reported elsewhere (Piatigorsky, Ozaki and Tyler, 1966), while the present account will deal mainly with exploration of the competition among amino acids.

That the rate of uptake of one amino acid may be inhibited by the presence of others has been shown in many experiments with intact cells of various organisms (see Wilbrandt and Rosenberg, 1961; Christensen, 1962, 1964; Johnstone and Scholefeld, 1965, for review). In general, the inhibition is found to occur between members of the same general class of amino acid and is interpreted as being due to a competition for transport across the cell surface.

Since sea urchin eggs are the subject of increasing numbers of investigations of amino acid incorporation into protein by the intact cells, it seemed to us desirable to determine whether or not such competition at the cell surface occurs with this material, too, and if so, to examine the interrelationships among the amino acids. While this work was in progress a preprint was received of an article by Mitchison and Cummins (1966) concerning the uptake of labeled valine and cytidine by sea

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urchin eggs at various stages of development. In tests with eggs at one hour after fertilization they report a marked inhibition of the uptake of C^{14}-valine by each of five neutral amino acids (L-leucine, DL-isoleucine, DL-alanine, DL-phenylalanine, DL-threonine) and slight inhibition by one basic amino acid (DL-lysine).

Our experiments show that these findings hold also for unfertilized eggs and provide further evidence, from measurements of both accumulation and subsequent incorporation of amino acid into protein, supporting that of Mitchison and Cummins that the inhibition operates as a competition for entrance into the cell. We have extended the measurements to include all twenty of the "coded" amino acids tested, with both fertilized and unfertilized eggs, for ability to inhibit both uptake and incorporation into protein of a neutral (valine), acidic (glutamic acid) and basic (arginine) amino acid. In the present article the amino acids that are termed basic are histidine, arginine and lysine. The acidic group includes aspartic acid, glutamic acid and their derivatives asparagine and glutamine. The remaining thirteen of the "coded" amino acids are placed in the neutral group.

Materials and Methods

Eggs were obtained from the sea urchin *Lytechinus pictus* by the method of KCl-injection, the suspension temporarily acidified to pH 5 to remove the gelatinous coat, and an aliquot removed for counting (Tyler and Tyler, 1966). For the tests of uptake and incorporation the eggs were incubated with the C^{14}-labeled amino acid and the C^{12}-amino acid being explored, in a total volume of 0.25 ml. of artificial sea water, at pH 8.0, in polystyrene test tubes, for the specified time and at 20° C. At the end of the incubation period a large excess (1 ml. of an ice-cold 0.1 *M* solution) of the C^{12}-amino acid, corresponding to the C^{14}-amino acid, was added as quencher. For the measurements of uptake the eggs were thoroughly washed with ice-cold artificial sea water and transferred with distilled water to filter papers which were rapidly dried and placed directly in the scintillation fluid [4] in which radioactivity was determined (Tri-Carb spectrometer) with about 50% efficiency. For the measurements of incorporation the same filter papers were rehydrated by transfer through absolute alcohol, 95% alcohol and 5% trichloroacetic acid (TCA). They were then processed, as usual (Tyler, 1966), with hot TCA, the alcohols, and ether, and transferred to the vials of scintillation fluid for determination of incorporation of the labeled amino acid into protein.

Results

1. *Inhibition of uptake of C^{14}-valine by an amino acid mixture*

As indicated above the initial experiments on the effect of additional amino acids on the incorporation of labeled valine were done in connection with tests of the action of dactinomycin. Table I gives the results of two experiments in which the incorporation of C^{14}-valine into protein was measured in the presence and absence of a mixture of amino acids (Borsook *et al.*, 1957) with and without dactinomycin. The inhibiting effect of the amino acid mixture is marked, regardless of whether or not dactinomycin is present. The latter had no significant effect on C^{14}-valine

[4] 2.88 g. PPO (2,5-diphenyloxazole) and 0.34 g. dimethyl POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene) per liter of toluene.

TABLE I

Action of an L-amino acid mixture and of dactinomycin** on incorporation of C^{14}-valine into protein by unfertilized eggs of L. pictus, incubated for 30 minutes at 20° C.*

Experiment	C^{14}-valine (sp. act. 185 C./*M*) μc./ml.	Counts per minute per 10^4 eggs			
		Without dactinomycin		With 0.015 mg./ml. dactinomycin	
		Without amino acid mixture	With amino acid mixture	Without amino acid mixture	With amino acid mixture
1	0.50	8327	216	10519	178
		9226	284	12112	173
2	0.42	1050	27	1078	14
		1701	24	1313	22

* Composition and final concentrations in mmoles/l.: Alanine, 0.33; arginine, 0.08; aspartic acid, 0.48; cysteine, 0.06; glutamine, 1.33; glycine, 0.89; histidine, 0.40; isoleucine, 0.05; leucine, 0.67; lysine, 0.30; methionine, 0.06; phenyl alanine, 0.26; proline, 0.23; serine, 0.28; threonine, 0.28; tryptophan, 0.05; tyrosine, 0.14.

** Gift of Merck, Sharp and Dohme, Rahway, N. J.; courtesy of Dr. H. B. Woodruff.

incorporation in these experiments. Differences between the two experiments in the absolute values for incorporation of C^{14}-valine may reflect differences in size of the endogenous free valine pool in the eggs.

The presence of the added amino acids did not, then, enable the eggs to show a stimulated incorporation of C^{14}-valine in response to dactinomycin, that had been previously noted with some batches of eggs of *Lytechinus* (see introduction).

2. *Pretreatment with amino acids*

In order to determine whether the inhibiting effect of the additional amino acids is on the accumulation of valine by the eggs or on its subsequent incorporation

TABLE II

Effect of pretreatment with an amino acid mixture (a.a. mix.) on the uptake of C^{14}-valine by unfertilized eggs of L. pictus, incubated for 1 hour at 20° C.*

Pretreatment for 1 hour in:	Counts per minute per 10^4 eggs			
	Total uptake		Incorporation into material precipitable by 5% trichloro-acetic acid	
	In presence of a.a. mix.	In absence of a.a. mix.	In presence of a.a. mix.	In absence of a.a. mix.
s.w.	80	23470	4	580
	100	27714	10	638
a.a. mix.	94	26442	8	1332
	132	27774	6	1338

* 0.53 μc./ml.; sp. act. 185 c./M.

into protein, tests were made on eggs that had been pretreated with the amino acid mixture and washed just before addition of the C^{14}-valine. Table II gives the results of one such experiment. The total uptake of C^{14}-valine, as well as the incorporation into protein, were determined. As the data show, preliminary exposure of the eggs to the mixture of amino acids has no effect on the subsequent uptake of the C^{14}-valine, either in the presence or in the absence of the C^{12}-amino acid mixture. But, uptake is almost completely suppressed by the amino acid mixture present during the period of incubation with the C^{14}-valine. The effect on uptake can, then, account for the inhibition of incorporation in the experiments shown in Table I.

The data of Table II also show inhibition of incorporation into protein in those eggs concurrently exposed to the amino acid mixture, regardless of prior exposure to the amino acid mixture. Furthermore there is an apparent increase in incorporation by those eggs exposed to the amino acid mixture before incubation with the C^{14}-valine alone. In three additional experiments an increase was obtained in one, while no appreciable difference was observed in the other two. At present, then, there is no consistent evidence that preincubation with other amino acids results in an increased incorporation of C^{14}-valine into protein.

Despite the washing following the pretreatment period the eggs probably retain most of the accumulated amino acids. This seems clear from experiments of other investigators (Nakano and Monroy, 1958; Mitchison and Cummins, 1966) and is indicated here by the large quantity of acid-soluble radioactivity remaining in the washed eggs. One may conclude, then, that retained amino acids do not influence the uptake of another amino acid, namely, valine. One or more of the amino acids in the added mixture evidently inhibit the uptake of valine when concurrently present in the medium. This was explored further with the individual amino acids and with fertilized as well as with unfertilized eggs.

3. *Effect of one amino acid on the uptake and incorporation of another*

(a) *C^{14}-valine*

Uptake, and incorporation into protein, of C^{14}-valine, C^{14}-glutamic acid and C^{14}-arginine by unfertilized and fertilized eggs were measured individually in the presence of an excess (*ca.* 3000 ×) of each of the other 19 "coded" amino acids. In some experiments the labeled amino acid was tested against the other 19 amino acids at the same time. In others about half of the amino acids were tested at one time, as noted in the legends for the figures. The results are represented graphically in Figures 1, 2 and 3. Tables III and IV present ratios of the average uptake of the labeled amino acid in the presence of the added C^{12}-amino acid to that in its absence. Ratios for incorporation are similarly presented. Table III includes results of an additional series of tests of incorporation (see footnote to table). In Figures 1, 2 and 3, for each experiment, the control values (indicated by NONE) are given at the top. These are followed by the values obtained for each of the added amino acids arranged in a decreasing (using the larger of each of the duplicate values) order of uptake.

