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Biochemical Patterns of Limnaea palustris Embryos

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BIOCHEMICAL PATTERNS OF

LIMNAEA PALUSTRIS EMBRYOS

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A Thesis

Presented to

The Faculty of the Department of Biology

The College of William and Mary in Virginia

———————

In Partial Fulfillment

Of the Requirements for the Degree of

Master of Arts

———————

By

Warren L. Rottmann

1967

APPROVAL SHEET

This thesis is submitted in partial fulfillment of

the requirements for the degree of

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PREFACE

A causal analysis of development depends, in part, upon a knowledge of the physico-chemical nature of a developing system. Historically, basic analytical biochemistry and cytochemistry have served as the foundation for further experimental investigations. The egg of the fresh-water pulmonate snail Limnaea is particularly well-suited for the study of descriptive and experimental morphology (Morrill, 1963a, 1963b, 1964a, 1964b; Raven, 1948, 1952, 1956, 1964). Its cytochemistry is also well known (Raven, 1945, 1946). Little, however, is understood about the biochemistry of developing Limnaea. Therefore, I elected to investigate the overall patterns of synthesis of three classes of macromolecules - ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and protein. The purpose of these investigations is to gain an insight into the biochemistry of development in Limnaea palustris. An attempt will be made to correlate the biochemical patterns with cytochemical and morphological observations. My technique is a quantitative method to illustrate qualitative relationships; the quantitative results are a function of the experimental parameters.

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ABSTRACT

The purpose of this study is to investigate some biochemical patterns during the development of normal and actinomycin-D-treated embryos of the pulmonate snail Limnaea palustris.

Rates of protein, RNA and DNA syntheses were determined at half-day intervals during development of Limnaea palustris. Incorporation of tritium-labelled leucine, uridine and thymidine was measured in the cold perchloric acid residue. In normal embryos the rates of protein, RNA and DNA syntheses are low and nearly constant throughout cleavage and gastrulation. As the gastrula develops into a trochophore, RNA and protein syntheses accelerate markedly. Protein synthesis "peaks" at the 2-day trochophore stage. A second surge of protein synthesis occurs at the 3-day stage – a period of marked embryogenesis and growth. Prior to this stage, there is a high rate of RNA synthesis.

Continuous treatment with actinomycin-D (100 ug./ml.) inhibits differentiation. The embryos arrest approximately between the 2 and 3-day stages. In the actinomycin-D-treated embryos uridine incorporation is not detected during the early stages. A low rate of incorporation occurs in the arrested embryos. Marked protein synthesis occurs during post-gastrula stages, but decreases with time. While the magnitude of the protein synthesis corresponds to the "2-day peak" of normal synthesis, it is delayed by approximately half a day.

The results suggest that through the 2-day stage, protein synthesis is under the control of "prescribed" RNA. Following this stage, genome-dependent RNA synthesis is required for further differentiation and growth.

SOME BIOCHEMICAL PATTERNS DURING THE DEVELOPMENT OF

NORMAL AND ACTINOMYCIN-D-TREATED EMBRYOS OF THE

PULMONATE SNAIL LIMNAEA PALUSTRIS

INTRODUCTION

The biochemistry of animal development is currently one of the most dynamic fields of biological endeavor (e.g. Weber, 1965). Systems of current interest include sea urchins (e.g. Gustafson, 1965; Gross, 1964), amphibia (Brown, 1964) and certain molluscs (Collier, 1965). Biochemical investigations, however, should be extended to other developmental systems. Additional comparative studies are needed to correlate various biochemical and morphogenetic patterns of development.

Biochemical studies have shown that early protein synthesis in sea urchins depends upon ribonucleic acids present in the unfertilized egg (e.g. Monroy, Maggio and Rinaldi, 1965; Denny and Tyler, 1964; Hultin, 1964; Gross, 1964). Furthermore, cytoplasmic particles containing messenger ribonucleic acid (m-RNA) have been isolated from early-cleavage sea urchin embryos (Spirin and Nemer, 1965). A marked increase in the synthesis of RNA and protein occurs at fertilization and increases through the blastula stage (e.g. Hultin, 1961; Elson, Gustafson and Chargaff, 1954; Comb and Brown, 1964). However, when DNA-dependent m-RNA synthesis is suppressed with actinomycin-D, protein synthesis only continues up to the blastula stage, suggesting that new m-RNA is needed for development past the blastula stage (Malkin, Gross and Romanoff, 1964; Nemer, 1964; Comb, et al., 1965; Gross and Cousineau, 1964).

A detectable change in RNA synthesis could not be demonstrated

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following fertilization in the amphibian Xenopus. During late cleavage, however, when DNA synthesis is rapid, RNA is synthesized (Brown, 1964). Although the maternal genome seemed to control the cells prior to gastrulation, newly synthesized gene products were required for gastrulation. Transfer and m-RNA were synthesized in specific regions of the blastula – particularly the presumptive areas for prechordal tissue, neural tissue and endoderm (Bachvarova and Davidson, 1966). Furthermore, rapid rates of ribosome and soluble protein syntheses began at Shumway stage 18, the muscular response stage (Brown and Caston, 1962). Until the swimming stage, no significant ribosome synthesis is necessary for further development (Brown, 1964). The data of Brown and Littna (1964) suggested that total RNA of Xenopus embryos does not increase significantly until post-hatching stages. These results support the hypothesis that early development is not dependent upon any marked RNA synthesis and that protein synthesis during early development is probably under the partial control of "prescribed" RNA's present in the unfertilized egg.

In the marine mud snail Ilyanassa, no net increase of RNA occurs until just prior to the histological differentiation into a veliger (Collier, 1961a). This stage is two days after gastrulation. However, the incorporation of P^{32} into RNA and the incorporation of C^{14}-leucine into protein occurs prior to the stage of net increase in RNA. Collier (1961a) suggested that P^{32} incorporation is an inadequate criterion for detecting the time of RNA synthesis. He tentatively concluded that little synthetic activity of protein and RNA occurs during the time of early development and that later syntheses are essential for histological differentiation (Collier, 1961a). Collier (1961b) suggested that

"morphogenetic factors" determine qualitative protein synthesis for differentiation. These may be mosaically localized in the egg, viz. in the polar lobe (Collier, 1961b). Indeed, "gene-activating factors" have been localized in this region (Davidson, et al., 1965).

Electrophoretic analysis of some hydrolytic enzymes of Ilyanassa demonstrated that at day four of development new enzymatic bands appear and that certain bands present at the earlier stages cannot be detected past this stage (Morrill and Norris, 1965). These phenomena correlate with the changes in protein and RNA syntheses at, and slightly previous to, this 4-day stage of development (Collier, 1961a). Similarly, electrophoretic studies indicated that several new enzymatic bands appear during day three of the development of Limnaea palustris. In this species, this is a notable stage in histo- and organogenesis. It has been suggested that these organ-specific bands are associated with morphological and functional differentiation (Norris and Morrill, 1964). Increase in RNA and protein syntheses prior to the 3-day "histogenesis" stage may occur in Limnaea as in Ilyanassa. Morrill (1964a) found that there is no significant change in total protein of the embryo or capsule albumen until the gastrula stage (approximately day one). Following gastrulation, the total embryonic protein increases at day two subsequent to the decrease of the albumen protein of the egg capsule (Morrill, 1964a).

The present study is designed to determine rates of syntheses of DNA, RNA and acid-insoluble protein, using tritium-labelled precursors during the normal development of L. palustris; and to test the effects of actinomycin-D on the biochemical and morphological patterns of this pulmonate snail. This antibiotic distinguishes periods of genome-

dependent RNA and protein syntheses by inhibiting formation of DNA-
dependent RNA (Goldberg and Rabinowitz, 1962).

MATERIALS AND METHODS

The incorporation of tritium-labelled precursors into the cold perchloric acid residue was used to indicate the rates of syntheses of DNA, RNA and protein. L-leucine-4, 5-H^3 (specific activity: 5.0 curies/ millimole, 5 uC/ml), thymidine-methyl-H^3 (specific activity: 6.7 curies/ millimole, 5 uC/ml) and uridine-5-H^3 (specific activity: 4.4 curies/ millimole, 5 uC/ml) were obtained from the New England Nuclear Corporation.

Egg masses were collected from a culture of *Limnaea palustris* maintained by Dr. John B. Morrill, College of William and Mary. Embryos used for single determinations were derived from one or two egg masses.

