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CARTERI F. NAGARIENSIS.

CITY UNIVERSITY OF NEW YORK, PH.D., 1979

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RNA SYNTHESIS IN THE DEVELOPMENT
OF VOLVOX CARTERI F. NAGARIENSIS

By

HOWARD S. CAPLEN

A dissertation submitted to the Graduate
Faculty in Biology in partial fulfillment of
the requirements for the degree of Doctor of
Philosophy, The City University of New York

1979

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

Basic aspects of RNA metabolism in Volvox carteri f. naqariensis were investigated by following patterns of incorporation of radioactive precursor into various classes of RNA molecules during different developmental stages. An ultrastructural study of development is presented to correlate morphological with biochemical results.

Several radioactive precursors were examined for possible use in RNA synthesis studies. Based on the criteria of efficiency of utilization and specificity of labeling, 2-³H-adenine was demonstrated to be the most suitable of these. Adenine appears to be taken up by Volvox from the standard growth medium, with considerable efficiency. The possibility that uptake of this molecule involves a carrier-mediated transport system is discussed.

Patterns of RNA synthesis at various stages of development were compared by an electrophoretic analysis of pulse-labeled total cellular RNA. The results suggest that the proportion of ribosomal RNA synthesized is greatest during part of the period of gonidial maturation and in post-inversion embryos. A preliminary measurement of the labeled precursor pool specific activity in gonidia and cleaving embryos confirms that these data reflect true differences

in the rate of RNA synthesis.

Polyadenylated RNA from Volvox carteri has been isolated and partially characterized. Electrophoretic profiles of total cellular poly(A)-associated RNA of Volvox spheroids indicate a heterodisperse distribution of size classes with the range extending from an apparent sedimentation value of approximately 10S to greater than 38S. The radioactive labeling kinetics of this material are typical for rapidly-turning-over RNA. The profiles of poly(A) RNA from different cell types show marked differences in average migration rate. Terminally-differentiated somatic cells contain a greater proportion of material of higher molecular weight than either gonidia (germ cells) or cleaving embryos. The poly(A) segments associated with cellular RNA, obtained by selective RNase digestion are heterogeneous in size as determined by gel electrophoresis with the largest tracts estimated to be 75 - 80 nucleotides long. Gonidia and embryos display the greatest degree of size heterogeneity while somatic cells show predominantly, the largest classes of poly(A) tract. It is apparent that gross changes in poly(A) RNA metabolism accompany development and cellular differentiation in Volvox.

The morphology of gonidia and embryos was compared in a transmission electron microscopy study. Changes in the

appearance and distribution of subcellular structures can be correlated with known and presumed changes in metabolism during the course of development.

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TABLE OF CONTENTS

LIST OF TABLES.....	x
LIST OF FIGURES.....	xi
INTRODUCTION.....	1
Opening Statement.....	1
RNA in Development.....	3
<u>Volvox</u> : A Model System for Studying Development and Differentiation.....	46
MATERIALS AND EQUIPMENT.....	52
METHODS.....	54
Growth and Maintenance.....	54
Synchronous Growth.....	55
Culture Conditions for Radioisotopic Labelling.....	56
Radioisotopic Labeling.....	56
Preparation of RNA Extracts.....	58
Final Purification of RNA.....	59
Isolation of Polyadenylated RNA.....	60
Isolation of Poly(A) Segments.....	62
Electrophoretic Analysis of RNA and Poly(A) Segments.....	62
RNA Rapid Microextraction Procedure for Gonidia and Embryos.....	64
Measurement of ATP Pools and Pool Specific Activity in Gonidia and Embryos.....	65
Electron Microscopy.....	68
Glassware and Solutions.....	69
RESULTS.....	70
Growth of Synchronous Cultures of <u>Volvox</u> <u>carteri</u> f. <u>nagariensis</u> Strain <u>HK-10</u>	70
Radioactive Precursor Labeling Kinetics.....	73
Isolation and Electrophoresis of RNA.....	90
RNA Synthesis in Development.....	93
Polyadenylated RNA in <u>Volvox carteri</u>	105
Ultrastructure of Development.....	125
DISCUSSION.....	146
Radioactive Precursor Labeling Kinetics.....	147
Isolation and Electrophoresis of <u>Volvox</u> RNA.....	149
RNA in Development.....	152

Polyadenylated RNA.....	160
ATP Pool Analysis.....	164
Ultrastructure of Development.....	166
Future Research.....	171
LITERATURE CITED.....	174

LIST OF TABLES

1. ATP Pool Size of Gonidia and Embryos.....	108
2. Specific Activity of ATP Pools in Gonidia and Embryos.....	109
3. Percentage of Total CPM as rRNA and tRNA CPM from Various Developmental Stages (See Figures 10 and 11).....	110

LIST OF FIGURES

1. Life Cycle of <i>Volvox</i>	72
2. Water Bath - Growth Chamber.....	75
3. Uptake and Incorporation of 2- ³ H-adenine and [5,6]- ³ H-uracil into <i>Volvox</i> and RNA.....	81
4. Uptake and Incorporation of 2- ³ H-adenine and ³³ P0 ₄ into <i>Volvox</i> Growing in SVM.....	83
5. Uptake and Incorporation of 2- ³ H-adenine and ³³ P0 ₄ into <i>Volvox</i> Growing in Phosphate-free SVM.....	85
6. Uptake and Incorporation of 2- ³ H-adenine.....	87
7. Kinetics of Uptake and Incorporation of 2- ³ H- adenine into <i>Volvox</i> Using Different Con- centrations of 2- ³ H-adenine.....	89
8. Electrophoretic Pattern of Total RNA.....	92
9. Light Micrograph of Purified Gonidia.....	95
10. Electrophoretic Patterns of Radioactively Labeled RNA Extracted During Different Stages of the Asexual Life Cycle of <i>Volvox carteri</i> f. <i>nagariensis</i> Strain HK-10.....	100
11. Percentage of Total RNA Radioactivity Incorporated in Ribosomal RNA and Transfer RNA.....	102
12. Standard Curve for the Luciferin-Luciferase Assay.....	107
13. Percent Poly(A)-associated RNA in Total RNA as a Function of Length of Labeling Period.....	113
14. Electrophoretic Patterns of Poly(A) RNA Isolated by Chromatography on Unsubsti- tuted Cellulose and Oligo(dT)-cellulose.....	117
15. Electrophoretic Patterns of Poly(A) RNA from Gonidia, Embryos, and Somatic Cells.....	119
16. Electrophoretic Pattern of Poly(A) Tracts from Total <i>Volvox</i> RNA.....	122
17. Electrophoretic Patterns of Poly(A) Tracts Isolated from Gonidia, Embryos, and Somatic Cells.....	124
18. Developing Gonidium.....	128
19. Nucleus of Developing Gonidium.....	131
20. Accessory Nucleolus.....	133
21. Cytoplasm of "Condensed" Gonidium.....	136
22. Cleaving Embryo.....	139
23. Detail of Embryonic Cell.....	141
24. Post-inversion Embryo.....	143
25. Mature Somatic Cell.....	145

INTRODUCTION

Opening Statement

It has been the purpose of embryologists and developmental biologists to understand the mechanisms that direct and drive the development of a multicellular organism from a single cell. Even in the simplest of cases, the least complex multicellular organisms, the degree of coordination of all of the events involved in the orderly appearance and arrangement of the parts of a new individual is much appreciated but the basis of regulation is little understood. Coordination is temporal and spatial. Each individual in the course of its development passes through a rigidly established sequence of intermediate stages involving cleavage, morphogenesis and cellular differentiation; the adult parts are not to be found in miniature in the egg ("preformed") waiting for the stimulus to grow. The spatial coordination involves the development of separate cell lines whose capacities for giving rise to distinct cell and tissue types may be traced back to the endowment of the various regions of the germ cell cytoplasm with different developmental potentialities. The best known instance of this is that which involves the setting aside of the germ plasma in eggs. If either the temporal or spatial patterns are disturbed the result is invariably abnormal development.

The types of programming just mentioned exist in the simplest of multicellular individuals. It is reasonable to suppose that such organisms would provide the most suitable material for studying elementary features of development. We, in this laboratory, as well as workers in several other laboratories have utilized Volvox in a systematic approach to the study of certain developmental phenomena. Since developmental processes are ultimately under the control of the genome, the study of both the genetics and biochemistry of development have been integrated. Volvox is well suited to such an approach. Its short generation time and haploid genome make it amenable to genetic analysis; the ability to grow it in large synchronous populations makes biochemical studies practical.