For the unfertilized eggs the two experiments of Figure 1 with C^{14}-valine show marked (greater than 50%) inhibition of uptake by SER, ARG, ASN, GLN, ALA,

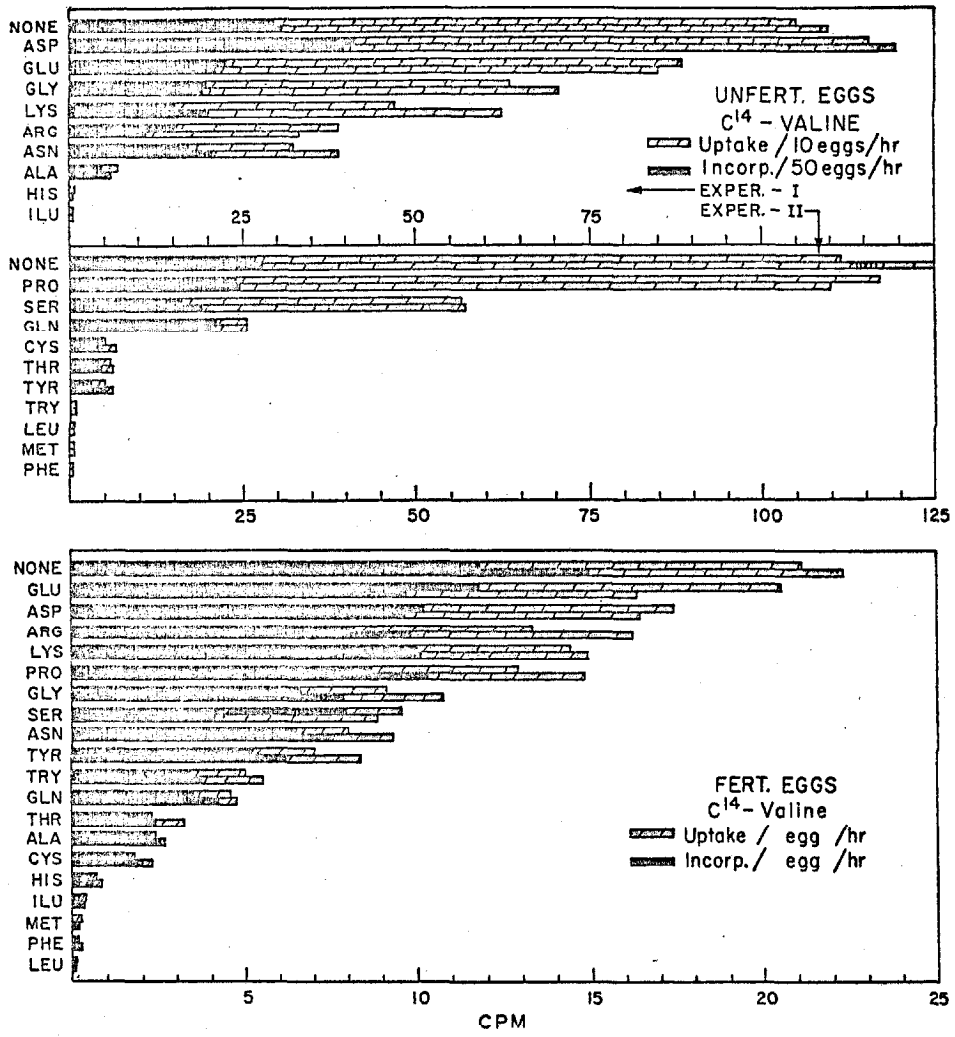

FIGURE 1. C^{14}-L-valine uptake, and incorporation into protein, by unfertilized and fertilized (one hour after fert.) eggs of *L. pictus* in presence of various individual C^{12}-L-amino acids. Incubation was for one hour in a total volume of 0.25 ml. of artificial sea water containing, per tube, 940 eggs (unfert., expt. I), 2860 eggs (unfert., expt. II) or 4314 eggs (fert.), and 0.83 μc./ml. of the C^{14}-valine (sp. act. 208.5 c./*M*). The added amino acids were each at 0.012 *M* except TYR which was at 0.0004 *M*. The tests were all run in duplicate and the individual results are represented by each member of the pairs of bars. For the unfertilized eggs the tests were done with 9 of the C^{12}-amino acids (expt. I) on one day and with the remaining 10 (expt. II) on another occasion, using eggs from a different female. In the experiment with the fertilized eggs all 19 of the C^{12}-amino acids were tested at one time. For the entries to the left of the figure: None = no added C^{12}-amino acid; ALA = alanine; ARG = arginine; ASN = asparagine; ASP = aspartic acid; CYS = cysteine; GLU = glutamic acid; GLN = glutamine; GLY = glycine; HIS = histidine; ILU = isoleucine; LEU = leucine; LYS = lysine; MET = methionine; PHE = phenylalanine; PRO = proline; SER = serine; THR = threonine; TRY = tryptophan; TYR = tyrosine; VAL = valine.

CYS, THR, TYR, HIS, ILU, LEU, MET, PHE, TRY, listed in decreasing order of the average values, as given in Table III. For all except the first three of these the inhibition of uptake is greater than 75%, and for all except the first four the inhibition of uptake is greater than 90%. The values for degree of inhibition of incorporation are similar to those for inhibition of uptake for each of the tested amino acids except for GLN where incorporation is much less inhibited (24 to 30%) than is uptake (79%).

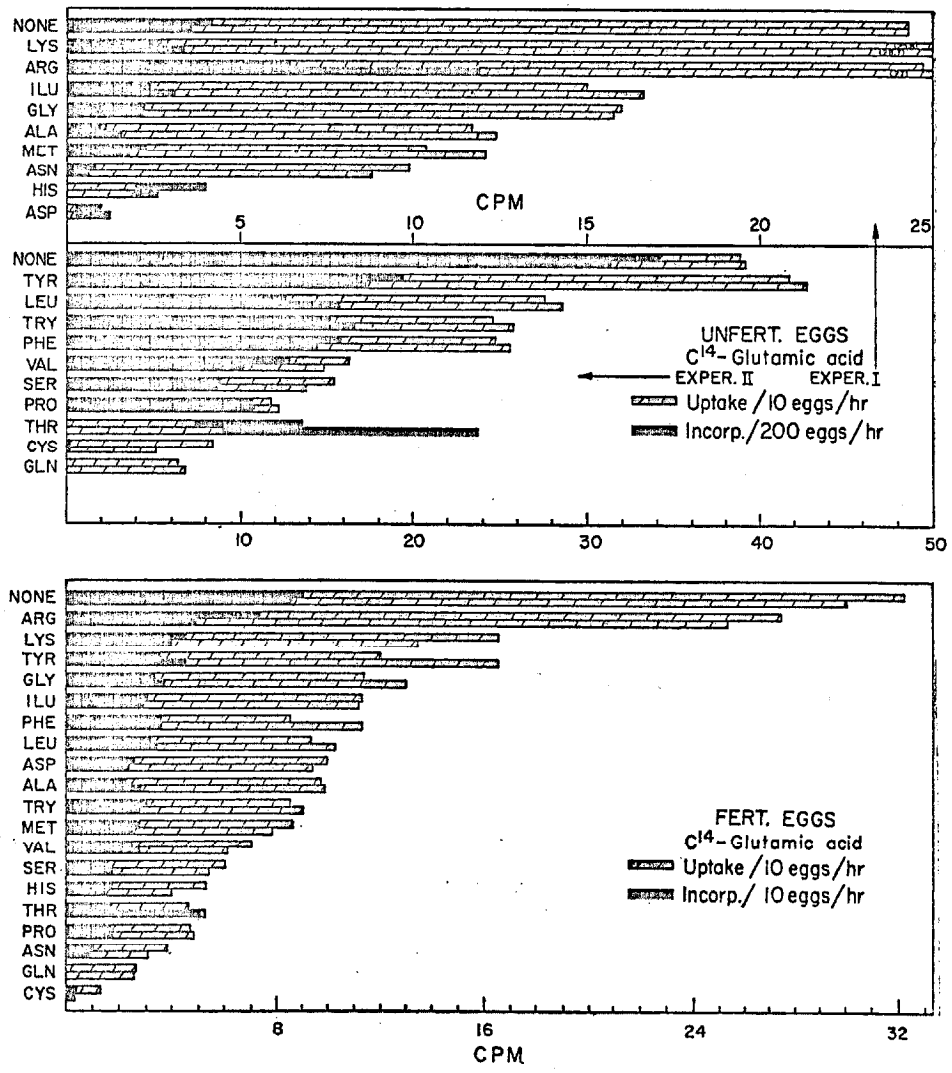

FIGURE 2. C^{14}-L-glutamic acid uptake and incorporation into protein; same description as for Figure 1, except that egg numbers were 3650 (unfert., expt. I), 1570 (unfert., expt. II), and 1190 (fert.).