Embryonic development was timed from the onset of first cleavage. Incubation and incorporation were carried out at $24° \pm 1°C$. Incorporation was investigated at half-day intervals from the 2-cell stage through the 4.5-day stage. At the proper stage, the embryos were manually removed from their jelly mass by rolling them out on filter paper. They were rinsed several times in sterile culture water. Thirty embryos were transferred to a disposable plastic cup (Analoe), the water removed, 0.5 ml of precursor added, and the embryos incubated for 2 hours at $24°C$. The incorporation was stopped with the addition of 70% ethanol. After 10 minutes, the embryos received two 10-minute washes of 0.5 ml sterile culture water. The embryos were decapsulated manually. Twenty-five embryos were transferred to a Millipore filter (pore size:

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0.45 microns) with the aid of a capillary pipette. The filter was placed on a Swinnex adapter connected to a vacuum system. This apparatus, diagrammed in Figure 1, was used to process up to twelve samples at the same time. A wash fluid was dropped onto the filter and whole embryos; the surface tension itself allowed the fluid to remain. Unincorporated precursor was removed from the whole embryos by washing 10 times (0.25 ml, for 2 minutes per wash) with ice-cold 0.2N perchloric acid (PCA) containing 1 mg/ml of unlabelled precursor. After each wash, the fluid was drawn off and collected by the vacuum system.

Subsequently, the washed embryos and filter were transferred to a scintillation-counting vial; 0.3 ml of Nuclear Chicago solubilizer (NCS) was added; and the material dissolved overnight at room temperature. A Vortex brand mixer was used to break up the filter and aid in dissolution.

Radioactivity was measured with a Nuclear Chicago liquid scintillation counter, Model Series 720. A toluene-base counting fluid (4 g of 2, 5-diphenloxazole, 80 mg of 2-p-phenylenebis (5-phenyloxazole) and 1 liter of toluene), found to be the most efficient in the counter, was used throughout. Background was determined using a "blank" vial consisting of counting fluid, 25 unlabelled embryos, 1 Millipore filter and 0.3 ml NCS-solubilizer. Although the dissolved filter imparted a yellow color to the scintillation fluid, counting efficiencies averaged between 10 and 15% for tritium. Vial efficiencies were determined by the internal standardization technique with a tritium-labelled toluene standard (New England Nuclear Corporation). The "channels-ratio" method could not be used for vials containing low activity. An important technical observation was the "excited state" of freshly prepared

Figure 1. Apparatus for washing embryos which have been incubated in radioisotopic precursors. Embryos are placed on a filter, supported on a Swinnex-brand adapter. Surface tension allows the ice-cold 0.2N perchloric acid containing 1 mg/ml unlabelled precursor to cover embryos and filter. The fluid is drawn off under vacuum and collected in side-arm test tubes. The manifold connection allows 12 samples to be processed simultaneously.

0.25ml "Wash"

Millipore Filter

Swinnex Adapter

Luer Hypodermic Needle

Stopper

Vacuum

Side·Arm Test Tube

2 x 4" Block

Manifold

Trap

Vacuum
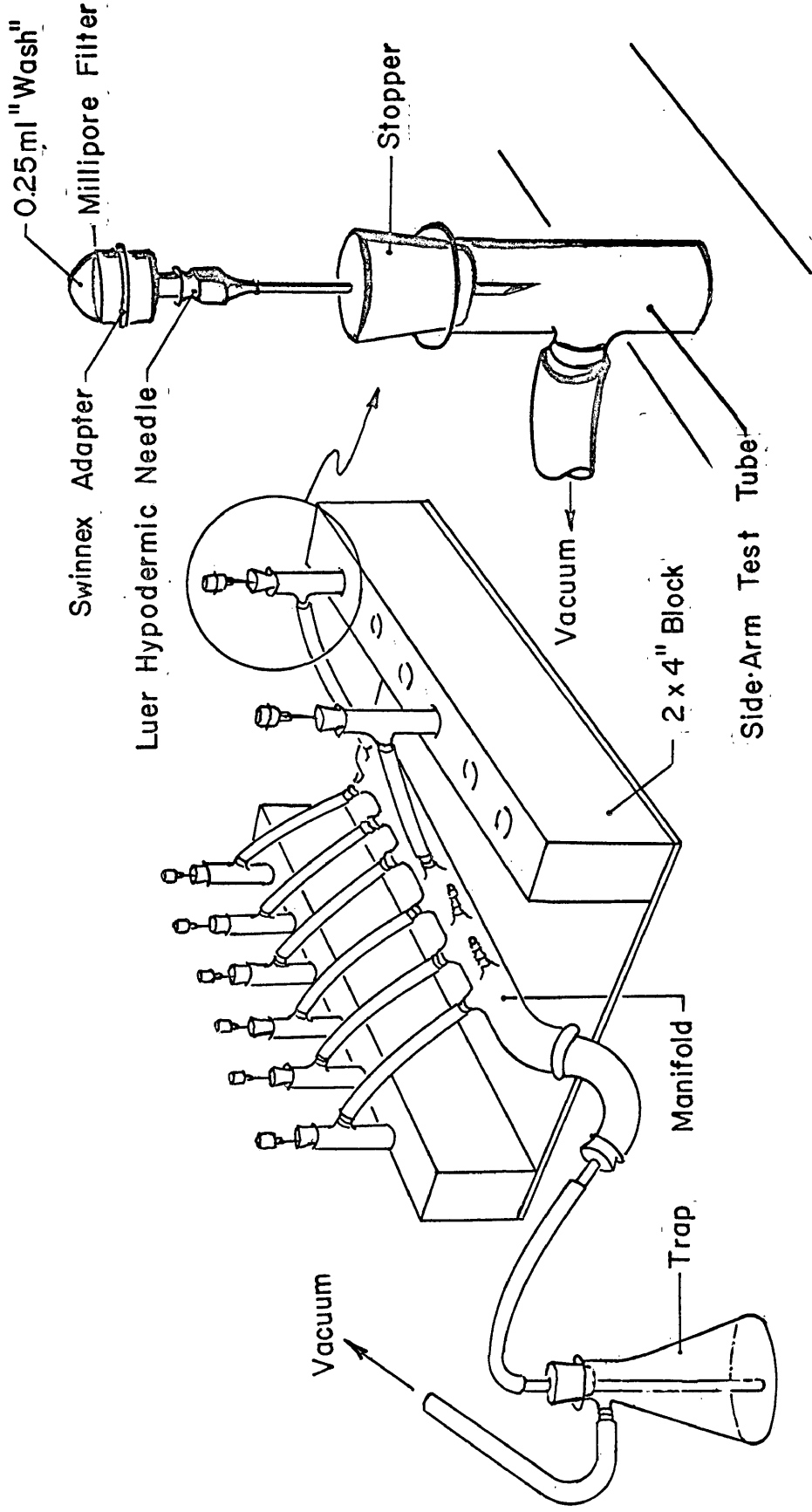
vials. Control experiments by Dr. R.E.L. Black indicated that this "excitation" is not due to the vial glass, to the NCS, to light exposure or to the shaken fluid. This excited state may arise from energy released during some exothermic reaction as the filter, embryos and solubilizer dissolve in the counting fluid. The "excitation" of the scintillation fluid decreased over a period of a week. The observed counts per minute then stabilized. Consequently, before any radioactivity measurements were made, freshly prepared vials were stored at room temperature in the dark for at least 9 days. All counting measurements were made at room temperature.

Similar techniques were used to study the effects of actinomycin-D (100 ug/ml) on morphogenesis and RNA and protein syntheses. The embryos were subjected to continuous treatment from the onset of first cleavage.

The results of the present investigation are based upon the measurement of residues of synthesized materials which were not solubilized by the 70% ethanol or cold 0.2N PCA. These residues are thought to be essentially acid-insoluble protein, RNA and DNA. Acid-insoluble materials lost through the Millipore filter, per se, could not be estimated. Tyler (1966) used a filter-paper absorption technique for the extraction of labelled protein for sea urchin embryos. The incubation mixture was plated onto Whatman #1 filters and extracted with trichloroacetic acid (TCA) and ethanol. The Millipore filter used in the present work has a much smaller pore size (0.45 u). The dead embryos appeared to remain intact on the filters. It would seem, therefore, that the filter retains most of the acid-insoluble materials.

Isotope incorporation studies should include controls for bacterial

contamination. This need was stressed in work on developing sea ur-
chins (Glisin and Glisin, 1964). Both the general procedure employed
and the development of Limnaea within a proteinaceous capsule prevented
microbial contamination without the use of antibiotics. Bacteriolog-
ically sterile L. stagnalis (Friedl, 1964) and Australorbis glabratus
(Chernin, 1957) can be obtained by "surface" sterilization of the em-
bryos, proving the efficiency of the capsule as a bacterial screen.
During the present experiments, encapsulated embryos were reared in
sterile culture water to keep bacterial-protist populations low. In-
corporation of precursor into the encapsulated embryos was stopped by
the addition of 70% ethanol. Since the dead embryos were decapsulated
and washed in sterile culture water before transfer to the filters,
any dead micro-organisms were probably left behind with the discarded
capsules.