The regulation of RNA metabolism is of crucial importance to development. The orderly synthesis, processing, transport, distribution, and degradation of different RNAs is responsible for normal cellular differentiation and morphogenesis. The study reported here was initiated to analyze the role of RNA metabolism in Volvox development. Since Volvox is a comparatively new organism to the biochemistry laboratory, there is still much work of a descriptive nature to be done. Patterns of RNA synthesis during the life cycle were studied and the nature of the polyadenylated RNAs of

Volvox was examined. It is hoped that this work will contribute to an understanding of the underlying molecular control mechanisms involved in Volvox development.

RNA in Development

Historical Background - Classical Embryology

The experimental approach to the study of development has changed in the last hundred years in step with technological advances in the laboratory. Biochemical and special cytological techniques have largely replaced direct observation with the naked eye and microscope. But the most fundamental questions concerning the development of multicellular organisms that were first asked long ago by embryologists are in essence the same ones asked now by developmental biologists.

With the emergence of the cell doctrine in the last century and the demonstration of nuclear equivalence and cytoplasmic localization early in this century the broad conceptual basis of development, which has given direction to all later experimental designs, was firmly established. It is largely the details which remain to be elucidated.

Earlier theories of development sought to explain embryogenesis largely by postponing the question entirely. The theory of preformation maintained that within every egg there resided an invisible miniature of a fully developed organism.

During development this embryo grew and became visible.

It was Charles Bonnet (1745) who demonstrated parthenogenesis in the greenfly and thereby showed that it was the egg alone, and not the sperm which fertilized it, that contained the preformed embryo. Within the developing embryo (if it were female) there were smaller eggs containing even smaller embryos and so on. Bonnet maintained, as did other 18th century preformationists, that the embryo of every organism, past, present, and future, had existed from the time of Creation and awaited only activation by fertilization.

Even as preformationism was gaining in popularity in the middle of the 18th century, the doctrine that would largely replace it had its genesis in the microscopic observations, made by C.F. Wolff (1759), regarding the formation of the chick embryo. The gradual emergence of the complex organ rudiments from simple membranes by a series of foldings, swellings, invaginations, and evaginations convinced Wolff that the embryo did not exist preformed in the egg. The egg, instead, contained the raw materials, uniformly distributed, for the formation of the structures of the mature organism. His ideas were not well received at their conception. The doctrine of epigenesis achieved wide acceptance only after embryologists near the turn of the 20th century were able to reinforce Wolff's direct observations with experimental evidence.

In 1892, Driesch demonstrated that when the first two blastomeres of a first cleavage sea urchin embryo were separated from each other they both were able to form complete embryos. He later showed that any of the four blastomeres of a second cleavage embryo had similar capacity. Driesch thus spoke of prospective potency - the ability of an isolated blastomere to form a whole embryo - exceeding prospective significance - the part of the embryo normally derived from that blastomere in situ. His work was repeated in amphibian embryos by Spemann (1901) and Schmidt (1933). This appeared to be solid confirmation of the concept of epigenesis and a refutation of Weismann's hypothesis (1889) that specific substances are divided into two halves of "unequal quality" at each nuclear division.

However, in contrast with the results of experiments done with sea urchins and amphibians were numerous observations made mainly, although not exclusively, on the embryos of lower invertebrates. They showed that even at the two-celled stage in these embryos the blastomeres did not have the same potential; isolated, they could only give rise to partial embryos. For example, Wilson (1892, 1904) in the annelid Nereis and the scaphopod mollusc Dentalium, Crampton (1896) in the gastropod mollusc Ilyanassa, and Conklin (1905) in the urochordate Styela found that from the first cleavage

on, the individual blastomeres of the cleaving embryo were restricted in their morphogenetic potential. Isolated blastomeres were capable only of giving rise to the parts of the adult that they normally generated in the intact embryo. The elegant isolated blastomere and cell lineage studies of these and other embryologists (reviewed by Wilson, 1925) led to the conclusion that there was a localization or asymmetric distribution of specific developmental determinants within the egg cytoplasm. These observations were the basis of a neopreformationist school of thought which arose in the latter part of the 19th century.

It appeared at the time that there were two fundamentally different types of animal development - the regulative type exemplified by sea urchins and amphibians and the determinant type of mainly lower invertebrates (Styela was a notable exception). The subsequent work of experimental and biochemical embryologists has demonstrated that there is really no qualitative difference between the two (reviewed by Davidson, 1976). The blastomeres of regulative embryos eventually show restricted morphogenetic potential, but it usually only appears in the blastomeres of the third cleavage division stage or later.

The conflict between epigeneticism and neopreformationism was resolved (at least philosophically) by the very

workers whose experimental results might have intensified the polarization. C.O. Whitman, whose seminal descriptive work (Whitman, 1878) had served the neopreformationist cause, absolutely rejected preformationism and sought a solution to the dilemma in an explanation of the equally important roles of cytoplasmic localization of morphogenetic potential and genetic activity in the nucleus during development (Whitman, 1895 a,b). For Wilson and Driesch the nucleus assumed a role of priority. The essence of Wilson's argument can be found in these words:

...the cytoplasm is essential to inheritance; for without a specifically organized cytoplasm the nucleus is unable to set up specific forms of synthesis...If the nucleus be the formative centre of the cell, if nutritive substances be elaborated by or under the influence of the nucleus while they are built into the living fabric, then the specific character of the cytoplasm is determined by that of the nucleus, and the contradiction vanishes. In accepting this view we admit that the cytoplasm of the egg is, in a measure, the substratum of inheritance, but it is so only by virtue of its relation to the nucleus, which is, so to speak, the ultimate court of appeal. The nucleus cannot operate without a cytoplasmic field in which its peculiar powers may come into play; but this field is created and moulded by itself. Both are necessary to development; the nucleus alone suffices for the inheritance of specific possibilities of development. (Wilson, 1896, p. 262.).

While Wilson's statement points up the interdependence of nucleus and cytoplasm it is evident that he was not aware, in 1896, of the real significance of the egg and cell cyto-

plasm in early development and differentiation. It is true that the character of the cytoplasm is determined by the nucleus, but in the egg it is not the oocyte nucleus as much as the maternal environment which establishes the organization of the egg cytoplasm; and in the young embryo, it is not the blastomere nucleus which gives the blastomere cytoplasm its particular character because this cytoplasm was imprinted with its traits by the egg nucleus. These facts became apparent to Wilson later on.

Those fundamental questions alluded to earlier can be phrased in terms of the nucleocytoplasmic relationship: What is the origin of the morphogenetic determinants that are distributed asymmetrically to the germ cell and embryonic cell cytoplasm? Where and how do they exert their effects in the cell? and What is the role of the nucleus in specifying the differentiated state of the cell? The answers to these and other questions posed eighty or more years ago are, today, still objects of the most intensive research in developmental biology.

Genome Structure and Function

Most evidence, classical and molecular, converges on one fact - the ability of the nucleus to determine the differentiated state of a cell depends not on any special set of genetic instructions that it contains but rather on a

particular subset of the entire genome that it selectively expresses. This is the essence of the theory of differential gene activity in development and cellular differentiation.

A basic implication of this argument is that every cell of a multicellular organism has an identical genetic complement. The early experiments by Driesch demonstrated that any of the individual nuclei of the cells of a cleaving embryo contained the information to program the development of a complete embryo. Spemann's (1928) elegant experiments with artificially constricted newt eggs extended Driesch's observations concerning the totipotency of cleavage nuclei. More recently, demonstrations that nuclei from fully differentiated Xenopus cells grown in culture can be transplanted to enucleated eggs to give normally developing tadpoles (Laskey and Gurdon, 1970) have supplied the strongest confirmation of the principle of nuclear totipotency.

Numerous direct measurements of the DNA content of nuclei have indicated that all of the differentiated somatic cells of an organism contain an identical amount of DNA. That the nuclear DNA content of embryonic and differentiated cells of the same species is informationally equivalent has been repeatedly demonstrated by DNA-DNA renaturation experiments (reviewed by Davidson, 1976). In the prototypic experiments, McCarthy and Hoyer (1969) observed that the DNAs

extracted from the cells of several adult mouse tissues were able to compete equally well with mouse L cell DNA for complementary sites in mouse embryo DNA.

There are exceptions to the principle of DNA constancy in development. In some cases these consist of selective losses of chromosomal material during development. For example, Boveri (1899) found that chromatin diminution occurred during early cleavages in Ascaris in the cells destined to form soma, the cells giving rise to the germ line retaining the full complement of chromatin. Similarly, programmed elimination of chromosomal material has been observed in certain dipteran insects (Geyer-Duszyńska, 1959; see Metz, 1938) but the phenomenon is, on the whole, rare.