For the fertilized eggs the amino acids that effect greater than 50% inhibition of both uptake and incorporation are the same as for the unfertilized eggs, except that the following are now brought just within this group: GLN (incorp.), GLY (uptake) and SER (incorp.). At the 75% level of inhibition the same amino acids

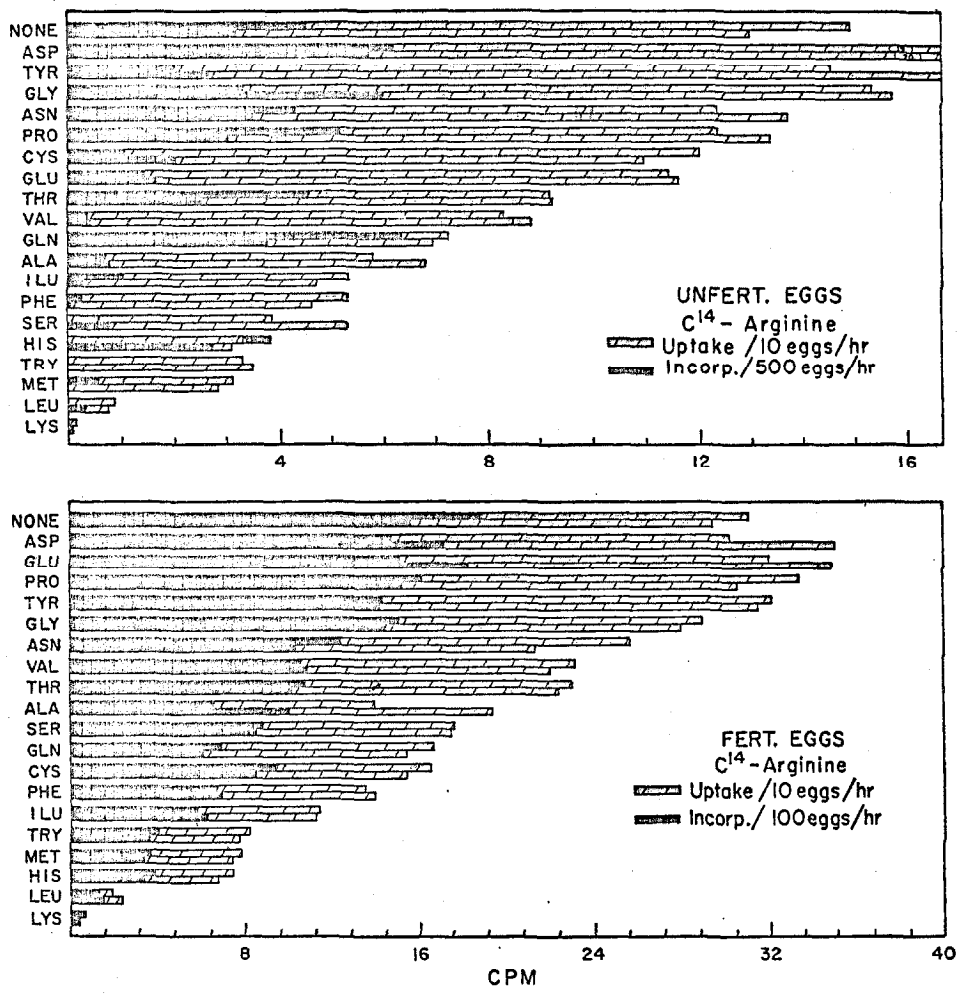

FIGURE 3. C^{14}-L-arginine uptake and incorporation into protein; same description as for Figure 1, except that egg numbers were 4170 (unfert.) and 4140 (fert.), that *L. anamesus* instead of *L. pictus* was used in the experiment with the unfertilized eggs and that the sp. act. of the C^{14}-arginine was 222 c./*M*.

are effective except for TYR (uptake and incorp.) and TRY (incorp.). Even at the 90% level of inhibition most of the inhibiting amino acids are the same as for the unfertilized eggs with respect both to uptake and incorporation, as comparisons of the values in Tables III and IV show.

The amino acids that effect the high (90% or better) degree of inhibition of uptake of C^{14}-valine, both for unfertilized (ALA, CYS, THR, TYR, HIS, ILU, LEU, MET, PHE and TRY) and for fertilized (CYS, HIS, ILU, LEU, MET and PHE) eggs all belong to the neutral group, with the exception of HIS which is only weakly basic. This holds also for the inhibition of incorporation into protein.

TABLE III

Influence of individual amino acids on the uptake and incorporation into protein of a neutral, an acidic and a basic amino acid by unfertilized eggs of Lytechinus pictus, incubated for 1 hour at 20° C.*

"Competing" C^{12}-amino acid at 0.012 *M*	Ratios of cpm's for mixture of C^{12}- and C^{14}-amino acid to cpm's for C^{14}-amino acid alone								
	C^{14}-L-Valine (3.9×10^{-6} *M*)			C^{14}-L-Glutamic acid (3.9×10^{-6} *M*)			C^{14}-L-Arginine (3.7×10^{-6} *M*)		
	Total uptake	Incorp.	Incorp.**	Total uptake	Incorp.	Incorp.**	Total uptake	Incorp.	Incorp.**
Alanine	0.07	0.17	0.08	0.49	0.34	0.42	0.45	0.22	0.35
Arginine	0.34	0.44	0.54	1.07	2.36	1.31	—	—	—
Asparagine	0.33	0.64	0.83	0.04	0.20	0.09	0.93	1.07	0.70
Aspartic acid	1.09	1.35	0.92	0.01	0.29	0.12	1.38	1.65	1.94
Cysteine	0.05	0.15	0.01	0.18	0.07	0.69	0.81	0.47	0.83
Glutamic acid	0.80	0.71	0.81	—	—	—	0.83	0.43	0.95
Glutamine	0.21	0.76	0.70	0.17	0.00	0.20	0.51	1.42	0.90
Glycine	0.62	0.64	0.80	0.65	0.55	0.49	1.11	1.32	0.68
Histidine	0.00	0.01	0.46	0.09	0.85	0.26	0.23	0.92	0.39
Isoleucine	0.00	0.01	0.02	0.65	0.70	0.47	0.36	0.19	0.14
Leucine	0.00	0.01	0.01	0.72	0.44	0.55	0.06	0.07	0.09
Lysine	0.51	0.58	0.60	1.12	0.79	0.51	0.01	0.35	0.06
Methionine	0.00	0.02	0.01	0.46	0.52	0.26	0.22	0.07	0.01
Phenylalanine	0.00	0.01	0.01	0.65	0.46	1.15	0.35	0.15	0.34
Proline	0.95	0.89	0.89	0.31	0.33	0.52	0.93	1.15	0.87
Serine	0.48	0.63	0.71	0.37	0.27	1.13	0.33	0.08	0.23
Threonine	0.05	0.17	0.13	0.21	0.57	0.72	0.66	1.00	0.46
Tryptophan	0.00	0.03	0.04	0.64	0.49	0.52	0.24	0.00	0.20
Tyrosine***	0.05	0.15	0.12	1.08	0.56	0.66	1.12	0.65	0.68
Valine	—	—	—	0.40	0.35	0.53	0.62	0.10	0.39

* *Lytechinus anamesus* used in experiments with C^{14}-L-arginine, columns 1 and 2. Eggs of this species resemble closely those of *L. pictus*.

** Separate experiment in which only incorporation into protein was measured.

*** At 0.0004 *M*.

(b) *C^{14}-Glutamic acid*

For the unfertilized eggs all but five (ARG, GLY, ILU, LYS and TYR) of the 19 C^{12}-amino acids cause greater than 50% inhibition of uptake or incorporation, or both, of the C^{14}-glutamic acid. With the fertilized eggs all but one (ARG) do so. The 75% (or more) inhibition level with unfertilized eggs is attained by ASN, ASP (uptake), CYS, GLN, HIS (uptake), and THR (uptake). At this level, for the fertilized eggs, these same amino acids, except for ASP, are effective as are also HIS (incorp.), PRO, SER, THR (uptake) and VAL (uptake). At the 90%

level with the unfertilized eggs there are ASN (uptake), ASP (uptake), CYS (incorp.) and GLN (incorp.). With the fertilized eggs 90% inhibition is given only by CYS and GLN.