Morphological observations were made on developing normal and
actinomycin-D-treated embryos at half-day intervals. In addition,
whole mounts were prepared by gluteraldehyde fixation, dehydration in
ethanol and clearing in toluene. Thin sections (10 μ) were prepared
by 1-hour gluteraldehyde fixation (0.5 ml of 50% gluteraldehyde in 3.5
ml of 0.1N cacodylate buffer, pH 7.2), paraffin sectioning and hema-
toxylin-eosin staining.

The Incorporation of Thymidine-methyl-H^3

Rates of thymidine incorporation were measured in normal embryos. The rates are plotted as a function of the stage of development (Figure 2). The resultant curve appears to be an exponential (logarithmic) function; i.e. rates of incorporation, per embryo, increase exponentially, as a function of time.

The first detectable increase in the rate of thymidine incorporation occurred at the 1.5-day stage (late gastrula), just prior to the development of the trochophore. Subsequently, thymidine incorporation rates increased rapidly (the slope of the curve represents acceleration).

The Incorporation of Uridine-5-H^3

Rates of uridine incorporation were measured in normal and actinomycin-D-treated embryos. The results, summarized in Figure 3, show that incorporation rates in normal embryos were low and nearly constant through the 1.5-day stage (late gastrula). Following this stage, a marked acceleration of uridine incorporation rates accompanied the development of the trochophore (2-day stage). As the normal trochophore persisted (2.0-2.5-day stages), rates of incorporation were high but constant. Development of certain adult primordia (2.5-3-day stages) may have been accompanied by a small increase in incorporation. Between the 3.5- and 4-day stages, normal morphological differentiation and

Figure 2. Rates of incorporation of thymidine-methyl-H^3
into DNA in normal L. palustris embryos at 24°C.
Each point represents 1 determination; the curve
passes through the mean value at each stage of
development. Gastrulation commences at approx-
imately 1.0 days; the trochophore develops by 2.0
days; adult organ primordia commence development
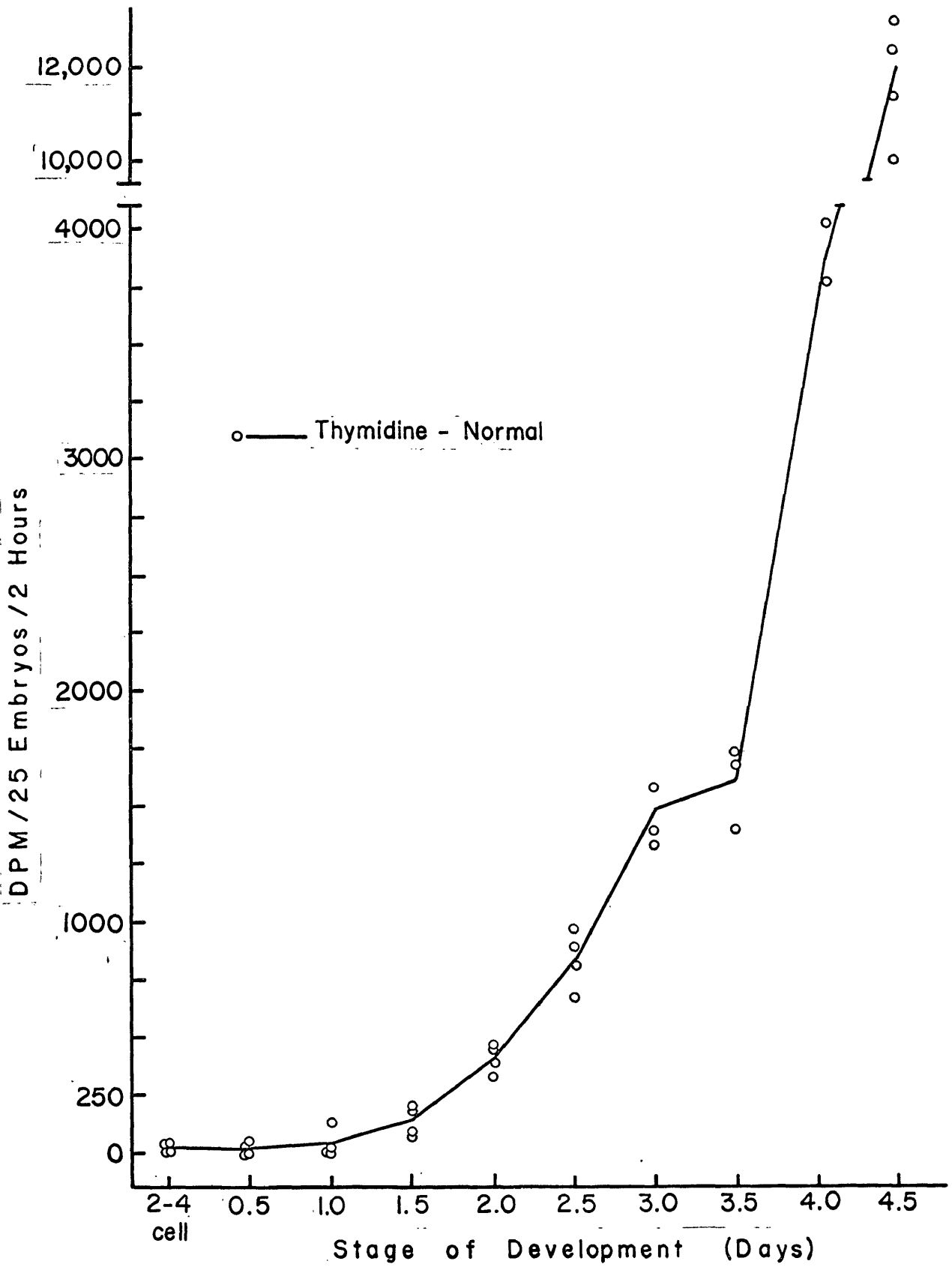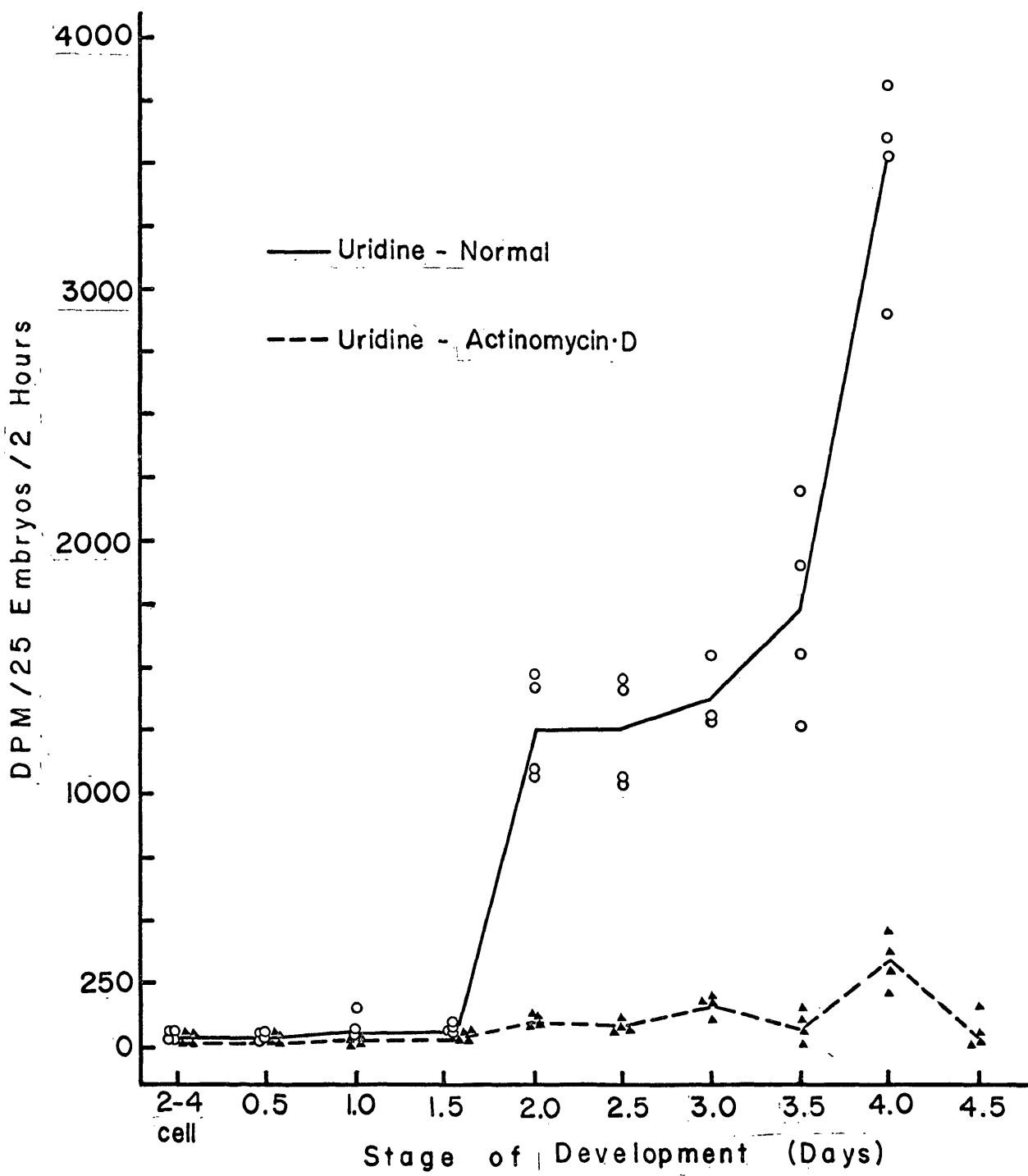and growth by 3.0 days.