In some well known instances, selected portions of the genome are replicated many times over in the absence of coincident replication of other parts of the genome. The best characterized of these involve the amplification of the cistrons for high molecular weight ribosomal RNA (rDNA) in the genomes of maturing oocytes of many animal species and in some lower eucaryotes (see Introduction - Oogenic Activity: Transcription and Fate of Oocyte RNAs). There are also reports of fluctuations in rRNA gene dosage related to developmental stage in Drosophila (e.g. Shibata, 1971) and tissue age in mammals (Gaubatz et al., 1976). Clearly these accounts

and others suggest that the DNA constancy rule, verified with respect to the genes for at least some messenger RNAs (e.g. Kohne and Byers, 1973) may not be wholly applicable at the rRNA gene locus. Still, this phenomenon constitutes only a special case of a quantitative change in the genome but not a qualitative one.

If all of the differentiated cells of an organism contain an informationally identical complement of nuclear DNA, then it remains to be explained why different cell types produce different kinds of proteins. It is conceivable that every differentiated cell transcribes all of the structural gene sequences in the genome and these are subjected to a post-transcriptional selection process which chooses the appropriate messenger RNAs for translation. While post-transcriptional processes are undoubtedly important factors in the regulation of gene expression they are not thought to be the primary control mechanisms.

The bulk of evidence favors direct transcriptional level control of gene expression in development and cellular differentiation; different cell types transcribe different populations of messenger RNAs. McCarthy' and Hoyer's (1964) early RNA-DNA competition hybridization experiments indicated that while the nuclear DNA content in each of the cell types of the adult mouse was identical, qualitatively different RNA

populations were transcribed from one cell type to another. The hybridization conditions employed in these experiments permitted the hybridization only of complementary repeated sequences and not the DNA sequences present in low copy number that are presumed to code for most, if not all, mRNAs. The experiments of Mizuno et al. (1973) with the sea urchin, specifically designed to test the diversity of transcribed repetitive DNA sequences at different developmental stages, showed an increase in the diversity of transcribed sequences as development and differentiation progressed. These observations have since been extended to comparisons of low copy number RNAs (RNA transcribed from unique DNA sequences) in differentiated cells (reviewed by Davidson and Britten, 1973).

The preceding discussion makes a case for the primacy of transcriptional level controls in the regulation of gene expression. In order to determine possible mechanisms of transcriptional control, it is necessary to understand something of the nature of chromatin and nucleotide sequence organization in DNA. There has been encouraging progress in these areas very recently, particularly with respect to chromatin structure (see reviews by Kornberg, 1977 and Felsenfeld, 1978).

The major components of eucaryotic chromosomes are DNA, histones, non-histone chromosomal proteins (NHC proteins),

and RNA. Four of the five major classes of histones are complexed with DNA in a repeating subunit called a nucleosome. In each such structure, a sequence of DNA, approximately 200 base pairs in length, is helically wrapped around a histone core consisting of two copies each of the slightly lysine-rich histones (H2A and H2B) and the arginine-rich histones (H3 and H4). Of particular relevance to the present discussion are findings that nucleosome structures are associated with actively transcribing DNA. For example, the globin gene in adult erythrocytes active in globin synthesis shows greater sensitivity to DNase I, a nuclease known to have a preference for nucleosome-associated DNA, than the one in fibroblasts where such synthesis is not occurring (Weintraub and Groudine, 1976). A similar observation has been made with transcriptionally active and inactive ovalbumin genes (Garel and Axel, 1976). This is not to say that inactive genes and heterochromatin are not associated with nucleosomes (they are), but rather that the nucleosome structure associated with active genes is modified without the loss of its histone components.

Most of the DNA in chromosomes is, in fact, associated with nucleosomes. The types of DNA sequences contained in nucleosomes include both single-copy and repeated sequences. Thus, it is not presently possible to make a correlation

between any specific class of DNA and its association with nucleosome structures (see Kornberg, 1977).

The non-histone chromosomal proteins, serving a variety of enzymatic and structural functions are found intimately associated with chromatin (see Elgin and Weintraub, 1975). DNA and RNA polymerases, nucleases, kinases, methylases, proteases, RNA transport proteins, and nuclear structural proteins constitute NHC protein components as do putative gene activators. It is this last group of proteins which has generated the most interest in the search for the primary selective regulators of transcription in cell differentiation. They will be discussed later in relation to hypotheses for the regulation of gene expression (see Introduction - RNA Synthesis and Processing and Regulation of Gene Expression).

The organization of nucleotide sequences within the genome remains to be fully elucidated. The Davidson group has done much to describe the organization of DNA in metazoan chromosomes (see Davidson, 1976 for a summary) and provide a model for explaining the basis of differential gene activity (Britten and Davidson, 1969).

The major portion of the genomes of animals and higher plants contains interspersed repetitive DNA sequences (Davidson, 1976; Walbot and Dure, 1976; Zimmerman and Goldberg, 1977).

In Xenopus laevis, 50-55% of the DNA consists of short repetitive sequence elements, an average of 200-400 nucleotides long, alternating with longer non-repetitive elements of 800 to several thousand nucleotides in length (Davidson et al., 1973). The "Xenopus pattern" of sequence organization appears to be of general occurrence in metazoans. A different pattern so far seen only in a few insect species and typified by Drosophila (Manning et al., 1975), contains interspersed repetitive and non-repetitive sequences which are both much greater in nucleotide length. Although they have been little investigated, the genomes of higher plants may conform to a modified "Xenopus-like pattern" (Walbot and Dure, 1976).

Suggestions that the non-repetitive or single-copy sequences, contiguous to the repetitive sequences, are codogenic structural genes have been confirmed in the sea urchin (Davidson et al., 1975). The implications of this observation in hypothetical models of genetic regulation will be discussed later (see Introduction - RNA Synthesis and Processing and Regulation of Gene Expression).

RNA Synthesis and Processing and Regulation of Gene Expression

In eucaryotic nuclei the major classes of RNA - ribosomal RNA, transfer RNA, and informational (heterogeneous

nuclear and messenger) RNA are transcribed from the DNA template by three different RNA polymerases (see Biswas et al., 1975 and Roeder, 1976 for general reviews). Each polymerase has a molecular weight of approximately 5.6×10^5 and consists of two large and six to eight smaller subunits. RNA polymerase I whose activity resides mainly in the nucleolus, is responsible for the synthesis of the precursor of high molecular weight ribosomal RNA. RNA polymerases II and III, located in the nucleoplasm transcribe informational and low molecular weight (tRNA and 5S rRNA) RNA, respectively.

Based on our current understanding of the mechanism of transcription in eucaryotes there are, theoretically, two general ways in which transcription can be selectively regulated. Either RNA polymerases can be so modified as to recognize only promoter sequences next to a specific set of structural genes or modification can occur at the chromatin-level, only permitting RNA polymerases to bind and transcribe DNA sequences corresponding to a certain set of structural genes. Most evidence favors the latter strategy over the former but both will be considered.

Selective transcriptional control is exercised^d in a very general way by different RNA polymerases as described above. Ribosomal high molecular weight RNA, heterogeneous nuclear RNA including messenger RNA sequences, and transfer and 5S

ribosomal RNAs are each transcribed by one of the three classes of RNA polymerase that have been identified in the nuclei of eucaryotic cells. A change in the activity of any one of these polymerases should affect the synthesis of the class of RNA which is reliant on that specific enzyme for its transcription. A differential increase or decrease in the activity of a particular class of RNA polymerase has been found in differentiated cells undergoing a change in physiological state (reviewed by Roeder, 1976). RNA polymerase levels do not, however, fluctuate significantly during the early embryonic development of Xenopus when there is clearly variation in the relative transcriptional rates of the major classes of RNA (Roeder et al., 1971; Roeder, 1974). Although enzyme concentrations do not change during the developmental periods studied, changes in the in vivo activity of one or more of the enzymes brought about by some transient polymerase stimulating (or repressing) proteins, have been implicated (for references see Roeder, 1976).

It would appear, however, that despite a comparatively complex subunit structure and a number of proteins which can modulate their activity, RNA polymerases lack the heterogeneity required for the sequence specific transcription of messenger RNAs. Until it is demonstrated otherwise, it will be generally acknowledged that differential gene expression

is determined by chemical or structural modifications of chromatin which determine the accessibility of specific sequences to transcription by RNA polymerases.