For the inhibition of uptake and incorporation of C^{14}-glutamic acid there again appears to be a relationship to type of amino acid. Thus strong inhibition is given by ASP, ASN and GLN which are all grouped in the acidic category. Only CYS and THR, of the neutrals, and HIS, of the basics, strongly inhibit uptake by the

TABLE IV

Influence of individual amino acids on the uptake and incorporation into protein of a neutral, an acidic and a basic amino acid by fertilized eggs of Lytechinus pictus, 1 hour after fertilization. Incubation was for 1 hour at 20° C.

"Competing" C^{12}-amino acid at 0.012 *M*	Ratios of cpm's for mixture of C^{12}- and C^{14}-amino acid to cpm's for C^{14}-amino acid alone					
	C^{14}-L-Valine (3.9×10^{-6} *M*)		C^{14}-L-Glutamic acid (3.9×10^{-6} *M*)		C^{14}-L-Arginine (3.7×10^{-6} *M*)	
	Total uptake	Incorporation	Total uptake	Incorporation	Total uptake	Incorporation
Alanine	0.11	0.18	0.32	0.29	0.55	0.48
Arginine	0.68	0.67	0.85	0.68	—	—
Asparagine	0.40	0.52	0.11	0.11	0.78	0.65
Aspartic acid	0.78	0.74	0.31	0.27	1.08	0.92
Cysteine	0.09	0.14	0.03	0.04	0.53	0.52
Glutamic acid	0.85	0.81	—	—	1.11	0.97
Glutamine	0.21	0.29	0.08	0.01	0.53	0.38
Glycine	0.46	0.54	0.39	0.38	0.94	0.85
Histidine	0.04	0.05	0.15	0.17	0.24	0.21
Isoleucine	0.02	0.02	0.37	0.33	0.38	0.36
Leucine	0.01	0.01	0.32	0.37	0.08	0.08
Lysine	0.67	0.75	0.48	0.48	0.02	0.03
Methionine	0.01	0.02	0.27	0.30	0.25	0.23
Phenylalanine	0.01	0.01	0.32	0.38	0.45	0.40
Proline	0.64	0.71	0.16	0.21	1.06	0.91
Serine	0.42	0.43	0.18	0.20	0.58	0.50
Threonine	0.13	0.17	0.16	0.38	0.75	0.61
Tryptophan	0.24	0.27	0.29	0.33	0.26	0.21
Tyrosine*	0.36	0.43	0.46	0.46	1.05	0.83
Valine	—	—	0.21	0.30	0.75	0.62

* At 0.0004 *M*.

unfertilized eggs, and these same amino acids plus PRO, SER and VAL are similarly effective with the fertilized eggs.

(c) *C^{14}-Arginine*

Inhibition greater than 50%, for the uptake and incorporation, or both, of C^{14}-arginine, was obtained with all of the added C^{12}-amino acids with the exception of ASN, ASP, GLU, GLY, PRO, THR and TYR for the unfertilized eggs and, in addition, CYS and VAL for the fertilized eggs. At the 75% level of inhibition, only ALA, HIS, ILU, LEU, LYS, MET, PHE, SER, TRY and VAL for the

unfertilized eggs and HIS, LEU, LYS, MET and TRY for the fertilized remain inhibitory. At the 90% level of inhibition of uptake and/or incorporation LEU, LYS, MET, SER, TRY and VAL remain for the unfertilized eggs while only LEU and LYS are effective in the fertilized eggs.

It is evident that amino acids categorized as acidic did not significantly inhibit the uptake of C^{14}-arginine. In fact, ASP showed a slight enhancement of uptake and incorporation for the unfertilized eggs but this effect was not repeated with the fertilized eggs. Only a few neutral amino acids appreciably inhibited C^{14}-arginine uptake. On the other hand, both basic amino acids, LYS and HIS, showed strong inhibition for both unfertilized and fertilized eggs.

4. *Effect of fertilization on uptake of amino acids and on incorporation into protein*

Apart from the inhibitory effects of added amino acids, the data of Figures 1, 2 and 3 also permit incorporation to be compared with uptake with regard to the changes they undergo upon fertilization for C^{14}-valine, C^{14}-glutamic acid and C^{14}-arginine. This information is summarized in Table V. It is clear that uptake of

TABLE V

Effect of fertilization on uptake of amino acids and on incorporation into protein by eggs of Lytechinus (from data of Figures 1, 2 and 3; average values of cpm's per 10^3 eggs for 1 hour incubation)

	C^{14}-Valine			C^{14}-Glutamic acid			C^{14}-Arginine		
	Uptake (U)	Incorp. (I)	I/U	Uptake (U)	Incorp. (I)	I/U	Uptake (U)	Incorp. (I)	I/U
Unfertilized	11361	576	0.05	3172	91.7	0.03	1398	7.2	0.005
Fertilized	21738	13398	0.62	3116	901	0.29	3029	173	0.057
Fert./Unfert.	1.91	23	12.4	0.98	9.8	9.67	2.17	24	11.4

all three of these amino acids is high in the unfertilized egg. Upon fertilization there is an approximately two-fold increase in uptake of valine and of arginine, and no appreciable change in uptake of glutamic acid, at the stated external concentrations. The data for incorporation, however, show the usual great stimulation that occurs upon fertilization. In the present experiments these amount to 23- to 24-fold for valine and arginine, and 10-fold for glutamic acid. If incorporation is expressed in terms of uptake (columns 4, 7 and 10 of Table V) then the increase upon fertilization is of the order of 10-fold for all three amino acids, at the indicated concentrations and incubation time.

These comparisons are made apart from considerations of possible feedback inhibition of uptake, particularly in the fertilized eggs, and of possible effect of depletion of labeled amino acid from the medium. The data of Mitchison and Cummins (1966) show that with C^{14}-valine at a concentration of 0.14 mM there is no appreciable feedback inhibition of uptake by fertilized sea urchin eggs during a period of one hour. The concentration of valine in the present tests (0.0039 mM) is very much less than this. Therefore, feedback inhibition is unlikely. While similar information is not available for glutamic acid and for arginine the present

data would indicate that feedback inhibition is not likely to have occurred to any very appreciable extent in these experiments.

With regard to depletion of the labeled amino acid from the medium, calculations from the data presented in Figures 1, 2 and 3 show that the average concentrations in the medium at the end of the incubation period are reduced by approximately 2% for glutamic acid, 5% for arginine and 40% for valine. It is only for valine, then, that the value for uptake by the fertilized eggs may be appreciably affected by depletion of the label. The 40% reduction by the end of the incubation period would mean an approximately 20% average decrease in uptake, assuming linearity between uptake and concentration. This does not require altering the above statement of an approximately two-fold increase upon fertilization.

The external concentration employed in tests of valine-uptake is about one-fifth that found by Mitchison and Cummins (1966) to give maximum rate of uptake with fertilized eggs of *Paracentrotus lividus*. These workers, using concentrations well above that giving maximum rate of uptake for fertilized eggs, report a considerable increase in uptake upon fertilization. This may be estimated from their Figure 1 to amount to 15- to 30-fold. It would appear, then, that the amino acid concentrations at which the present measurements were made were in a range at which the uptake rate relative to the maximum attainable for the unfertilized egg was higher than that for the fertilized egg. This may also mean that the maximum rate is reached at lower concentrations for unfertilized than for fertilized eggs.

Discussion

The present results provide information of use in studies of changes in protein synthesis upon fertilization and early development of sea urchin eggs. The demonstration by Mitchison and Cummins (1966), with fertilized sea urchin eggs, of the ability of one amino acid to inhibit the accumulation of another, has been confirmed, and the tests have been extended to include all twenty of the "coded" amino acids in the presence of a characteristic neutral, acidic and basic amino acid in both unfertilized and fertilized eggs. The analysis has shown that competition occurs primarily between amino acids that belong to the same group. However, these interrelationships are not exclusive and there is a certain degree of overlap.

As noted in the introduction there have been many studies (*e.g.*, Wilbrandt and Rosenberg, 1961; Scholefeld, 1961; Jacquez, 1961a, 1961b; Christensen, 1962, 1964; Christensen *et al.*, 1962; Oxender and Christensen, 1963; Johnstone and Scholefeld, 1965; Guroff *et al.*, 1964; Larsen *et al.*, 1964; Spencer and Brody, 1964; Adamson *et al.*, 1966; Alvarado, 1966) with cells of various other kinds of organisms, in which the influence of one amino acid on the uptake of another has been examined. Competition is found to occur largely within the separate groups but there are many exceptions. The same general conclusions apply to the results of our experiments.