Figure 3. Rates of incorporation of uridine-5-H^3 into RNA in normal and actinomycin-D-treated embryos at 24°C. Each point represents one determination; the curves pass through the mean values at each stage of development. See text for a description of the development of normal and actinomycin-D-treated embryos.

growth were rapid and were accompanied by acceleration of rates of uridine incorporation.

Actinomycin-D-treated embryos incorporated uridine at constant low rates during early development, as did normal embryos. Contrasting with normal development, the significant acceleration of incorporation after the late gastrula in treated embryos was suppressed. There was, however, an increase in the rate of incorporation in the treated 4-day old embryos, which were morphologically retarded.

The Incorporation of L-Leucine-4, 5-H[3]

Rates of incorporation of l-leucine were measured in normal and actinomycin-D-treated embryos. During normal development, rates of incorporation were low and nearly constant through the 1.5-day (late gastrula) stage (Figure 4). Then the incorporation rate accelerated during development of the trochophore (2.0-day stage). During the persistence of the trochophore (2.0-2.5) rates of incorporation were somewhat lower. Following the 3.0-day stage, incorporation rates again accelerated. The latter period was characterized by marked histo- and organo-differentiation and growth.

Continuous treatment with actinomycin-D (100 ug/ml) significantly affected the rates of l-leucine incorporation and arrested development at the trochophore stage. Rates of incorporation (through the first half day) were higher in the treated embryos than in the controls. Since no overlap occurred in the range of data between controls and experimentals, this is likely to be significant. This phenomenon was not investigated further, but is discussed later. Rates of incorporation in the treated embryos remained low through the first 2.0 days

Figure 4. Rates of l-leucine-4, 5-H^3 into protein in normal and actinomycin-D-treated embryos at 24°C. Each point represents one determination; the curves pass through the mean values at each stage of development. See text for a description of the development of normal and actinomycin-D-treated embryos.
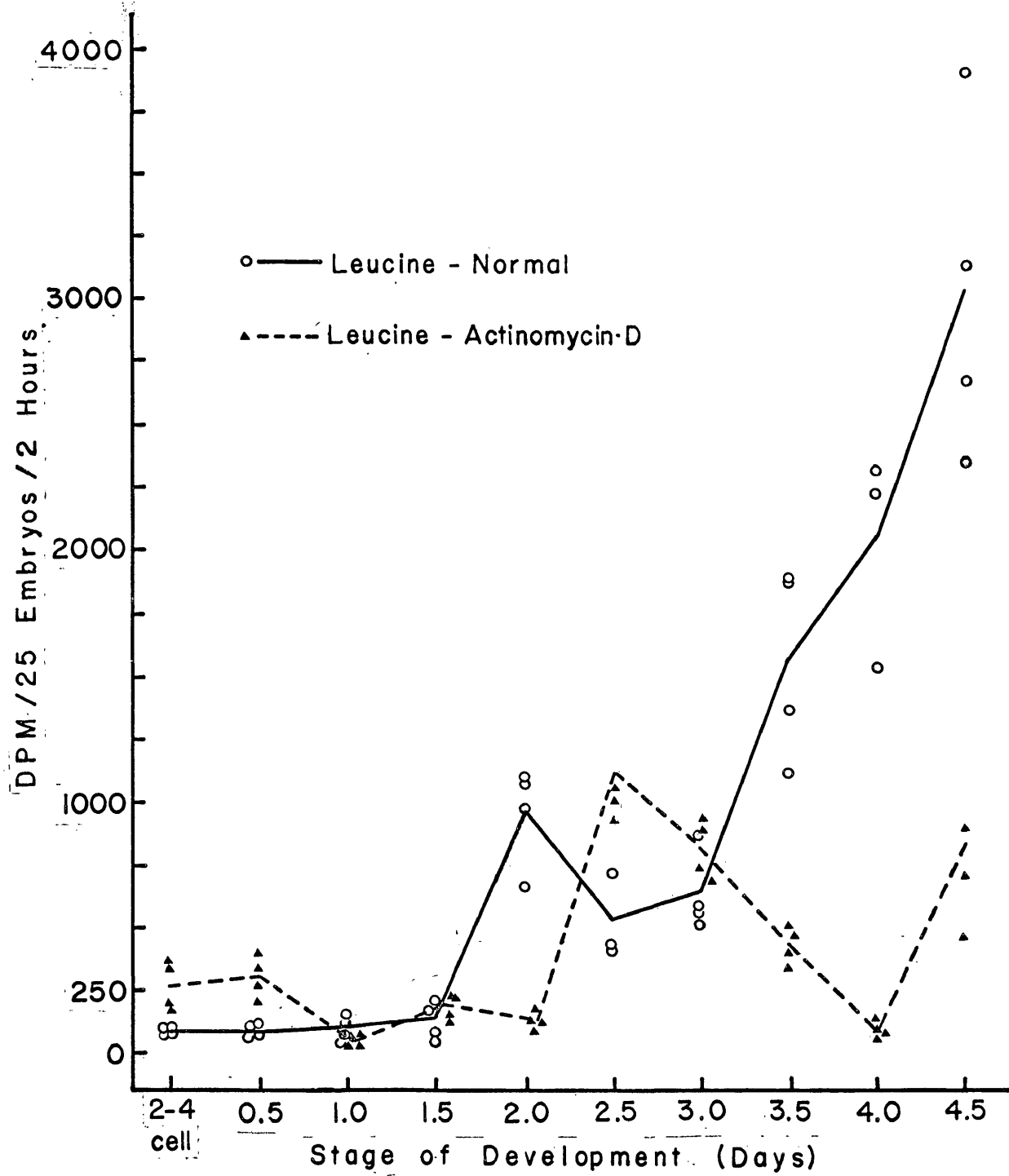
of development. Following this time, there was a significant acceleration of leucine incorporation. The high rate of incorporation achieved by 2.5 days decreased through the fourth day of development. While the "peak" of the incorporation rate was of the same order of magnitude as the rate in the 2.0-day normal trochophore, the period of acceleration and morphogenesis was delayed by half a day. In both normal and treated embryos the period of initial acceleration was associated with the development of the trochophore. As mentioned, actinomycin-D (100 ug/ml) arrested overall development at this stage. The inhibitor prevented the secondary increase of leucine incorporation rates normally associated with adult-tissue differentiation and growth.

Morphological Observations

Development of normal and actinomycin-D-treated embryos from the same egg mass was observed at half-day intervals. Whole mounts and sections of embryos were also prepared.

Although actinomycin-D stimulated rates of leucine incorporation in the early cleavage period, it did not affect the rates of the second and third cleavages. Gastrulation occurred after 1.0 days of development in both normal and treated embryos. Both types of embryos showed active ciliary motion after 1.5 days when the larval endoderm cells enlarged and the gastropore became smaller in the controls. In contrast, 1.5-day-old treated embryos had a large gastropore and had no large endodermal cells. By the second day, control embryos developed into definitive trochophores, complete with velum, stomodeum, albumen cells and protonephridia. A small shell gland, a well-developed gut and a small foot were present by the third day of development of

normal embryos.

Development in actinomycin-D-treated embryos past the gastrula stage differed from the controls. The treated embryos showed little development beyond the trochophore stage. A stomodeum and velum were visible after 2.5 days of development. Unlike the normal trochophore, the large albumen cells were reduced in size. Occasionally, albumen cells and tiny shell glands were observed in 3.0-day embryos. By 6 days of age, protonephridia of the treated embryos exhibited crystalline concretions detected in polarized light. These embryos usually died before 8 days of age. Their morphological development had been arrested at a trochophore-like stage.