The chromatin components which have been most recently and strongly implicated in gene-specific regulation of transcription are the non-histone chromosomal (NHC) proteins. Although no one-to-one relationship between an identifiable NHC protein and a particular gene product has been demonstrated, there is enough circumstantial evidence to connect at least a portion of the NHC proteins to differential gene transcription (reviewed by Stein et al., 1974 and Elgin and Weintraub, 1975). Paul and Gilmour (1968) showed, in chromatin reconstitution experiments, that irrespective of the tissue source of the DNA or histone components, NHC proteins extracted from each specific tissue caused the transcription in vitro of qualitatively different sets of RNA molecules as assayed by RNA-DNA competition hybridization. Many in vivo studies have demonstrated a correlation between a change in the proliferative or differentiated state of cells and quantitative or qualitative changes in the NHC protein components of their nuclei (for reviews and references see Stein et al., 1974 and Baserga, 1975).

More recently the role of NHC proteins in the developmental program of early embryos has received attention.

Di Berardino and Hoffner (1975) found that late gastrula nuclei in Rana pipiens embryos accumulated large quantities of non-histone proteins from the cytoplasm. When these nuclei were transplanted into enucleated eggs the proteins migrated back to the cytoplasm.

Briggs and Justus (1968) isolated a protein involved in the maternal effect o mutation in Ambystoma, the Mexican axolotl (Humphrey, 1966; Briggs and Cassens, 1966). Brothers (1976) further investigated this o⁺ substance which is synthesized during oogenesis. It resembles the presumed regulatory proteins in its behavior. The o⁺ substance was found to be stored in the blastomere cytoplasm during cleavage and was then evidently taken up by the nuclei in the cells of the early blastula. Nuclear transplantation experiments revealed that o⁺ substance had induced a stable change in early blastula nuclei making them competent to support continued normal development. Female homozygous Ambystoma mutants unable to synthesize o⁺ substance produce eggs which, upon fertilization, are not capable of development beyond the blastula stage.

Finally, NHC proteins have been implicated as essential factors in steroid hormone action in target tissues. Steroid hormones become associated with protein receptors in the cytoplasm of target cells. These complexes move to the nucleus

where they bind to chromatin and ultimately induce the transcription of specific messenger RNA molecules. Tsai and Hnilica (1971) with cortisol and Barker (1971) with estradiol demonstrated that the steroid-receptor complex specifically bound a minor non-histone protein component of chromatin. Studies of the interaction of the progesterone-receptor complex with chromatin in the cells of chick oviduct tissue (O'Malley and Means, 1974) have given strong evidence that hormone receptor complexes bind NHC proteins which are responsible for the activation of particular structural genes.

An external or cytoplasmic signal, however it is transmitted to the genome, often leads to substantial quantitative and qualitative changes in transcription. How this is accomplished has proven to be an extremely complex problem in even the simpler eucaryotes. Hypotheses to explain control of genetic expression during development and differentiation have depended for their conceptual foundations on knowledge concerning genetic regulatory systems in procaryotes (e.g. the lac operon) and DNA sequence organization in eucaryotic chromosomes (see Introduction - Genome Structure and Function).

A major difficulty arising with eucaryotic gene regulatory models based too closely on procaryotic systems, such as the lac operon, is that while many bacterial structural gene sequences are transcribed coordinately as parts of polycis-

tronic messages, eucaryotic transcripts are, as far as is known, always monocistronic. The structure of the eucaryotic genome requires the integrated activation of a large number of non-contiguous genes, an arrangement which cannot be adequately provided for with simple procaryote-like models.

The discovery that repeated sequence DNA is dispersed throughout the genome and adjacent to structural genes (Davidson et al., 1973; Davidson et al., 1975) has provided support for a model of genomic transcriptional control based loosely on the operon concept. Britten and Davidson (1969; Davidson and Britten, 1973) have proposed that repeated sequences next to structural genes act as regulatory genes; they are roughly analogous to the promotor and operator regions of procaryotic operons. These regulatory or receptor loci respond to sequence specific activators which might be either RNA molecules or proteins coded for at integrator loci elsewhere in the genome. The activators are synthesized in direct response to specific inducing agents (e.g. a steroid hormone or a cytoplasmic determinant). Functionally or developmentally related structural genes are coordinately activated because they share a common repeated sequence (receptor locus) family. Thus, a specific activator will recognize a group of repeated DNA sequences adjacent to all of these loci. It has in fact, been demonstrated that the gastrula-stage mes-

senger RNAs of sea urchins are transcribed from structural genes which share a specific subset of all repeated sequence families (see Davidson et al., 1977). To further support their model and add substance to it, the Davidson group has proposed a new function for heterogeneous nuclear RNA (hnRNA) (Davidson et al., 1977). It is maintained that the large hnRNA molecules found in the nucleus are not, for the most part, mRNA precursors as is commonly supposed. They are, instead, the transcription products of the integrator loci. These hnRNA molecules are known to contain repeated DNA sequence transcripts. It is suggested that they are rapidly cleaved by nuclear endonucleases and the repeated sequence transcripts that are liberated, serve as the putative activator RNA molecules.

There is considerable circumstantial evidence to support this model for transcriptional regulation (although it undoubtedly can be used to support alternative hypotheses). Several of the model's elements as they were originally hypothesized have subsequently been experimentally verified, as for example, the location of repeated sequences adjacent to structural genes and the observation that coordinately activated structural genes share a common family of repeated sequences. The model also implicitly provides a role for non-histone chromosomal proteins as the initial inducers

which stimulate the integrator genes.

Future efforts (if they have not already begun) should be directed toward isolating the activator RNA molecules (or proteins) and demonstrating, in vitro, that a particular set of these activators can promote the transcription of a specific group of structural genes. It would then remain to show that a given inducer is responsible for the synthesis of a particular family of these activators.

A fundamental question posed in this dissertation is to what degree is control of gene expression effected at the transcriptional level. Not enough is presently known about the mechanisms governing post-transcriptional processing to assess its importance in the regulation of gene activity. It has been shown that ribosomal RNA which does not find its way into functional ribosomes will not be properly processed and is degraded without ever leaving the nucleus (Cooper, 1973; Toniolo and Basilico, 1976; Bowman and Emerson, 1977). The same might be true for improperly processed messenger RNA sequences that were not destined to be translated. Very recently, evidence was presented (Wold et al., 1978) which indicated that the nuclear RNA of adult sea urchin tissue contained virtually all of the sequences which were found in blastula polysomal messenger RNAs. The most profound implication of these results is that processing of nuclear RNA and

not differential gene transcription is mainly responsible for the ultimate expression of distinct sets of genes in different cell types. This type of regulation was earlier proposed by Kijima and Wilt (1969) and Aronson and Wilt (1969). The investigators in the study reported above, acknowledge some difficulties in interpreting their data, so until there is further supporting evidence the significance of their findings cannot be truly assessed.

The remainder of this section briefly reviews the processing of the major species of RNA in eucaryotes. Emphasis is placed on processing events which are likely to regulate the production of functional RNA molecules.

The high molecular weight ribosomal RNAs (25-28S and 17-18S) are both transcribed from a single large precursor molecule which is cleaved by endonucleases during processing (reviewed by Perry, 1976). The genes for rRNA are localized in one or several clusters in the genome and the genes within a cluster are separated by non-transcribed DNA spacers. Each transcript contains, in addition to the sequences for the mature rRNAs, an interspersed, transcribed spacer sequence which is eliminated during subsequent processing. It varies in molecular weight from about 2.8×10^6 in lower eucaryotes to 4.5×10^6 in higher eucaryotes. Most of the ribosomal structural proteins as well as other proteins are associated

with the initial transcript (Kumar and Warner, 1972; Shepherd and Maden, 1972). Methylation of those portions of the precursor which will be processed into the mature rRNAs (Greenberg and Penman, 1966), is required for proper cleaving of the molecule (Vaughan et al., 1967). The actual processing which gives the 17-18S and 25-28S rRNAs takes place in the nucleolus in a pre-ribosomal particle. As mentioned above, rRNA molecules which are not properly processed are rapidly degraded; rRNA processing may be important in the regulation of ribosome formation (Cooper, 1973; Bowman and Emerson, 1977). There are two further rRNA processing steps which are not found in all organisms. The first of these is a break in one of the immediate precursors to the larger (28S) rRNA which leaves attached to the 28S rRNA a non-covalently bound, heat-dissociable 5.8S rRNA (e.g. Fujisawa et al., 1973; Helser and McLaughlin, 1975). The other, so far found almost exclusively in various protozoa and protostomian animals, is a "hidden" break introduced at or near the center of the large rRNA which results in fragmentation upon denaturation (Bostock et al., 1971; Ishikawa and Newburgh, 1972; N. Rubin, personal communication); the significance of these breaks is unknown. Little is known of the processing of pre-5S rRNA, if any exists.