The concentration of the competing amino acid in each of our tests with valine is many thousands of times higher than that at which, according to Mitchison and Cummins (1966), the maximum rate of uptake is attained. This is probably true also for glutamic acid and arginine although the plateau levels for these have not been determined. We may infer, then, that the experiments reveal all instances in which a particular amino acid has some appreciable ability to compete for entrance

into the cell with the three amino acids tested. The correlations between the uptake of an amino acid and the incorporation into protein are very good for the unfertilized eggs and even better for the fertilized eggs where the values are higher and variation is correspondingly lower. Thus, the inhibition that one amino acid effects on the incorporation of another evidently takes place at the uptake site. That this site operates independently of the sites of protein synthesis is suggested by the wide divergences between uptake and incorporation with respect to the changes in these properties that are observed upon fertilization.

As noted above, and as is summarized in Table V, the unfertilized eggs exhibit a relatively high capacity for uptake of the three test amino acids, and the increase upon fertilization is evidently rather small. The high amino acid uptake rate of the unfertilized egg contrasts with other uptake systems studied in sea urchin eggs. For example, phosphate uptake (Whiteley, 1949; Whiteley and Chambers, 1961) and nucleoside uptake (Nemer, 1962; Piatigorsky and Whiteley, 1965; Mitchison and Cummins, 1966) are very strongly suppressed, as is the transport of many other substances in the unfertilized sea urchin egg (*cf.* Monroy, 1965; Rothschild, 1956).

Apart from the theoretical considerations that are of interest in the transport of amino acids into cells, one may utilize the data presented here, in combination with measurements of the maximum rates at which the various labeled amino acids are incorporated into protein, to specify the more effective mixtures of amino acids for labeling nascent protein in sea urchin eggs. Measurements of rates of incorporation of individual labeled amino acids as a function of concentration have been made on eggs of *Lytechinus* at one hour after fertilization (Tyler, 1965b and unpublished). From these measurements the presently available values for the (approximately) maximum incorporation into protein, in mμmoles incorporated in one hour by 10^4 eggs (and the values for the external concentrations, in μmoles/ml., at which maximum or near maximum incorporation is first attained given in parentheses) are as follows: ALA-2.3(30), ARG-2(>60), ASN-0.5(2.8), ASP-1.7(>1.9), CYS-CYS-0.3(sat. in s.w.), GLU-4.5(40), GLN-2.6(0.1), GLY-1(3.8), HIS-0.5(0.1), ILU-3.8(0.3), LEU-1.6(0.03), LYS-0.5(120), MET-0.2(0.03), PHE-0.4(0.1), PRO-1(>0.24), SER-2(8), THR-2.5(1.0), TRY-2(sat. in s.w.), TYR-0.2(0.4), and VAL-3.3(0.1).

Depending upon how the various parameters are evaluated and combined a number of highly effective mixtures may be formulated. The general procedure is to maximize incorporation into protein while minimizing effects of competition among the amino acids. It is assumed that the labeled amino acids would be available at about the same specific activity. One example of a group of amino acids that would yield high radioactivity of nascent protein is: ILU, ARG, GLU and PRO. The addition of other amino acids would tend to reduce incorporation by virtue of competition of uptake. However, one may substitute for each of these certain other "competing" amino acids that have reasonably high values of incorporation when tested individually. For example, if VAL were substituted for ILU there would not be a very great over-all change in the values for incorporation given by the mixture. Similarly ASP could be substituted for GLU without large effect, as could LYS for ARG. Obviously, there are many more complex mixtures and substitutions that might be formulated, but since the present tests were made

with only 57 of the 380 possible combinations, further assessment of the most effective mixtures does not seem warranted at this time.

Summary

1. Tests were made of the uptake and incorporation into protein of a neutral (C^{14}-valine), an acidic (C^{14}-glutamic acid) and a basic (C^{14}-arginine) amino acid in the presence of a mixture of other amino acids and in the presence of a great excess (3000-fold) of each of the other "coded" amino acids by unfertilized and fertilized eggs of *Lytechinus pictus*.

2. The results showed competition occurring principally among amino acids belonging to the same group. For C^{14}-valine the amino acids that effected strong inhibition (90% or greater) of uptake with unfertilized eggs were ALA, CYS, THR, TYR, HIS, ILU, LEU, MET, PHE and TRY, and with fertilized eggs were CYS, HIS, ILU, LEU, MET and PHE. For C^{14}-glutamic acid 90% inhibition of uptake was given by ASN and ASP with unfertilized eggs and by CYS and GLN with fertilized eggs. Finally, strong inhibition of C^{14}-arginine uptake was demonstrated by LYS and LEU with both unfertilized and fertilized eggs. Similar results were obtained in the corresponding tests of incorporation into protein. The inhibitory effects on incorporation are, then, attributable to competition for uptake.

3. In contrast to the relatively low capability of the unfertilized egg to incorporate amino acid into protein it possesses a relatively high ability to accumulate amino acids from the surroundings. For C^{14}-valine and C^{14}-arginine, the uptake rate by the unfertilized egg was approximately half of that of the fertilized egg, while for C^{14}-glutamic acid the pre- and post-fertilization rates of uptake were approximately the same.

4. The percentage of accumulated C^{14}-amino acid that was incorporated in one hour into protein in these experiments with valine, glutamic acid and arginine was 5, 3 and 0.5, respectively, in the unfertilized eggs and 60, 30 and 6, respectively, in the fertilized eggs. When expressed in terms of uptake, and assuming no large change in the pool of free amino acid in the egg, there is an approximately 10-fold increase in incorporation into protein upon fertilization for each of these three amino acids.

5. The results, also, enable formulations to be made of the kinds of combinations of labeled amino acids that would be the more highly effective in labeling nascent proteins of sea urchin eggs. One such combination would be ILU, ARG, GLU and PRO with each of these being replaceable by certain alternative "competing" amino acids as indicated in the text.

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APPENDIX 2

AMINO ACID DISPLACEMENT AND ITS USE FOR AUGMENTATION OF INCORPORATION OF LABELED AMINO ACIDS INTO PROTEIN IN UNFERTILIZED AND FERTILIZED EGGS OF THE SEA URCHIN LYTECHINUS PICTUS

INTRODUCTION

Studies of protein synthesis involving the uptake and incorporation of labeled amino acids by intact sea urchin eggs are necessarily complicated by the presence of an endogenous pool of free, non-radioactive amino acids. One effect of the pool is to lower the specific radioactivity of an administered labeled amino acid. The amount of labeled amino acid that would be incorporated into protein is thus lower the larger the pool-size of the corresponding amino acid. Precedures that would reduce the pool-size would then effectively increase the specific radioactivity of the particular proteins under investigation. This should facilitate measurements of protein synthesis, especially in cases where relatively limited amounts of labeled amino acids of not very high specific activity are available. This is, of course, subject to the provision that the reductions in pool-size do not exceed the levels at which the concentration of the particular amino acid becomes rate-limiting. There would, presumably, be an optimum concentration at which the lowering of rate of protein synthesis by reduction of the endogenous pool-size is maximally compensated by the increase in specific radioactivity that is obtained.

The literature of cell physiology abounds with experiments showing

that the size of the intracellular pool of free amino acids, and of other substances, can be altered by various procedures. Special interest attaches to experiments in which the administration of one particular substance displaces a related one from the cell.

It is known in the first place that various small molecular substances can be lost from isolated cells. Thus, experiments (Lockart and Eagle, 1959; Eagle, 1963) with cultured mammalian cells have demonstrated that amino acids, as well as other materials necessary for growth, readily leak out of cells cultured at a low population density. Experiments with rabbit erythrocytes (Park, Post, Kalman, Wright, Jr., Johnson and Morgan, 1956) and human erythrocytes (Rosenberg and Willbrandt, 1957) have shown that xylose and glucose, respectively, can be made to leave the cells against a concentration gradient by the addition of the same sugar or of closely related sugars to the medium of the cell suspension. Investigations with cultured Ehrlich ascites carcinoma cells (Heinz, 1954; Heinz and Walsh, 1958) have shown that preloading the cells with glycine or with other amino acids that competitively inhibit the uptake of glycine, promotes an increase in the rate of additional glycine uptake. The increase in glycine influx has been interpreted as an increase in the rate of glycine exchange by the pre-loaded cells. It is possible, then, to effect the displacement of one substance from intact cells by the presence of the same or of another, related material in the medium of the cell suspension (see Willbrandt and Rosenberg, 1961; Christensen, 1962, Eagle, 1963, for reviews).