Normal embryos differentiated and grew rapidly after 3.0 days of development. The shell grew over nearly half of the body; the foot became muscular and active; eyes began developing; and pigmentation occurred by 3.5 days. Further differentiation and growth were rapid. The adult kidney was present; the heart beat commenced; the eyes were pigmented; and the shell covered most of the body by the 4.5-day stage.

Thus, normal embryos developed in two phases. The first phase consisted of the development of the trochophore. This stage, which persisted for approximately one day, was followed by a dynamic phase of differentiation and growth of adult tissues and organs. In contrast, actinomycin-D-treated embryos only developed to a trochophore-like stage. The appearance of this form was retarded by approximately half a day when compared with normal development.

Summary of the Results

The results of the precursor-incorporation experiments (Figure 5) show that during the early development (up through 1.5 days) of normal embryos, rates of incorporation of L-leucine, uridine and thymidine were low and nearly constant. This period of development included cleavage and gastrulation. As the late gastrula developed into a trochophore (1.5-2.0 days), rates of incorporation accelerated. After this, while the rates of incorporation of thymidine continued to increase steadily, acceleration of leucine and uridine incorporation decreased. While the trochophore persisted (2.0-2.5 days), rates of leucine and uridine incorporation were high, but did not continue to accelerate. Then when the trochophore developed adult structures (after 2.5-day stage), rates of leucine and uridine incorporation again accelerated. Rapid acceleration of leucine and uridine incorporation occurred after 3.0 days of development. Rapid differentiation and growth of the embryo also occurred after 3.0 days.

Continuous treatment of L. palustris embryos with actinomycin-D (100 ug/ml) prevented the rapid increase of incorporation rates of uridine which was associated with either trochophore development or adult-tissue differentiation and growth. The acceleration of the rates of leucine incorporation occurred as the treated embryos developed into trochophore-like larvae which arrested at this morphological stage. However, an increase in the rate of leucine incorporation occurred in these arrested embryos (4.0-4.5 days of development) after a small but significant increase in the uridine incorporation rate (3.5-4.0 days of development).

Figure 5. Summary of the rates of precursor incorpora-
tion. Curves are plotted from mean values
of at least 3 determinations. Incorporation
of leucine, uridine and thymidine into pro-
tein, RNA and DNA, respectively, are pre-
sented for normal and actinomycin-D-treated
embryos at 24° C.
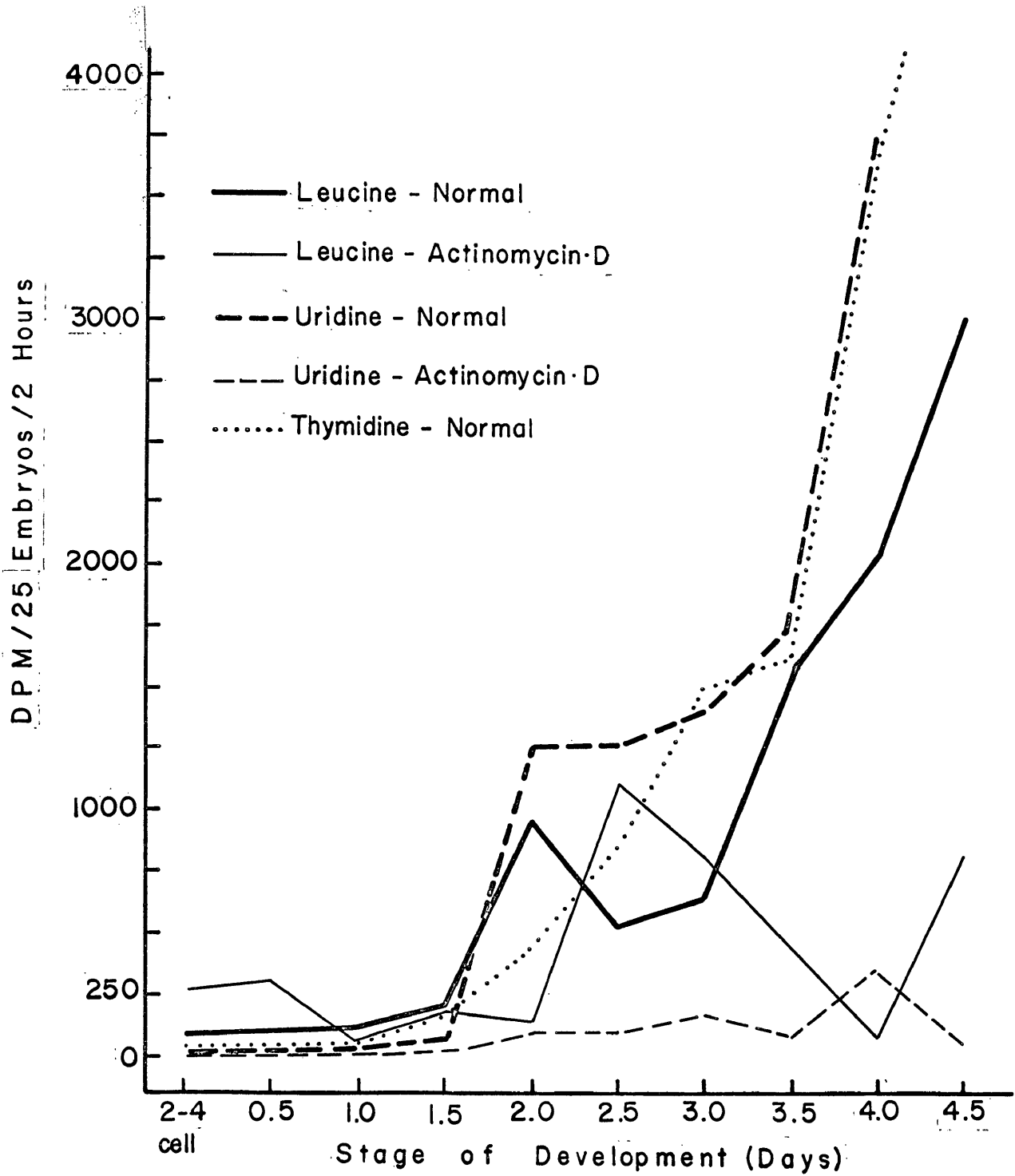
DISCUSSION

An interpretation of the results is based on three assumptions:
1) Rates of precursor incorporation reflect rates of RNA, DNA and acid-insoluble protein syntheses (see Appendix II for discussion of precursor incorporation); 2) Actinomycin-D suppresses DNA-dependent RNA synthesis; and 3) Protein synthesis is ultimately controlled by DNA and mediated by RNA. In view of these assumptions, the results suggest that surges of protein and RNA syntheses correlate with both trochophore development and the differentiation/growth of adult tissues. Since actinomycin-D suppresses DNA-dependent RNA synthesis, the RNA which mediates protein synthesis for trochophore development must be synthesized before first cleavage (time of the onset of treatment). This suggests that protein synthesis, associated with trochophore development, is under the control of "prescribed" RNA. Actinomycin-D arrests development at a trochophore-like stage and suppresses RNA synthesis. This suggests that further differentiation and growth, from trochophore to adult, requires DNA-dependent RNA synthesis.

According to this interpretation, the first phase of acceleration of the rate of RNA synthesis is not entirely causally related to trochophore development, since a trochophore-like larva develops in the absence of this acceleration of RNA synthesis. Because the trochophore is not completely normal, some DNA-dependent RNA synthesis may be required, assuming no secondary effects of the inhibitor (see Appendix III for discussion of actinomycin-D effects). The bulk of normal RNA

synthesis at this stage must be associated with the differentiation/ growth phenomena which occur a day later. This suggests that a major portion of the RNA synthesis during trochophore development is utilized during later protein synthesis.

Patterns of protein and RNA syntheses suggest that development of L. palustris is bi-phasic. Raven (1946) made the same conclusion from his cytochemical studies of Limnaea. Recently, Bedford (1966) drew similar conclusions from her cytochemical studies on the prosobranch gastropod Bembicium. Both workers contend that in each germ layer there are two distinct classes of cells which arise during development: 1) larval cells which cease mitosis and synthesis and serve as locomotor and nutritive sites; 2) cells which give rise to adult structures, rich in DNA and RNA and active in protein synthesis. Raven (1963) claimed that primary differentiation, giving rise to larval tissues, depends upon the segregation of morphogenetic factors during early development and that gastrulation stimulates the production of new causal factors for adult differentiation. The present investigation suggests that in L. palustris these morphogenetic factors are, in part, prescribed RNA's and that gastrulation induces genome-dependent RNA synthesis. This synthesis effects protein synthesis required for secondary differentiation and growth.