Messenger RNAs are all apparently derived for a category of larger nuclear molecules designated heterogeneous

nuclear (hn)RNA. The ratio of the size of hnRNA to mRNA may be close to unity as in Achlya, the water mold (Timberlake et al., 1977) or as great as 10 to 20 in mammalian cells (Holmes and Bonner, 1973; Derman and Darnell, 1974). The functions of the non-messenger sequences found in hnRNAs are unknown although a large portion of them in most or all hnRNAs in higher eucaryotes consist of interspersed sequences derived from repetitive DNA (e.g. Holmes and Bonner, 1974; Smith et al., 1974). Aside from excision of the codogenic messenger sequences from hnRNA precursors there are several other steps involved in processing.

Tracts of polyadenylic acid are added post-transcriptionally to the 3' end of nuclear RNA molecules and are found in cytoplasmic messenger RNA (Edmonds et al., 1971). There is evidence that existing 3' poly(A) tracts may be further extended in the cytoplasm (Derman and Darnell, 1974; Slater and Gillespie, 1973; Wilt, 1973). The function of polyadenylation has not been elucidated; it does not appear to be required for ribosome recognition, binding, or translation since experimentally deadenylated mRNAs are capable of being translated (Huez et al., 1974) although translational efficiency may be reduced (Doel and Carey, 1976). Nonpolyadenylated and deadenylated RNAs when tested in an oocyte translation system are degraded more rapidly than polyadenylated species,

suggesting that poly(A) may confer stability on the mRNA molecule (Marbaix, et al., 1975). It has also been suggested that poly(A) sequences may serve as cues for the specific cleavage of mRNA sequences from hnRNA (Derman and Darnell, 1974) or that they are necessary for the transport of functional mRNAs from the nucleus to the cytoplasm (Darnell et al., 1971). The apparent absence of poly(A) tracts from certain classes of mRNA, most notably the histone messages, has fostered the opinion by some that polyadenylation has no function in RNA metabolism. (Recently, it has been found that at least a portion of histone mRNA in Xenopus does contain 3' polyadenylate tracts (Levenson and Marcu, 1976; Ruderman and Pardue, 1978).) It seems unreasonable to suppose that a significant portion of cellular energy would be devoted to a vestigial metabolic function.

Whether they are polyadenylated or not, hnRNAs and mRNAs in eucaryotes generally contain a peculiar nucleotide structure at their 5' termini called a "cap" (Furuichi et al., 1975; Rottman et al., 1974; Surrey and Nemer, 1976). It consists of a terminal 7-methylated guanylate residue in a 5'-5' triphosphate linkage with the second nucleotide residue (general structure: $m^7GpppXpYp$, where X, X and Y, or neither, are ribose-methylated). Kinetic evidence indicates that the cap structures attached to hnRNAs are conserved during pro-

cessing and are linked to the functional mRNAs derived from them (Salditt-Georgieff et al., 1976; Perry and Kelley, 1976). The role of the cap structure in the binding of mRNA to the ribosome has been demonstrated (Both et al., 1975; Shimotohno et al., 1977) but the nature of the interaction between cap and ribosomal subunit (and/or initiation factor) is not clear. The cap may simply protect the mRNA from degradation by a 5'-exonuclease (Shimotohno et al., 1977). However, experiments which demonstrate in vitro inhibition of translation in the presence of 7-methylguanosine-5'-monophosphate, a cap analogue, suggest that this 5'-terminal structure has a more active role in the initiation of translation (Hickey et al., 1976).

Messenger RNA is methylated internally as well as at the 5'-terminal cap. The function of this internal methylation is unclear. It may not be necessary for the formation of functional mRNAs since some mRNA molecules are not internally methylated (Perry and Scherrer, 1975).

Messenger RNA molecules have generally been considered to be derived from much larger hnRNA molecules by specific endonucleolytic scission. The precise location of the messenger sequence in the hnRNA molecule has been the subject of several studies. Experiments which have demonstrated close association of the messenger sequence with either the

5'-terminal cap structure (Perry and Kelley, 1976; Salditt-Georgieff et al., 1976) or the 3'-polyadenylate tract (Darnell et al., 1973; Lodish et al., 1973) along the RNA strand, have been used as evidence for the 5' proximal or 3' proximal location of the messenger sequence in the hnRNA molecule. The finding of Perry and Kelley (1976) that the cap in hnRNA is conserved throughout the processing of mRNA, sharply contradicts data that places the mRNA sequence adjacent to the 3'-polyadenylate segment which also appears to be conserved during processing (Darnell et al., 1973; Lodish et al., 1973).

Recent investigations of viral mRNA processing indicate that portions of a single mRNA sequence may be transcribed from non-contiguous DNA sequences (Berget et al., 1977; Chow et al., 1977). According to the most reasonable hypothesis that has been proposed (Berget et al., 1977) a single structural gene transcript contains the parts of an entire messenger sequence separated by intervening non-codogenic sequences. During a single processing step the intervening sequences are excised and the partial mRNA sequences are ligated together to form a continuous messenger. It has been suggested that this mechanism could account for the apparently contradictory data regarding the location of the messenger sequence in the hnRNA molecule; parts of the sequence might be derived from several regions of the hnRNA molecule. These so-called

"split genes" have been found to code for the RNAs of several proteins - ovalbumin (Breathnach et al., 1977), immunoglobulin (Brack and Tonegawa, 1977) and β -globin (Jeffreys and Flavell, 1977; Kinneburgh et al., 1978). This processing system requires an enzyme with ligating activity to join the various partial sequences; no such enzyme activity has yet been identified.

An important factor in the formation of functional messenger RNA is the immediate and continuing association of the RNA transcripts with proteins. Heterogeneous nuclear RNA, free cytoplasmic mRNA, and polysomal mRNA are always found in ribonucleoprotein particles (RNPs) (Spirin, 1978) termed "informosomes" (Spirin et al., 1964; reviewed by Preobrazhensky and Spirin, 1978). The attachment of protein begins while the RNA is still being transcribed (Miller and Hamkalo, 1972; Malcolm and Sommerville, 1974). Nuclear RNA, free cytoplasmic mRNA, and polysome-associated messenger ribonucleoprotein particles each have a unique protein composition, suggesting that proteins with different functions are added and removed as RNA molecules move from one intracellular compartment to the next. Heterogeneous nuclear RNA is associated with a class of regularly arranged proteins with possible structural and stabilizing roles (Beyer et al., 1977). The enzymes required in mRNA processing apparently constitute a part of the nuclear

RNPs. An RNA cleaving nuclease (Niessing and Sekeris, 1970) and a poly(A)-polymerase activity (Niessing and Sekeris, 1972) have been found and poly(A)-binding proteins involved in mRNA transport (Schwartz and Darnell, 1976) and stabilization (Muller et al., 1978) have been identified in such complexes. Free cytoplasmic ribonucleoprotein particles (the original informosomes) contain completely processed mRNA molecules and a set of proteins whose functions have not been characterized. They may contain some proteins which prevent the mRNA from engaging in translation (Preobrazhensky and Spirin, 1978) until the appropriate time. This is the concept of the "masked" messenger (see Introduction - Oogenic Activity: Transcription and Fate of Oocyte RNAs). Activation may involve changes in a portion of the protein moiety of the RNP induced by a regulatory protein also associated with the RNP. For example, it has been found that certain of the proteins of these particles are capable of being phosphorylated (Egly et al., 1972) and a cyclic AMP-dependent protein kinase has been purified from free cytoplasmic RNPs (Egly et al., 1976). There may be other proteins in the RNPs more directly involved in binding to ribosomes and in translation. Recently several translation initiation and elongation factors have been found to tightly bind RNA (Vlasik et al., 1978; Ovchinnikov et al., 1978). Of course, the capacity to bind RNA does not neces-

sarily mean that these factors are normal constituents of RNPs.

Transfer RNA in eucaryotes is derived from a slightly larger precursor transcript of 100-140 nucleotides (reviewed by Perry, 1976; also see Smith, 1976). The precursor molecules are transcribed from clustered, iso-coding genes (genes coding for the same tRNA) (Feldman, 1976; Clarkson et al., 1978). The transcripts are very rapidly transported to the cytoplasm and the discovery of enzymes in the cytoplasm capable of cleaving the precursor to the size of a functional tRNA molecule (Smillie and Burdon, 1970; Siddiqui and Chen, 1975) has led to speculation that tRNA processing may occur in the cytoplasm (Perry, 1976). Methylation and the conversion of uridine to pseudo-uridine conversion is apparently necessary for the proper functioning of the tRNA molecule but the role of methylation is unknown. The tRNA molecule can function without this modification but at somewhat reduced efficiency (Amalric et al., 1977). The C-C-A sequence involved in the aminoacyl linkage is added to the tRNA molecule by a nucleotidyl transferase (see Smith, 1976).