Experiments with sea urchin eggs that demonstrated a competition for uptake of amino acids occurring principally among amino acids belonging to the same group (Mitchison and Cummins, 1966; Tyler, Piatigorsky and Ozaki, 1966; see Appendix 1 of this thesis), suggested that the uptake of an amino acid in this system might also be related to the couterflow of other amino acids of the same general type. In the competition experiments, data were obtained (Table II of Tyler, Piatigorsky and Ozaki, 1966; see Appendix 1 of this thesis) which indicated that pretreatment of unfertilized eggs of Lytechinus pictus with a mixture of non-radioactive amino acids might cause an increase in the subsequent incorporation of C^{14}-valine into protein. It was considered of interest, then, to study further the displacement of an amino acid during the uptake of other individual amino acids by unfertilized and fertilized sea urchin eggs, since it might provide one means by which the endogenous pool of specific amino acids in intact eggs may be partially reduced. The present study demonstrates that C^{14}-valine can indeed be displaced from intact unfertilized and fertilized sea urchin eggs by the addition, to the surrounding sea water, of one or another of the neutral amino acids. It shows also that the basic and acidic amino acids are not effective in the displacement of C^{14}-valine from the eggs.

MATERIALS AND METHODS

Eggs from the sea urchin Lytechinus pictus were shed into artificial sea water by injecting potassium chloride into the perivisceral cavity

of the animal. The gelatinous layer of the eggs was removed by temporary adjustment of the suspension to pH 5. The number of eggs was then counted in an aliquot of the suspension (Tyler and Tyler, 1966a).

For the tests on the displacement of C^{14}-L-valine from unfertilized and fertilized eggs, preloading with C^{14}-L-valine, washing by centrifugation in artificial sea water and subsequent incubation of the eggs with the individual C^{12}-L-amino acids were performed as given in the legend to Figure 1. After incubation a sample of the supernatant sea water of the preloaded eggs was transferred to filter paper strips to dry and its radioactivity measured by scintillation counting (Tri-Carb spectrometer) giving about 50% efficiency for C^{14} measurements and about 3% efficiency for H^3 measurements. For measurements of amounts of labeled amino acid in the eggs these were washed 4 times by centrifugation and transferred with distilled water to strips of filter paper. The method used for these measurements has been described elsewhere (Tyler, 1966).

RESULTS

1) Displacement of C^{14}-L-valine from intact unfertilized and fertilized eggs by various C^{12}-L-amino acids.

The results of tests of the ability of individual amino acids to promote the loss of radioactivity from eggs preloaded with C^{14}-valine are graphically illustrated in Figure 1. The length of the horizontal bars in the figure represents the amount of radioactivity that was

Fig. 1. C^{14}-L-VALINE DISPLACEMENT FROM INTACT UNFERTILIZED AND FERTILIZED EGGS OF LYTECHINUS PICTUS IN PRESENCE OF VARIOUS INDIVIDUAL C^{12}-L-AMINO ACIDS. Eggs were preloaded with C^{14}-L-valine by exposure to 1 μc /ml. of C^{14}-L-valine (sp. act., 208.5 c/M) in artificial sea water at 20°C for an hour (unfertilized eggs, upper graph) or a half-hour (fertilized eggs, 30 minutes after fertilization, lower graph). The preloaded eggs were washed 4 times by centrifugation in artificial sea water. Unfertilized (10,160 eggs/tube) and fertilized (17,780 eggs/tube) preloaded eggs were incubated, in duplicate tubes, for one hour at 20°C in a total volume of 0.5 ml. of artificial sea water with individual C^{12}-L-amino acids each at 0.028 M, except tyrosine which was at 0.00032 M. The supernatant sea water was then assayed for radioactivity as given under Materials and Methods. The individual results of the duplicate determinations are represented by each member of the pairs of bars.

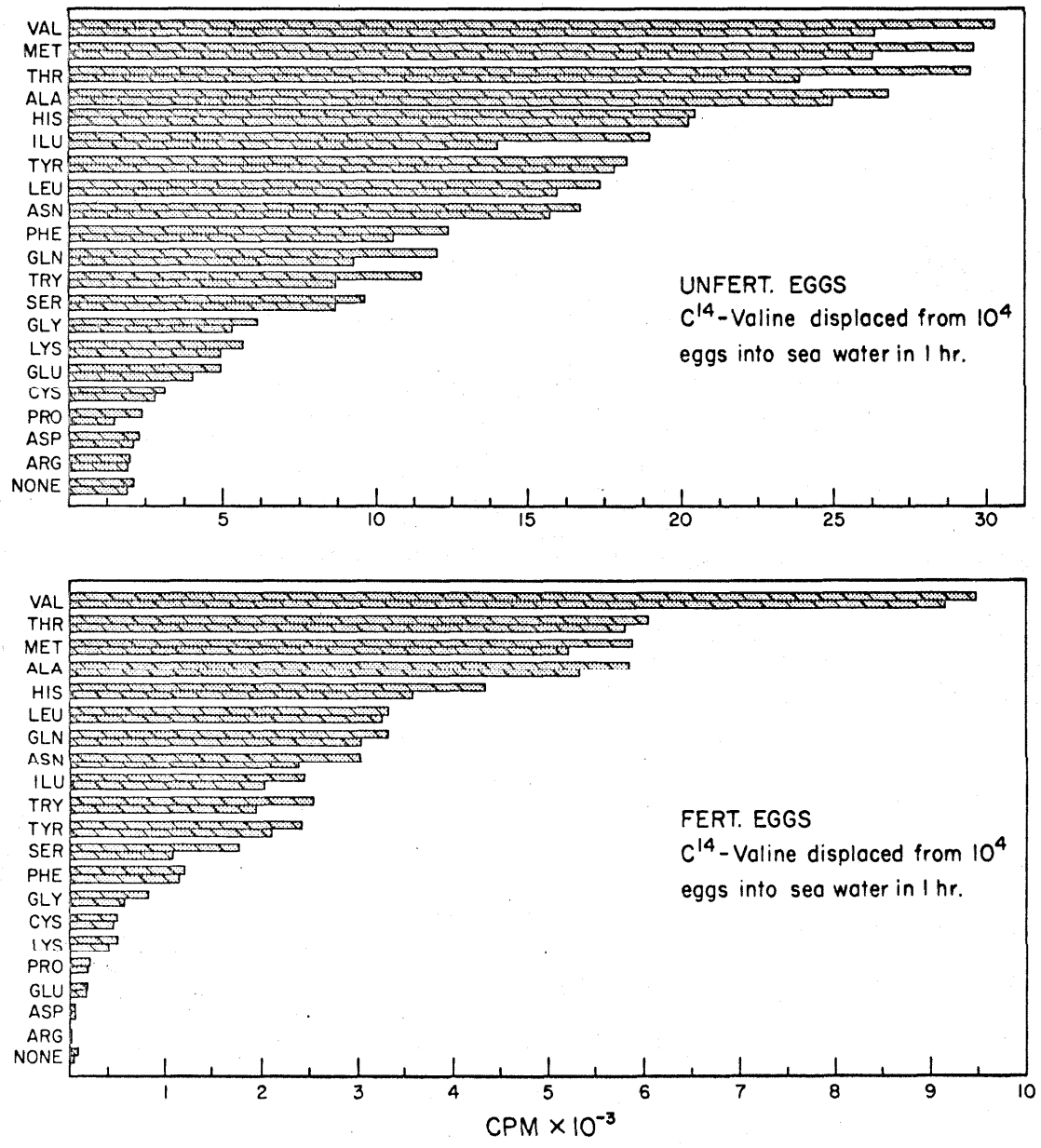
VAL
MET
THR
ALA
HIS
ILU
TYR
LEU
ASN
PHE
GLN
TRY
SER
GLY
LYS
GLU
CYS
PRO
ASP
ARG
NONE
5
10
15
20
25
30
UNFERT. EGGS
C14-Valine displaced from 10^4
eggs into sea water in 1 hr.
VAL
THR
MET
ALA
HIS
LEU
GLN
ASN
ILU
TRY
TYR
SER
PHE
GLY
CYS
LYS
PRO
GLU
ASP
ARG
NONE
1
2
3
4
5
6
7
8
9
10
CPM × 10^-3
FERT. EGGS
C14-Valine displaced from 10^4
eggs into sea water in 1 hr.