At the present time, there is only circumstantial evidence for the synthesis and presence of prescribed RNA's in the uncleaved molluscan egg. Cytochemical studies indicate that during early phases of oogenesis RNA is synthesized in the nucleolus (Bretschneider, 1948; Cowden, 1958, 1961; Bedford, 1966) and that the nucleolar contents are released into the cytoplasm (Bretschneider and Raven, 1951; Raven, 1958).

During later stages of oogenesis the RNA activity of the nucleolus decreases while that of the cytoplasm increases. Cowden (1966), working with several different molluscs, observed that a change in cytoplasmic RNA-protein complexes prior to yolk synthesis occurred and that histone-like proteins were associated with ribosomes. He suggests that histone-like proteins may mask RNA messages and may control the release of masked RNA messages under the influence of proteases during development. The occurrence of masked m-RNA's (polysomes) has been reviewed for several developmental systems (Berman, 1967; Grant, 1965; Kaulenas and Fairbairn, 1966; Spirin, 1966). These reviewers have proposed that information for early development is stored in the unfertilized egg cytoplasm in masked m-RNA-ribosome complexes and that these are released at different stages following fertilization.

Cytochemical studies support the idea that prescribed RNA's may be present and mosaically segregated during early development. In the fertilized uncleaved egg of Limnaea (Raven, 1948), of the pulmonate Succinea putris (Jura, 1959) and of Bembicium (Bedford, 1966), high concentrations of RNA are associated with the outermost cortical plasm. In addition, the so-called beta and gamma yolk granules of Limnaea contain RNA. The beta granules are rich in ribonuclease-sensitive RNA (Raven, 1945). Collier (1960) points out that this RNA may be due to a shell of granules adhering to the surface of the yolk granules as reported by Berthier for the egg of Planorbis. The gamma granules contain less stainable ribonuclease-insensitive RNA (Jura, 1959; Raven, 1945). Since ribonuclease insensitivity has been used as a criterion for distinguishing masked RNA (Humphreys, Penman and Bell, 1965), it would be interesting to know if the gamma granules contain informational RNA.

During cleavage the micromeres stain intensely for RNA (Bedford, 1966; Raven, 1946). In Limnaea darkly staining RNA granules fuse to form large bodies in each of the four macromeres of the 24-cell embryo. These RNA bodies pass into the fourth quartette of micromeres (Raven, 1946; Minganti, 1950). This segregation of RNA bodies may play an important role in the differentiation of the trochophore since the fourth quartette of micromeres gives rise to the primary mesomere and larval structures such as the protonephridia and larval digestive gland of the trochophore (Verdonk, 1965).

While there is some cytochemical evidence of RNA synthesis during the cleavage of pulmonates, this synthesis is minor compared to the total amounts of RNA detectable in the cytoplasm (Jura, 1959; Raven, 1946). Indeed, Raven (1948) claimed that the total concentration of RNA decreases during early development. In Chiton (Cowden, 1961), Ilyanassa (Collier, 1961a) and L. palustris (present study) little or no RNA synthesis is detected before gastrulation.

The effects of metabolic inhibitors on molluscan development support the idea that major protein and RNA syntheses only occur after gastrulation. Barbituric acid, a suppressor of RNA synthesis, arrested the development of the pulmonate Planorbis at the trochophore stage, but did not affect earlier development (Sherbet and Lakshmi, 1964a). Actinomycin-D, up to a concentration of 200 ug/ml did not affect cleavage or gastrulation of L. stagnalis (Geilenkerken, 1967). These inhibitor studies also suggest that major RNA synthesis is not required for early development in these molluscs. Chloramphenicol (an inhibitor of ribosome-directed protein synthesis) arrested the development of Planorbis at the gastrula, suggesting that major protein synthesis

also is not required for early development (Sherbet and Lakshmi, 1964b). Present results support this idea. While uridine and leucine incorporation into L. palustris embryos is constant and low during early development, incorporation rates only accelerate after gastrulation (Figure 5). In addition, actinomycin-D did not affect cleavage or gastrulation, nor did it prevent protein synthesis during these stages. These results suggest that the minimal protein synthesis during early development is under the control of prescribed RNA's.

This lack of major protein and RNA syntheses during cleavage or early gastrulation in molluscs contrasts with the developmental patterns of echinoderms and amphibia (Collier, 1961a) and may be due to the spatial distribution of cytoplasmic "informational" material associated with their determinate type of cleavage (Markert and Cowden, 1965; Verhey and Moyer, 1965).

The results of the present experiments indicate that in L. palustris the bulk of RNA synthesis during gastrulation is not associated with primary differentiation of larval organs and that major RNA synthesis begins during the late gastrula and occurs before the appearance of adult organ primordia. Gastrulation in molluscs marks a time of RNA synthetic activity in the ectoderm and endoderm as detected cytochemically (Bedford, 1966; Jura, 1959). DNA becomes Feulgen positive during interphase in Limnaea; DNA granules become closely associated with the nucleoli (Raven, 1946). RNA synthesis begins during Ilyanassa gastrulation, one day before a detectable net increase in RNA and the appearance of organ primordia (Collier, 1961a). Contrarily, Cowden (1961) found no cytochemical evidence for RNA synthesis before the trochophore stage of Chiton. He concluded that newly synthesized

RNA plays no role in primary differentiation of molluscs and that the RNA functioning during primary differentiation must be produced during oogenesis.

One may wonder why RNA synthesis may be associated with molluscan gastrulation, but is not functional at that time. In reviewing his experiments on heat shock and lithium treatment, Raven (1963) stressed the importance of gastrulation during the development of _Limnaea_. Exogastrulae (gastrulae with everted archentera) possess larval organs, but never adult structures. He concluded that new causal factors during gastrulation are necessary for the differentiation of adult tissues (Raven, 1963). These causal factors may include 1) the stimulation of RNA synthesis necessary for secondary differentiation and growth and 2) RNA synthesis associated with the induction of the shell gland (Raven, 1952) and possibly other organs. The results of the present studies are in accordance with the idea of a stimulation of RNA synthesis after gastrulation. After late gastrulation, _L. palustris_ embryos incorporate uridine at high rates (Figure 5). However, this RNA is not causally related to the development of the trochophore, which will develop even when the synthesis of RNA is blocked with actinomycin-D. It would seem that RNA synthesized during the development of the trochophore becomes functional at a later time in development. Many examples of ontogenetic prescribing of RNA's have been reviewed for other species (Berman, 1967).

The results of this investigation indicate that newly synthesized RNA is necessary for the differentiation and growth of adult organs, but not for larval structures. Interestingly, the onset of protein and RNA syntheses in _L. palustris_ trochophores which accompanies

differentiation and growth of the adult organ primordia coincides with the appearance of new enzymatic bands in zymogram patterns of embryo extracts (Morris and Morrill, 1964). An increase in dipeptidase activity and a measureable increase in total embryo protein and in the utilization of capsule albumen also coincide with this stage of development (Morrill, 1964a). Therefore, the period of adult differentiation and growth is associated with high rates of RNA synthesis and both quantitative and qualitative protein synthesis. Because actinomycin-D arrested development at the trochophore stage, it would be interesting to know how much of the arrested development is caused by 1) inhibited synthesis of enzymic proteins involved in the utilization of the albumen reserves and 2) inhibited synthesis of proteins directly involved in the differentiation and growth of adult organ primordia.

APPENDIX I

Control Experiments for Technique

At the onset of this project, a standard homogenization-extraction technique was employed to detect incorporated precursor. The small number of embryos available for each determination (25-50) proscribed rigorous fractionation procedures. The encapsulated embryos were washed briefly and then homogenized. Cold perchloric acid was used to precipitate a residue. The supernatant was drawn off after centrifugation. The residue was resuspended in fresh acid. These steps were repeated up to 15 times. The acid-insoluble fraction was subsequently dissolved and counted. This extraction procedure, designed to remove the unincorporated precursor, failed to displace all of it. The control experiment consisted of incubating dead encapsulated embryos in labelled precursor. The "trapped" radioactivity of the various control conditions is presented in Figure 6. Since treatment with the detergent sodium desoxycholate failed to remove all of the trapped material, the homogenization technique was abandoned. An investigation of the nature of the trapping was not pursued.