Oogenic Activity: Synthesis and Fate of Oocyte RNAs

The development of a multicellular organism begins before the onset of embryogenesis. The mature animal egg contains the macromolecules and food reserves to carry the embryo

through the initial embryogenic stages. Oogenesis is a period of intense synthetic and genetic activity. Food reserves, such as yolk proteins, are elaborated from precursors contributed by accessory tissues of the mother and stored for later use. Proteins and RNA are synthesized in extraordinary quantities and, like the food reserves, are accumulated for subsequent use in the young embryo.

But the maturing egg is not merely a warehouse from which the embryo draws the food reserves and informational and structural molecules it needs for early development. It is the framework within which the early embryo is constructed and the way in which particular structures and molecules are distributed within the egg cytoplasm determines the orderly development of an organism.

This section examines first the nature of genetic activity in the oocyte and second the cytoplasmic localization of morphogenetic determinants. The importance of RNA in both of these developmental phenomena will become obvious.

Patterns of RNA synthesis during oogenesis, in various animal species, are very consistent, although they differ in detail. In metazoan oocytes the different classes of RNA and many proteins are synthesized and stored in sufficient quantities to support development through the initial cleavage period. The high metabolic demands of rapid cleavage division

divert cellular resources which could otherwise be utilized in full-scale RNA and protein synthesis. Ribosomal RNA and transfer RNA are produced in the oocyte to provide the translational machinery for the synthesis of large quantities of proteins during cleavage. The proteins are coded for, in large measure, by messenger RNAs that are also produced during oogenesis and stored until needed.

The synthesis of transfer RNA begins very early in oogenesis in Xenopus oocytes (Thomas, 1974) and it continues throughout the entire oogenic period. As noted previously the genes for each of the major tRNA species are repeated in the Xenopus haploid genome an average of 200 times, although the repetition frequency for the genes of each tRNA class varies (Clarkson et al., 1973). The redundancy of tRNA genes (increased four-fold because the pre-meiotic oocyte contains four genome-equivalents of DNA) and a sustained high rate of synthesis throughout oogenesis permits the accumulation of sufficient quantities of tRNA to support protein synthesis in the early embryo, even in the absence of amplification of the tDNA sequences in the oocytes (Ford, 1971; Denis and Mairay, 1972). Less information is available for patterns of tRNA synthesis in other species. However, the rate of tRNA synthesis in the sea urchin must be very great as suggested by the experiments of Nemer and Bard (1963). They demonstrated that

homogenates from unfertilized eggs could support high levels of in vitro incorporation of amino acids into protein although the rate of protein synthesis in the unfertilized egg itself is extremely low. Thus, tRNA availability was not the limiting factor in protein synthesis in the sea urchin egg. Since there appears to be, at most, only a low level of tRNA synthesis during the early cleavage period (Gross et al., 1965; O'Melia and Villee, 1972), it must be concluded that, as is true with the other major classes of RNA, tRNA is synthesized during oogenesis and stored for later use in protein synthesis during cleavage.

The gene coding for the precursor of both high molecular weight ribosomal RNAs is reiterated to varying extents in the genomes of all organisms (reviewed by Tartof, 1975). The haploid genome of Xenopus laevis contains 450 tandem repeats of the rDNA sequence located in the nucleolar organizer region (Brown and Weber, 1968). In the pre-meiotic oocyte nucleus containing four times the haploid DNA content, there are expected to be nearly 2000 rDNA genes.

This number of templates is apparently not adequate to meet the oocyte's demand for rRNA. Beginning in pre-meiotic oogonia and continuing through the pachytene stage of meiosis I in the oocyte, the rDNA genes are selectively replicated (amplified) (Brown and Dawid, 1968). The germinal vesicle of

a Xenopus oocyte may hold 1500 extra-chromosomal nucleoli, each containing several sets of the rDNA genes. Amplification of the rRNA cistrons has been well documented in other amphibians (see Davidson, 1976), fish, molluscs, worms, insects (reviewed by Gall, 1969 and Tobler, 1975), and unicellular eucaryotes (see Tartof, 1975 for a review; Gall, 1974; Findly and Gall, 1978). The necessity for this rDNA amplification in developing oocytes is apparent when it is noted that the mature Xenopus oocyte contains over 10^{12} ribosomes (Perkowska et al., 1968). According to the calculations of Perkowska it would require over 450 years to synthesize the rRNA for this many ribosomes with only the somatic cell complement of rDNA (ca. 900 genes). Amplification makes production of this enormous number of ribosomes possible in a span of six months. There is no evidence for amplification of the genes for ribosomal proteins. The most reasonable explanation for this is that the messenger RNAs coding for ribosomal proteins are, in effect, amplified by repeated translation.

Ribosomal RNA is not synthesized in significant quantities until after the active amplification of rDNA is complete. The rate of rRNA synthesis is at a maximum during the mid-vitellogenic period and then declines as the oocyte reaches final maturation (reviewed by Davidson, 1976).

In certain insect species characterized by meroistic oogenesis (see Davidson, 1976), oogenic RNA is synthesized not in the oocyte but in the nurse cells which surround it. This RNA is then fed to the oocyte via large intercellular bridges. In Drosophila, for example, there are fifteen nurse cells surrounding an oocyte and each is 1024-fold polytene. Ribosomal RNA synthesis begins early and continues throughout oogenesis (Mermod et al., 1977). It is obvious that this strategy in some insects can accomplish the same purpose as gene amplification in other organisms.

The genes coding for 5S rRNA are known not to be amplified (Wegnez and Denis, 1972) but they are highly redundant within the genome. There are approximately 24,000 copies of the 5S rDNA sequence per haploid Xenopus genome (Brown and Weber, 1968). The pattern of 5S rRNA synthesis in the oocyte parallels that of transfer RNA, beginning in the very immature oocyte and continuing throughout oogenesis. Newly synthesized 5S rRNA is incorporated into a ribonucleoprotein particle along with tRNA (see above) and then released when required for use in oocyte ribosomes (Mairy and Denis, 1972). It is interesting to note that the Xenopus oocyte synthesizes a specific variety of 5S rRNA which differs from the molecule synthesized in somatic cells by six out of 120 nucleotides (Brownlee et al., 1974); a similar phenomenon has been reported

in Tinca, a freshwater fish (Denis and Wegnez, 1977). The functional significance of this occurrence is unclear, although it has been observed that the oocyte 5S rRNAs of both organisms are more heat-stable than the corresponding somatic cell 5S rRNAs (Denis and Wegnez, 1977).

The patterns of messenger RNA synthesis and utilization during oogenesis and early embryogenesis are more complex than had originally been surmised from early descriptive and kinetic studies. The growing oocyte must synthesize all of the messenger RNAs for its own enormous protein synthetic requirements as well as the maternal messenger RNAs active in early embryogenesis. The prevalent view has been that the most important period of mRNA synthesis coincides with the "lampbrush" chromosome stage in the actively growing oocyte. Lampbrush chromosomes with their characteristic extended lateral loops of duplex DNA (Gall, 1963) arises during the protracted diplotene stage of meiosis I in Xenopus oocytes and at various other oogenetic stages in other animal oocytes (structure and function reviewed by Davidson, 1976). That lampbrush chromosomes are the sites of incredible transcriptional activity is well documented (see Davidson, 1976). The RNA transcripts are most likely hnRNAs based on their physical properties (Sommerville and Malcolm, 1976) and it might be reasonably concluded that mRNAs are derived from these trans-

cripts. The maternal messages are stored in an inactive form complexed to proteins (see Introduction - RNA Synthesis and Processing and Regulation of Gene Expression) and utilized only after fertilization in the early stages of embryogenesis.

It has been found, however, that the young pre-lampbrush stage (pre-vitellogenic) Xenopus oocyte contains the same quantity of poly(A) mRNA as is found in the unfertilized egg (Rosbash and Ford, 1974). There is no further net accumulation of messenger RNA in the oocyte during the lampbrush and subsequent stages although poly(A) RNA continues to be synthesized during these stages (Rosbash and Ford, 1974). The most consistent explanation for this data is that poly(A) mRNA produced on lampbrush chromosomes turns over in the cytoplasm and is not stored to a significant extent as maternal messenger RNA. This conflicts with the observations of Ford et al., (1977) who found the poly(A) RNAs of Xenopus oocytes to be very stable. The presence of lampbrush chromosomes in amphibian oocytes, thus, presents a paradox: what purpose is served by the lampbrush stage with its enormous transcriptional activity, if the pre-lampbrush oocyte has already accumulated the maximum quantity of maternal mRNA sequences found in the unfertilized egg?