Figure 1.

present in the sea water after the preloaded eggs were exposed for one hour to the non-radioactive amino acid listed to the left of the bars along the ordinate. It is clear that in the absence of added amino acid the eggs retain most of their radioactivity. On the other hand, the presence of some kinds of amino acids in the medium, notably neutral ones, results in appreciable accumulation of label in the sea water. Other amino acids, principally acidic and basic ones, have very little effect on the accumulation of radioactivity in the medium.

Table I summarizes the data expressed as the ratio of cpm's in the sea water after one hour incubation in the presence of a C^{12} amino acid to the total cpm's in the eggs and the medium. The amino acids added to the medium were all at concentrations many times higher than that at which their incorporation is saturated (Tyler, 1965b; Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis), with the exception of arginine, proline and tyrosine. Arginine was approximately at the half-saturating concentration and proline and tyrosine were just below the saturating concentration with respect to their incorporation into protein.

The percentage of radioactivity lost from the unfertilized eggs (Exps.1 and 2) after incubation with a non-radioactive amino acid is consistently higher than that lost from the fertilized eggs (Exp. 3). This is explained by the lower incorporation of the accumulated C^{14}-valine into protein by the unfertilized eggs than by the fertilized eggs with the result that there is a larger free pool of endogenous C^{14}-valine in the former than in the latter. The relative amounts of radioactivity displaced from the eggs by different C^{12}-amino acids, however,

TABLE I

Influence of Individual C^{12}-Amino Acids on the Displacement of C^{14}-L-Valine from Intact Unfertilized and Fertilized eggs of *Lytechinus pictus*[a]

"Displacing" C^{12}-amino acid at 0.028 M	Ratios of CPM's in sea water to CPM's in sea water and eggs		
	Unfertilized eggs		Fertilized eggs
	Expt. 1	Expt. 2	Expt. 3
Alanine	0.49	0.41	0.28
Arginine	0.04	0.01	0.00
Asparagine	0.31	0.21	0.14
Aspartic acid	0.04	0.02	0.00
Cysteine	0.06	0.05	0.03
Glutamic acid	0.08	0.08	0.01
Glutamine	0.20	0.17	0.16
Glycine	0.11	0.09	0.04
Histidine	0.39	0.40	0.20
Isoleucine	0.32	0.49	0.12
Leucine	0.32	0.28	0.17
Lysine	0.10	0.09	0.0
Methionine	0.54	0.54	0.28
Phenylalanine	0.22	0.23	0.06
Proline	0.03	0.03	0.01
Serine	0.17	0.15	0.07
Threonine	0.51	0.57	0.30
Tryptophane[b]	0.19	0.22	0.11
Tyrosine	0.35	0.42	0.11
Valine	0.53	0.54	0.47

[a] These data (Expt. 1 and 3) were obtained from the tests (average values of duplicate determinations) shown in Fig. 1. An additional experiment (Expt. 2) was conducted under the same conditions stated in the legend to Fig. 1 except that incubation with the C^{12}-L-amino acids was at cell density of 8540 eggs per tube.

[b] At 0.00032 M.

are approximately the same in the experiments with the unfertilized as in those with the fertilized eggs. Thus it is likely that the mechanism by which one amino acid displaces another is the same before and after fertilization.

The amino acids that caused greater than 50% displacement of the accumulated C^{14}-valine in unfertilized eggs are VAL, MET and THR. These three amino acids, along with ALA, were also the most effective in displacing the accumulated C^{14}-valine from the fertilized eggs. In the case of the fertilized eggs, however, somewhat less than 50% of the accumulated radioactivity was displaced from the eggs by VAL, MET, THR and ALA. This is attributed to the considerable incorporation of C^{14}-valine into protein by fertilized eggs.

Greater than 10% but less than 50% displacement of labeled valine in unfertilized eggs was obtained by incubation with ALA, ILU, HIS, TYR, LEU, ASN, PHE, TRY, GLN and SER, listed in decreasing order of the average values of the two experiments given in Table I. A similar result was obtained with the fertilized eggs except that VAL, MET and THR were included in this list, as discussed above, and that PHE and SER were omitted since they displaced slightly less than 10% of the accumulated label.

GLY, LYS, GLU, CYS, ASP, PRO and ARG displaced only 10% or less of the radioactivity accumulated by unfertilized eggs. PHE and SER were also contained within this group in the tests with the fertilized eggs.

These experiments show that C^{14}-valine, a neutral amino acid, can be displaced from intact unfertilized and fertilized eggs by other

neutral amino acids. On the other hand, acidic and basic amino acids, such as ASP, GLU, ARG and LYS, do not appreciably remove C^{14}-valine from the eggs. Several other amino acids, namely, PRO, GLY and CYS, are categorized as neutral but, however, lack the capacity to displace appreciable amounts of C^{14}-valine from the eggs.

2) Evidence concerning mechanism of displacement of C^{14}-L-valine by the added neutral amino acids.

The amino acids that displace C^{14}-valine from intact eggs are, for the most part, the same as those that can competitively inhibit the uptake of C^{14}-valine (Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis), namely, neutral amino acids compete with and inhibit the uptake of valine by sea urchin eggs while acidic and basic amino acids have very little influence on the uptake of valine. The only significant exception is CYS which potently inhibits the uptake of C^{14}-valine by unfertilized and fertilized eggs but only displaces a small amount of C^{14}-valine from preloaded eggs.

An experiment was performed to test to what extent the radioactivity present in the medium after incubation of the preloaded eggs with neutral amino acids reflects a shift of a cell-medium equilibrium by competition for uptake of C^{14}-valine by the added amino acids, and to what extent the external radioactivity represents C^{14}-valine physically displaced from the egg by the uptake of the added amino acid. The experiment consisted of preloading eggs with C^{14}-valine followed by incubation at 20°C in either 10.0 ml. or 0.4 ml. of artificial sea water. At the

same time samples of the preloaded eggs were incubated in 0.4 ml. of individual solutions of an acidic (C^{12}-L-glutamic acid), a basic (C^{12}-L-arginine) and a neutral (C^{12}-L-methionine) amino acid, as well as in 0.4 ml. of a solution of C^{12}-valine, all in artificial sea water. The eggs were continually stirred to enhance exchange of C^{14}-valine between cell and environment. After one hour the eggs were centrifuged and an aliquot of the medium was assayed for its radioactive content.

The results of the experiment are given in Table II. Both C^{12}-valine and C^{12}-methionine displaced appreciable amounts of C^{14}-valine from the eggs. During the hour, however, neither C^{12}-glutamic acid nor C^{12}-arginine were effective in displacing C^{14}-valine from the eggs.

Important information comes from the tests of incubation in the two different volumes of sea water without added amino acid. Although the preloaded egg suspensions of 10.0 ml. were 25 times more dilute than those of 0.4 ml., the sea water of the more dilute suspensions contained only 3.4 and 5 times the amount of radioactivity found in the medium of the more concentrated suspensions of the unfertilized and the fertilized eggs, respectively. From these figures it appears that the progress towards equilibrium is relatively slow.

Thus, it would seem that the significant displacement of C^{14}-valine from the eggs obtained by the addition of neutral amino acids cannot be explained solely by a competition for reentry of C^{14}-valine. The evidence suggests that the efflux of C^{14}-valine from the preloaded eggs is too slow to account for its large accumulation in the medium in the

TABLE II

Influence of Dilution and of Individual Neutral, Acidic and Basic Amino Acids on the Displacement of C^{14}-L-Valine From Unfertilized and Fertilized Eggs of Lytechinus pictus.[a]

"Displacing" C^{12}-amino acid at 0.028 M	Volume (ml)	CPM's in sea water for 10^4 eggs after 1 hour		Ratios of CPM's in sea water to CPM's in sea water and eggs	
		Unfertilized	Fertilized	Unfertilized	Fertilized
Sea water	0.4	85;90	10;4	0.01	0.00
Sea water	10.0	286;315	35;35	0.02	0.00
Arginine	0.4	73;137	8;9	0.01	0.00
Glutamic acid	0.4	48;48	15;14	0.00	0.00
Methionine	0.4	4390;9458	1947;1856	0.26	0.20
Valine	0.4	3304;4316	2199;1984	0.22	0.22

[a]Experimental procedure as given in the legend to Fig. 1 except that there were 3104 unfertilized and 3768 fertilized eggs per tube. Unfertilized and fertilized (1 hr. after fert.) eggs were preloaded with C^{14}-L-valine (sp. act. 208.5 c/M) for one hour.

presence of competing neutral amino acids. While this should be directly tested by measurements of the kinetics of influx and of efflux of valine from sea urchin eggs, it appears, from the present evidence, that the non-radioactive "displacing" amino acid interacts directly with the preloaded egg in a way to effect the release of C^{14}-valine.