A "precursor-displacement" technique was tested next. At first, encapsulated embryos were "washed" on filters with cold acid and unlabelled precursor. This method was also inadequate. Consequently, 1- and 3-day embryos were decapsulated manually (removed from capsule and albumen) and subjected to precursor displacement on the filters. To determine non-specific incorporation (trapping) of the radioisotope,

some embryos (1-day stage) were chilled to 4°C, while others were killed with ethanol before they were exposed to the radioactive precursor. The alcohol-killed embryos were incubated for 2 hours at 24°C. The chilled embryos were incubated for 2 hours at 4°C. The presence of radioactivity in the washed embryos was considered to be non-specifically incorporated precursor. The displacement technique removed nearly all of the unincorporated precursor under the control conditions (Figure 6). Comparison of techniques indicates that the albumen is the site of precursor trapping.

In order to determine effects of concentration on the rates of incorporation, 1-day gastrulae were incubated in various concentrations of C^{14}-valine (specific activity: 0.2 curies/millimole) for various lengths of time. The incorporated radioactivity is presented in Figure 7 as a function of incubation time and concentration. The slope of a line at a single concentration represents the rate of incorporation. Apparently, after an early acceleration, rates of incorporation become linear. Initial exposure to the precursor has a brief stimulatory effect, while higher concentrations have a prolonged stimulatory effect on rates of incorporation. These results indicate that the quantitative observations are a function of the precursor concentration.

Figure 6. Amount of radioactivity not removed from
control embryos by three extraction tech-
niques. Embryos were killed with 70% eth-
anol before incubation with precursor.
Radioactivity residual after extraction is
"trapped." L, l-leucine-H^3; U, uridine-H^3;
T, thymidine-H^3.

COUNTS PER MINUTE / 25 EMBRYOS

350
340
330
320
190
180
170
160
150
40
AVERAGE 30 BACKGROUND
20
10

HOMOGENIZED 15·PCA EXTRACTIONS

HOMOGENIZED 10·PCA & DOC EXTRACTIONS

DECAPSULATED, WASHED ON MILLIPORE 10 X WITH PCA PLUS UNLABELLED PRECURSOR
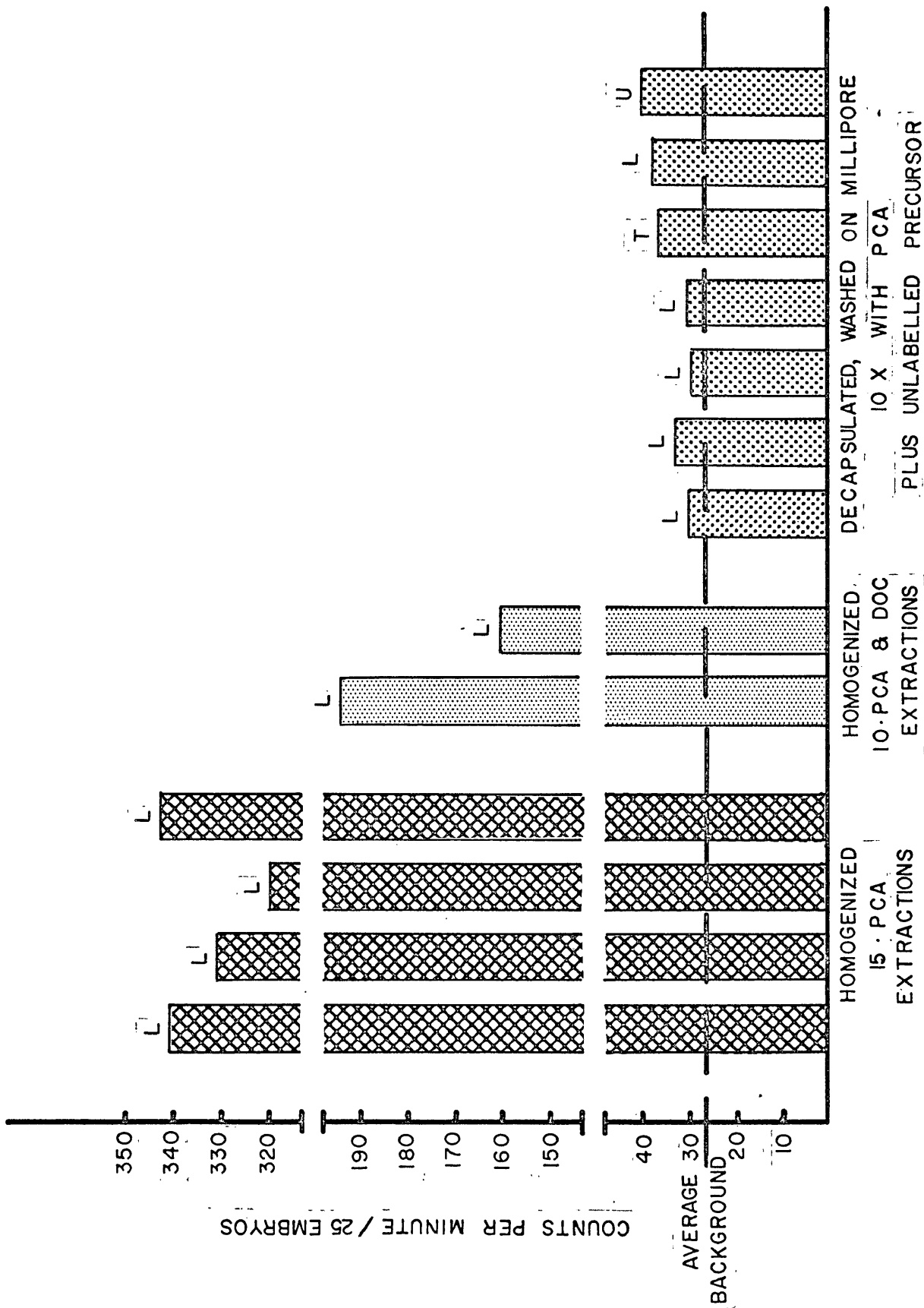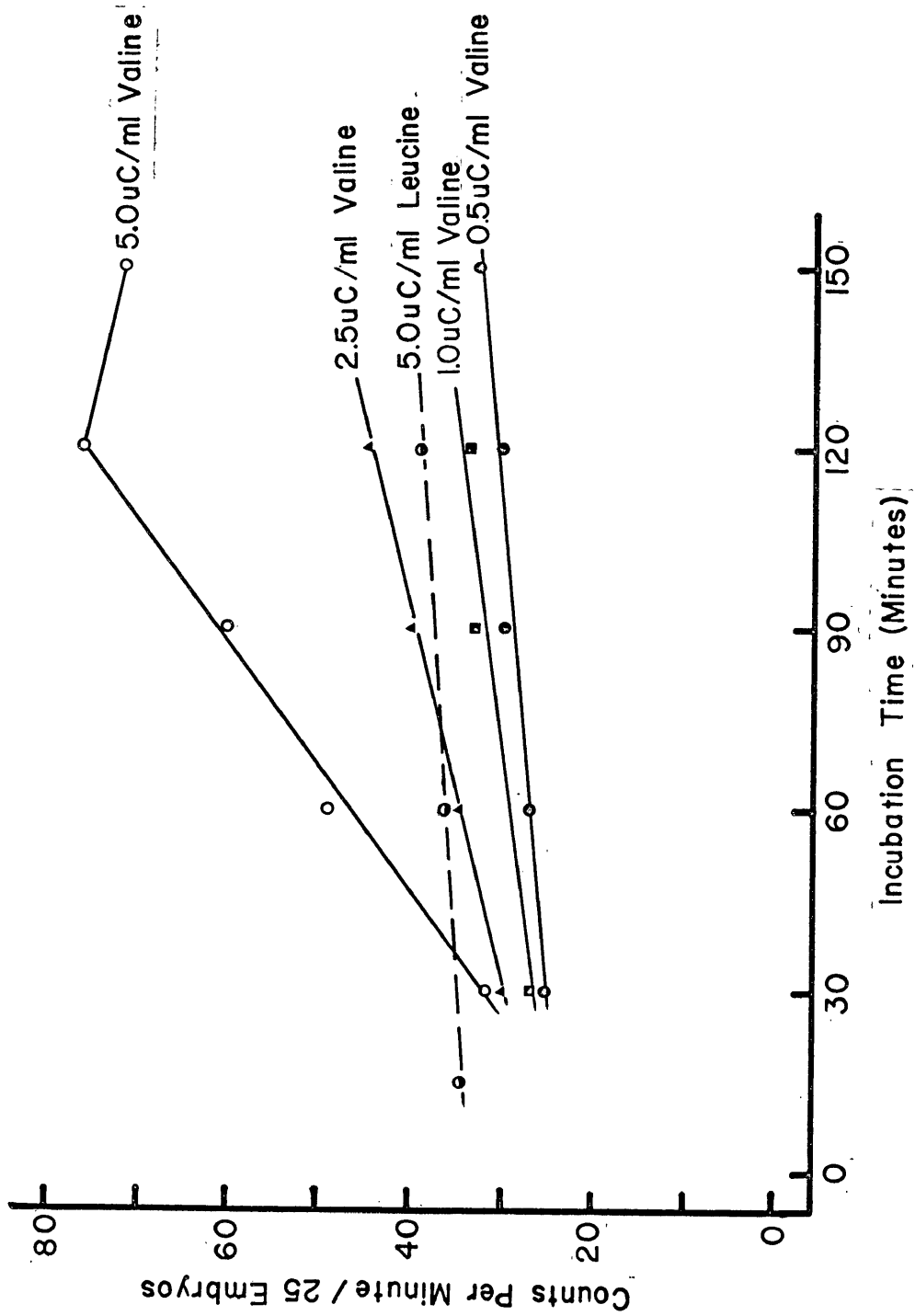
Figure 7. Effect of precursor concentration on rates of C^{14}-valine incorporation. Gastrulae were incubated for periods of 30, 60, 90, 120 and 150 minutes. Slopes of the lines represent the rates of amino acid incorporation.