It is, of course, probable that much of the messenger RNA transcribed in the oocyte is also translated in it. This

appears to be the case in sea urchin oocytes (Hough-Evans et al., 1977). The lampbrush period in the sea urchin oocyte is comparatively long, many times longer than would be necessary to transcribe the quantity of stored maternal mRNA found in the unfertilized egg (Davidson, 1976). According to Davidson the cytological evidence demonstrates that lampbrush chromosomes in sea urchin oocytes are transcribing at near maximum rates. It follows that mRNA in the oocyte must be turning over. Much of it is involved in protein synthesis in the oocyte and some is being stored as maternal messenger. It is probable that many of the same mRNA sequences in the oocyte are utilized in both ways (Hough-Evans et al., 1977) as, for example, the mRNAs for histones, tubulins, and so-called "housekeeping" enzymes.

Is there, in fact, any storage of maternal mRNA or is the oocytic mRNA found in the fertilized egg and early embryo, a surviving population of the constantly turning-over mRNAs in the oocyte? Stored maternal mRNA should be unusually stable in its native form and environment, i.e., it should have a longer half-life than oocyte mRNA in general. Ford et al. (1977) demonstrated that poly(A) mRNAs synthesized in pre-vitellogenic Xenopus oocytes are extremely stable, having half-lives exceeding two years. There is little evidence on this point from the oocytes of other species. Dworkin and

Infante (1978; also see Humphreys, 1971) surmised the existence of a stable mRNA population in sea urchin oocytes based on their conclusions that the mRNA synthesized in unfertilized eggs could not, by itself, account for a sharp increase in the number of polysomes after fertilization.

Maternal mRNA is stored in ribonucleoprotein particles (see Introduction - RNA Synthesis and Processing and Regulation of Gene Expression). Spirin et al. (1964) and Spirin and Nemer (1965) first demonstrated the existence of such particles in fish and sea urchin embryos and hypothesized their role as "masked" forms of maternal messages. In one well documented case in sea urchins, the messages for histones were found in cytoplasmic RNPs (Gross et al., 1973). They are inactive in translation in the mature oocyte but upon fertilization become associated with polysomes (Woods and Fitschen, 1978). Inhibition of translation in the oocyte could be due either to the lack of some factor required for translation, to an inhibitor of ribosome mRNA complex formation, or to some feature of the ribonucleoprotein particles themselves which make them untranslatable. The available evidence makes the first two propositions untenable; the translational machinery of the oocyte can carry on protein synthesis as well as that in the fertilized egg (see above). It has instead been suggested that the RNPs are kept inactive

in oocytes by a labile component, such as a protein which is removed after fertilization, thus activating the maternal mRNA (see Introduction - RNA Synthesis and Processing and Regulation of Gene Expression; Monroy et al., 1965; Mano and Nagano, 1970). This hypothesis has recently been tested (Jenkins et al., 1978) in unfertilized sea urchin eggs. Ribonucleoprotein particles isolated under precisely controlled environmental conditions were non-translatable in a cell-free protein synthesizing system. These particles could be activated by changing the isolation procedure; varying the Na^+ concentration in isolation buffers resulted in translatable RNPs (although the investigators do not suggest that this is the physiological basis of activation). The maternal RNPs found in embryos must be translatable since they are the only possible source of the mRNA associated with the polysomes whose numbers increase sharply after fertilization. Dworkin and Infante (1976) concluded that the RNPs in embryos were not stable and thus did not behave as the "masked" mRNA complexes in oocytes. These results most likely indicate that previously inactive maternal RNPs had been activated (and consequently de-stabilized) for translation after fertilization.

Oogenic Activity: RNA and Cytoplasmic Localization

The role of cytoplasmic localization of morphogenetic

factors in the formulation of early concepts of development was discussed earlier (see Introduction - Historical Background - Classical Embryology and see Wilson, 1925) and there have been recent comprehensive reviews of various aspects of the subject (see Beams and Kessel, 1974; Eddy, 1975; Davidson, 1976). The mechanisms by which the cytoplasmic determinants are distributed to specified regions of the egg cytoplasm far in advance of the morphogenetic events that they determine remains a wide open question in embryology. The nature of these determinants and their mode(s) of action have, however, been studied with considerable success. There is a substantial amount of evidence to suggest the involvement of RNA in many localization phenomena.

The most extensively studied instance of cytoplasmic localization is that of the germ cell determinants, particularly in insects and amphibians. Clear cytological evidence of its presence is provided by the polar granules which are found in the posterior region of the egg cytoplasm. Hegner (1911) first demonstrated the special function of this area in the egg of the beetle Leptinotarsa. If the polar cytoplasm of the egg was destroyed with a hot needle before the cleavage nuclei had reached the peripheral cytoplasm during blastoderm formation, a morphologically normal insect developed but it was sterile; germ cells were found to be absent

from the adult gonads. These experimentally operated organisms resemble a naturally occurring maternal effect mutant in Drosophila called grandchildless (gs) (Spurway, 1948). The offspring of homozygous gs females are sterile, having no germ cells. The eggs of these females also lack granules in the polar cytoplasm (Fielding, 1967). Cytoplasmic inclusions with a germ cell-determining function have been found in a variety of organisms (reviewed by Beams and Kessel, 1974; Eddy, 1975).

The first clue concerning the chemical composition of the polar granules in insect eggs was the observation that Hegner's results could be mimicked by selective UV-irradiation of the pole plasm in Drosophila eggs (Geigy, 1931). Smith (1966) demonstrated that frog eggs, similarly treated with UV, gave rise to sterile adults but the effects of the lesions in experimental eggs could be reversed by injecting polar cytoplasm from untreated eggs. Smith also noted that the wavelengths of UV light having the greatest sterility-inducing effect were the wavelengths to which nucleic acids were most sensitive.

After the cleavage nuclei move to the periplasm in Drosophila eggs in anticipation of blastoderm formation, polar granules are seen associated with polysomes (see Gehring, 1973). It has therefore been inferred that polar

granules contain maternal mRNAs which are the actual primary germ cell determinants.

UV-irradiation of specific regions of the egg cytoplasm before nuclear migration may affect the development of adult somatic structures as well as germ cell formation. When the lateral periplasm of early cleavage Drosophila eggs was subjected to UV-microbeam irradiation, the adults which developed showed defects and deletions in various adult thoracic and abdominal structures (Nothiger and Strub, 1972).

The most compelling evidence for the involvement of RNA molecules in cytoplasmic localization phenomena has been obtained in the dipteran midge Smittia. Local UV-irradiation of the anterior pole cytoplasm of the pre-blastoderm cleavage egg was shown to cause a defect in which the normal anterior structures of the embryo were missing and were replaced by structures typical of the posterior region (Kalthoff, 1971). The UV damage which caused the "double abdomen" embryos could be repaired by subjecting UV-treated eggs to certain wavelengths of visible light, suggesting a photoreactivation event involving nucleic acids (Kalthoff, 1973). More recently the putative determinant(s) has been shown to be RNase sensitive (Kandler-Singer and Kalthoff, 1976). The anterior poles of eggs immersed in a solution containing RNase were gently punctured. A large percentage of the embryos which developed

suffered from "double abdomens." Control solutions containing either of two peptide fragments, obtained by treating native RNase with TCA, did not cause defects while solutions containing a mixture of the two RNase fragments were active "double abdomen" inducers. Kandler-Singer and Kalthoff^f suggested that the morphogenetic determinant was an mRNA that was translated during cleavage.

It may be postulated that these cytoplasmically localized RNA molecules are translated into proteins which move to the nucleus and induce the expression of specific genes, thereby initiating the differentiated state (see Introduction-Genome Structure and Function). The RNA molecules may alternatively be viewed as the activator RNA molecules in the Britten-Davidson gene regulation model, which are responsible for the selective activation of batteries of genes (see Introduction - RNA Synthesis and Processing and Regulation of Gene Expression). However, until RNA components involved in cytoplasmic localization are isolated and identified the mechanisms of their action will remain a matter of speculation.