3) Incorporation of C^{14}-L-amino acids into protein by unfertilized and fertilized eggs after pretreatment with C^{12}-L-amino acids.

The data listed in Table III show that pretreatment of unfertilized (columns 2-4) or fertilized (columns 3-5) eggs with one or more amino acids often results in elevated incorporation of different labeled amino acids into protein. The amount of additional incorporation of labeled amino acid into protein varied in different experiments, ranging from 1.03- (experiment 4) to 2.04-fold (experiment 1) and from 1.00- (experiment 2) to 1.31-fold (experiment 3) for unfertilized and fertilized eggs, respectively. Preliminary tests of this type reported elsewhere (Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis) also gave variable results ranging from no appreciable effect to a 2.19-fold increase of C^{14}-valine incorporation into protein by unfertilized eggs after pretreatment for one hour with an amino acid mixture lacking valine. That the increase in incorporation of the C^{14}-valine into protein after pretreatment is not attributable to an increase of uptake was shown in the competition experiments, and confirmed in the present ones. These results, then, are interpreted to mean that the endogenous specific activity of a labeled amino acid given

TABLE III

Incorporation of C^{14}-L-Amino Acids into Protein by Unfertilized and Fertilized[a] Eggs of Lytechinus pictus after Pretreatment With C^{12}-L-Amino Acids

Expt. No.	Incorporation of labeled amino acids into protein by 10^4 eggs					
	CPM of unfertilized eggs			CPM of fertilized eggs		
	Control	Pretreated	Pretreated Control	Control	Pretreated	Pretreated Control
1[b]	5552 5740	11465 11538	2.04	56766 56139	66813 63206	1.15
2[c]	2409 2237	2911 2607	1.21	14912 9271	15157 9070	1.00
3[d]	695 855	1132 1044	1.40	13501 11208	15582 16841	1.31
4[e]	3197 2897	3424 2828	1.03			

[a]Experiments 1, 2 and 4, pretreated 30 min. after fertilization; experiment 3, pretreated 60 min. after fertilization.

[b]Pretreatment with a mixture of C^{12}-L-methionine, C^{12}-L-lysine and C^{12}-L-glutamic acid, each at 0.017 M for 1 hr. at 20°C, followed by incubation for 30 min. at 20°C with a mixture of C^{14}-L-valine (sp. act., 208.5 c/M), C^{14}-L-arginine (sp. act., 222 c/M) and C^{14}-L-aspartic acid (sp. act., 164 c/M) each at 0.5 μc/ml in a total volume of 1.0 ml of artificial sea water.

[c]Pretreatment with C^{12}-L-threonine at 0.1 M for 1 hr. at 20°C followed by incubation for 30 min. at 20°C with H^3-L-valine (sp. act., 870 c/M) at 5 μc/ml in a total volume of 1.0 ml of artificial sea water.

[d]Pretreatment followed by incubation for 20 min., as in experiment 2.

[e]Pretreatment followed by incubation for 15 min., as in experiment 2.

to the eggs can be made somewhat higher by a preliminary depletion of the corresponding non-radioactive amino acids from the eggs by pre-treatment with another non-radioactive amino acid belonging to the same group.

DISCUSSION

The present results show that C^{14}-valine can be displaced from intact unfertilized and fertilized eggs as a consequence of the uptake of non-radioactive valine or of other neutral amino acids. However, basic and acidic amino acids are relatively ineffective in the removal of valine from intact eggs. It has been assumed, in the interpretation of these tests, that the radioactivity displaced from the preloaded eggs is indeed C^{14}-valine. The radioactivity in the sea water can be evaporated to dryness. In addition all the radioactivity is soluble in 5% trichloroacetic acid. Nevertheless, it has not been chemically identified.

That incubation of eggs preloaded with C^{14}-valine at a population density 25 times lower than that of another equivalent suspension results in only a small difference in the amount of C^{14} that appears in the surrounding medium indicates that sea urchin eggs normally tightly bind most of their amino acids. In fact, marine invertebrates in general efficiently accumulate and retain amino acids (Stephens and Schinske, 1961; Stephens and Virkar, 1966). This is in contrast to the situation in cultured mammalian cells where low population densities result in leakage of nonessential amino acids, as well as other materials, at a

faster rate than the cell can synthesize them (Lockart and Eagle, 1959; Eagle, Piez and Oyama, 1961; Eagle and Piez, 1962; see Levintow and Eagle, 1961; Eagle, 1963, for reviews). The mammalian cells will not grow in dilute suspensions, then, unless the medium is enriched by the addition of the appropriate amino acids. This difference between cells of mammals and those of marine invertebrates is not altogether surprising in view of the fact that mammalian cells are adapted, in vivo, to very high population densities while sea urchin eggs must survive as single cells in an ocean.

Thus, the evidence suggests that the accumulation of radioactivity in the medium of the preloaded eggs exposed to certain individual amino acids is largely a consequence of a linked exchange flux rather than the outward diffusion of the C^{14}-valine which would subsequently be in competition for uptake with the second amino acid added to the sea water (Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis). It appears, however, that some C^{14}-valine may diffuse from the eggs and, therefore, a competition for uptake would contribute in part to the accumulation of C^{14}-valine outside of the eggs. The observation that cysteine, a powerful inhibitor of valine uptake, did not effect the removal of appreciable quantities of C^{14}-valine from the eggs provides additional evidence that the displacement of C^{14}-valine cannot be entirely explained by a competitive inhibition of C^{14}-valine influx without affecting its efflux.

Christensen, Riggs, Fischer and Palatine (1952) and Riggs, Walker and Christensen (1958) advanced the hypothesis on the basis of a number of observations that potassium efflux may be linked with amino acid

influx in Ehrlich mouse ascites carcinoma cells. It is unlikely that potassium exchange is connected with amino acid uptake or displacement from sea urchin eggs since Tyler and Monroy (1959) have shown that potassium is not readily exchanged by eggs until after fertilization. Yet, the present study has shown that amino acid exchange, as well as uptake (Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis), occurs to a considerable extent with unfertilized eggs.

Whatever the mechanism by which an exogenous amino acid induces the outward flow of another amino acid from intact eggs, the net result is a partial depletion of the endogenous pool of certain amino acids. This then potentially provides a method to elevate incorporation of specific, labeled amino acids into protein without affecting protein synthesis. As noted in the Introduction, if depletion goes very much beyond the point where the amino acid concentration is limiting, inhibition of protein synthesis would result. On the other hand, partial depletion of an amino acid should, theoretically, raise the endogenous specific activity of an administered amino acid. Since the pool of free amino acids is likely to differ from one batch of eggs to another, the effectiveness of the pretreatment in optimally depleting the pool of a particular amino acid will correspondingly differ. Furthermore, it is likely that the sea urchin egg responds to this deficiency and begins to synthesize the exhausted amino acid, possibly by the release of feedback inhibition and repression of enzyme synthesis, as is well documented in microorganisms (see Umbarger, 1961a, b; Vogel, 1961) and as may also occur in animal cells (Wilson and Pardee, 1964). Nonetheless,

labeling experiments performed before the eggs have completed restoring the amino acids to the partially depleted pool may result in an increased incorporation of the labeled amino acid into protein presumably without affecting the synthesis of protein.

In the present experiments unfertilized eggs were made to double the incorporation of labeled amino acid into protein by preliminary treatment of the eggs with a non-radioactive, non-competitive mixture of amino acids followed by exposure to a C^{14}-labeled mixture of non-competitive amino acids of the type presumably displaced from the eggs by the initial treatment. Such enhanced incorporation of radioactively labeled amino acid is more marked in tests with unfertilized than with fertilized eggs. This is possibly explained by the faster rate of synthesis of new amino acids by fertilized eggs which thus replenish the partially exhausted pool.

The utilization of this technique, then, may be most fruitfully applied to the study of in vivo protein synthesis by unfertilized eggs. The proteins of unfertilized sea urchin eggs can be significantly labeled by coupling this method of partial amino acid pool depletion with the subsequent labeling of protein by an assortment of non-competing amino acids given to the eggs at a concentration of maximal uptake per amino acid (Tyler, 1965b; Tyler, Piatigorsky and Ozaki, 1966, see Appendix 1 of this thesis). This procedure has already been of value in establishing that unfertilized eggs possess a population of polyribosomes active in the synthesis of protein (see part IV of this thesis). It may also eventually contribute to our knowledge concerning the types of proteins being synthesized by unfertilized sea urchin eggs.

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