Counts Per Minute / 25 Embryos

Incubation Time (Minutes)

5.0uC/ml Valine

2.5uC/ml Valine

5.0uC/ml Leucine

1.0uC/ml Valine

0.5uC/ml Valine

APPENDIX II

The Use of Radioactive Precursors

The success of an experiment depends upon the validity and precision of the experimental techniques. Since the biochemical procedures used in these experiments have been simplified, an evaluation of them is pertinent. The first assumption was that the tritium of the precursors remained attached to the precursor, which, in turn, was incorporated into acid-insoluble protein, RNA or DNA. L-leucine-4, 5-H^3 was used to denote acid-insoluble protein synthesis. This amino acid has been used extensively for this purpose (Hultin and Bergstrand, 1960; Gross and Cousineau, 1964; Spirin and Nemer, 1965). It is a relatively unreactive compound, synthesized mainly from pyruvate and giving rise to no other amino acids. It is a major component of elastin, myoglobin, myosin, globular proteins and certain histones (Needham, 1965). Since the biochemical activity of leucine is restricted mainly to protein synthesis, the incorporated tritium essentially reflects protein synthesis. Because the proportion of leucine varies according to the nature of the protein synthesized, leucine incorporation may not exactly reflect overall protein synthesis. In addition, the relative incorporation of various amino acids differs during development. Their individual rates of incorporation may not accurately reflect protein synthesis (Silver and Comb, 1966; Tyler, 1966).

Thymidine-methyl-H^3 was used to detect DNA synthesis. Since the tritium label is on the methyl group, conversion of thymidine to uridine

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or cytidine would require demethylation, hence, loss of the label.
The label could only be incorporated into RNA if thymidine were con-
verted to 5-methyl cytidine. This ribonucleoside is only found in
trace amounts in soluble RNA (Needham, 1965). Since nearly all of the
unincorporated precursor can be washed away, it is reasonable to be-
lieve that the radioactivity reflects newly synthesized, acid-insol-
uble DNA.

Uridine-5-H^3 incorporation was used to measure RNA synthesis.
It has often been used for this purpose (e.g. Gross, Malkin and Moyer,
1964; Spirin and Nemer, 1965). Uridine may be metabolically converted
to cytosine or thymidine (Needham, 1965). If this occurred, it might
be incorporated into DNA. Indeed, Collier (1963) has shown that the
utilization of uridine for DNA synthesis represents a major metabolic
pathway in the *Ilyanassa* embryo.

The conversion of uridine into thymidine requires the addition of
a methyl group at the 5-position. Such a transmethylation would re-
quire the loss of the tritium label from that position in uridine. The
label would probably be transferred to an acid-soluble hydrogen-accep-
tor, such as nicotinamide adenine dinucleotide (NAD). Therefore, the
use of uridine-5-H^3 obviates the possibility of the incorporation of
the label into DNA via thymidine synthesis.

Uridine may be metabolically converted to cytidylic acid and cyto-
sine (Kosower, 1962). In this case, the tritium label could be re-
tained and incorporated into DNA, via cytosine. Collier's data sug-
gested that this conversion in *Ilyanassa* was of minor importance (less
than 20% of all converted uridine) as compared with conversion to thy-
midine (Collier, personal communication, 1966). In L-tissue culture

cells, the conversion of uridine to cytosine was on the order of 25%
over a 2-hour period (Rake and Graham, 1962). Thus, we may assume
that uridine-5-H^3 incorporation in the Limnaea embryo represents RNA
synthesis.

It is difficult to evaluate quantitatively the observed rates of
precursor incorporation because the values of the rates of incorpora-
tion are, in part, a result of the experimental parameters. Although
all incubations were carried out at 24° C, a common experimental temp-
erature for L. palustris (e.g. Morrill, 1964a), these embryos will de-
velop over a range of temperatures and absolute rates of development;
hence, syntheses would vary accordingly. In addition, the concentra-
tion of precursor also affects the rates of incorporation (Figure 7).
The stimulatory effect of high precursor concentrations has also been
noted in Neurospora (Zalokar, 1961) and in sea urchin embryos (Markman,
1961; Mitchison and Cummins, 1966). Furthermore, the type of precur-
sor may yield different incorporation patterns (Tyler, 1966). These
and other parameters make the results relative rather than absolute.
I have, therefore, chosen to regard the data as quantitative measures
which express qualitative relationships.

APPENDIX III

RNA Synthesis and the Action of Actinomycin-D

Kadowaki and Maruo (1966) showed that the degree of suppression of RNA synthesis depends on the concentration of inhibitor. The results of the present investigation indicate that RNA synthesis is not totally suppressed by continuous treatment (Figure 3). The concentration of 100 ug/ml was selected because it arrested L. palustris embryos at a trochophore-like stage. It was assumed that syntheses previous to this stage were actinomycin-D insensitive.

Actinomycin-D is known to suppress DNA-directed RNA synthesis (Goldberg and Rabinowitz, 1962). It does, however, have other effects. For example, high concentrations of this inhibitor suppress DNA synthesis (Hurwitz, et al., 1962; Rothstein, Fortin and Sonneborn, 1966). Chromosomal breakage and growth suppression may also result from treatment (Fraccaro, et al., 1966). It may cause a decrease in cell polysome content which is independent of the inhibition of RNA synthesis (Revel, et al., 1964). General toxic effects unrelated to RNA synthesis can contribute to the suppression of protein synthesis (Soeiro and Amos, 1966). Likewise, other types of suppression of protein synthesis may be unrelated to the effect on template RNA synthesis. In this case, suppression can be eliminated with the administration of glucose (Honig and Rabinowitz, 1965). Lowry and Williams (1965) have shown that this secondary metabolic effect is not the result of the suppression of purine nucleotide (ATP or GTP) formation.

While side effects may occur, this compound exhibits a variety of actions directly on RNA synthesis. In addition to suppressing m-RNA synthesis (Goldberg and Rabinowitz, 1962), it will also inhibit the synthesis of soluble RNA (Merits, 1965). Several workers have found that ribosomal RNA synthesis is more sensitive than m-RNA and transfer RNA syntheses (Perry, 1963; Tamaoki and Mueller, 1966; Tyndall, et al., 1965). In view of these findings, one cannot use actinomycin-D to suppress any single class of RNA selectively; and experimental data must be interpreted with each case.

Actinomycin-D stimulation of the rate of leucine incorporation during cleavage was observed in the present experiments (Figure 4). This effect has been described in lens protein synthesis (Papaconstantinou, et al., 1966), in alkaline phosphatase synthesis (Nitowsky, et al., 1964; Rosen, et al., 1964), during bacterial synthesis of certain enzymes (Coleman and Elliot, 1964; Pollock, 1963), and during early protein synthesis of sea urchin development (Gross, Malkin and Moyer, 1964). Two hypotheses have been proposed to explain this phenomenon: 1) Stimulation may result from an increased availability of ATP for protein synthesis. This ATP would otherwise be used for RNA synthesis (Pollock, 1963). 2) Stimulation may occur because of the suppression of the synthesis of repressor proteins (Garren, et al., 1964).

Because of the limited amount of embryonic material, I could not experimentally segregate the classes of RNA synthesized. Various mechanisms of the translational control of protein synthesis and development have been reviewed recently (Berman, 1967; Singer and Leder, 1966). The reviews emphasize that a rigorous analysis is essential to distinguish the nature of the control of protein synthesis in question.

Actinomycin-D cannot be satisfactorily used to discern these classes. Without the necessary data for such an analysis, I cannot definitely conclude which types of RNA are specifically synthesized or prescribed during _Limnaea palustris_ development.

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