Volvox: A Model System for Studying Development and Differentiation

Since developmental processes are ultimately under the control of the genome, the more that is known of the genetics of an experimental organism, the more useful will it be to

the examination of developmental events at the molecular level. Several organisms have figured prominently in the growth of developmental biology. Of these, Drosophila has been the most advantageously employed with the above point in mind. Very little is known of the genetics of other organisms commonly employed as developmental systems. Volvox, although it has only recently received significant attention from developmental biologists, offers the potential for a simultaneous biochemical and genetic analysis of development. The purposes of this study were to examine basic aspects of RNA metabolism in Volvox and establish a set of standard procedures for continued research in the biochemistry of development of this organism.

Details of the morphology and development of Volvox carteri have been thoroughly reviewed (Starr, 1970; Kochert, 1975). Several varieties of this species have been identified and studied but the one employed for most research in this laboratory has been Volvox carteri f. nagariensis Iyengar, female strain HK-10 (Starr, 1969).

The asexual spheroid consists of 3-5000 small biflagellate somatic cells and 10-16 large reproductive cells (gonidia). Each gonidium gives rise to a new spheroid which, in its turn, is released from the parent and becomes a member of the next generation of spheroids.

The genesis of a spheroid from a gonidium involves a tightly programmed series of morphogenetic events. The important stages of the developmental life cycle are shown diagrammatically in Figure 1 and are described in the Results.

There are significant similarities between the development of a Volvox spheroid and the embryogenesis of many higher multicellular organisms. The growth of a gonidium (Stages 1 and 2, Figure 1) recalls the extensive maturation process in a metazoan oocyte. RNA and protein synthesis rates are very high during the gonidial growth period (Yates, 1974; Kochert, 1975; this study) as they are during oogenesis. The ability of all stages of embryogenesis to proceed in the absence of illumination (Caplen, personal observation) suggests that the gonidium accumulates food reserves and stored energy in the same way that the oocyte prepares for the energetic demands of early embryogenesis by amassing its reserves largely in the form of yolk.

The cleavage pattern of the young Volvox embryo has been carefully described (Janet, 1923; Kochert, 1968). The changing orientation of cleavage planes with each successive synchronous division results in a cleavage pattern reminiscent of the spiral type characteristic of early development in many lower vertebrates. The first five synchronous cleavage divisions give rise to morphologically identical daughter

cells. At the next division the anterior-most sixteen cells in the embryo each yield two cells unequal in size. The larger of each of these cells becomes a gonidial initial thus establishing the germ cell line. The position of gonidial initials in the embryo and consequently of gonidia in the spheroid is rigidly established by the localization of germ cell determinants in the maturing gonidium. Kochert and Yates (1970) have shown that mature gonidia exposed to UV irradiation of intensity sufficient only to penetrate the peripheral cytoplasm develop into spheroids which lack one or more gonidia at sites corresponding to the irradiated region. Recalling earlier UV irradiation studies with the eggs of insects and amphibians which demonstrated the existence and nature of the germ plasm, a similar localization of germ cell determinants in the mature gonidium is thus implied.

The inversion process following cleavage is the major morphogenetic event in Volvox development. The mechanism of inversion is based strictly on coordinated changes in the shapes of embryonic cells (Kelland, 1969; Pickett-Heaps, 1970; Viamontes and Kirk, 1977) and in this way differs fundamentally from gastrulation which involves migration of groups or layers of cells over one another. Still, inversion and gastrulation are analogous in that both give basic form to

the developing organism.

Only after inversion is completed do the somatic cells of the Volvox spheroid manifest full differentiation characterized by the appearance of both flagella and the synthesis and secretion of matrix material; they also become mitotically inactive. Embryonic cells of metazoans similarly show, at most, only partial differentiation when still actively dividing, while complete differentiation in adult cells generally coincides with the cessation of mitotic activity. By contrast, the fully differentiated cells of adult plant tissue, in many cases, retain the capacity to reproduce and such cells may be isolated and give rise to entire new plants (Steward et al., 1952; Halperin, 1966).

My brief comparison of development in Volvox and the metazoan is not intended as an argument for phylogenetic kinship between the two groups. All evidence indicates that the Volvocales and metazoa arose independently from unicellular ancestors. The similarities do, however, suggest that with the same fundamental building blocks and information system (e.g. the constituents of eucaryotic cells and the universal genetic code) these phylogenetically divergent groups have evolved clearly analogous morphogenetic strategies for embryogenesis.

As pointed out above, the real value of a research or-

ganism may lie in its accessibility to diverse experimental approaches. To the cytological and biochemical studies of Volvox development may be added genetic studies. The isolation of developmental mutants representing morphogenetic defects at every important stage of embryogenesis (Sessoms and Huskey, 1973) makes Volvox an especially suitable subject for coordinated biochemical and genetic studies. Volvox provides a simple and functional model for studying mechanisms of control in development and cellular differentiation.

MATERIALS AND EQUIPMENT

Commercial Sources

Radioisotopes - 2-³H-adenine (27 Ci/mmol), (5,6)-³H-uracil (40 mCi/mmol), 5-³H-uridine (26.7 Ci/mmol), K₂ ³³PO₄ (500 Ci/ml), Liquifluor, and Protosol were supplied by New England Nuclear, Boston, MASS.; glycylglycine, Trizma Base, Protease Type VI, Firefly Lantern Extract (FLE-250), diethylpyrocharbonate, ATP (disodium salt), and Sigmacel by Sigma Chemical Co., St. Louis, MO; agarose (Grade B) and Miracloth (a product of Chicopee Mills) by Calbiochem, La Jolla, CA; pancreatic ribonuclease A, ribonuclease T₁, and DNase (EP, RNase-free) by Worthington Biochemical, Freehold, NJ; acrylamide (5512), N, N'-methylene bis-acrylamide, 7x7 cm electron microscope film sheets (160 8124) developer (D-19), fixer, and propylene oxide by Eastman Kodak, Rochester, NY; sodium dodecyl sulfate (SDS), phenol, toluene, trichloroacetic acid, and ammonium persulfate by Fisher Scientific Co., Pittsburgh, PA; diamond knife for thin sectioning and Ludox AM by E.I. Dupont, Wilmington, DE; oligo (dT) cellulose (T-3) by Collaborative Research, Inc., Waltham, MASS; Nitex monofilament nylon mesh filter screens by Tobler, Ernst, and Traber, Co., Elmsford, NY; No. 3 paper filter discs (2.4 cm) and glass fiber (GF/A) filters by Whatman, Ltd., England; glutaraldehyde and materials for Maraglas, Polyscience, Inc., Rydal, PA; ultramicrotome

(MT-2) by Ivan Sorvall, Inc., Norwalk, CT; electron microscope (EM9S-2) by Carl Zeiss, Ober Kochen, West Germany; microfuge (Model 152) by Beckman Instruments, Spinco Division, Palo Alto, CA; silica gel thin layer chromatography plates by Quantum Industries, Fairfield, NJ; slab gel electrophoretic apparatus by E.C. Apparatus Co., St. Petersburg, FLA; N, N, N', N'-tetramethylethylene-diamine and electrophoretic apparatus by Canalco, Rockville, MD; and Ilfoprint Processor (1501), activator, stabilizer, and Ilfoprint single weight glossy paper by Ilford, England. All other chemicals used were of laboratory or reagent grade.

METHODS

Growth and Maintenance

Volvox carteri f. nagariensis, female strain HK-10 (obtained from the Algal Culture Collection of R.C. Starr, then at Indiana University) was used for all experiments described in this study. Axenic stock cultures were maintained at about 25°C in 16 x 100 mm screw cap culture tubes containing 10 ml of synthetic Volvox Medium (SVM) adjusted either to pH 7 (Provasoli and Pintner, 1959) or pH 8 (Starr and Jaenicke, 1974). Illumination was provided to the cultures by "cool-white" fluorescent tubes at an approximate intensity of 400 foot-candles, and was programmed for a 16 hr:8 hr light-dark cycle. Transfers of stock cultures were made twice monthly.

The larger stock and working cultures required for most experimental work were normally started by inoculating aliquots of stock cultures or single spheroids into larger vessels containing sterile SVM. One hundred, 300, and 500 ml cultures (in 250 ml, 500 ml, and 1 l Erlenmeyer flasks) were grown with continuous bubbling by cotton-filtered "house" air.

Asynchronous populations used in some earlier studies were obtained by transferring aliquots from a stock tube

culture to larger vessels containing sterile SVM pH 7 and continuing growth on a 16 hr:8 hr light-dark illumination cycle at an intensity of 400-500 foot-candles and at room temperature (25°C).

Synchronous Growth

Synchronous populations with a generation time of 48 hr were established by inoculating single spheroids into 300 or 500 ml of sterile SVM pH 8 and continuing growth on a 32 hr light-16 hr dark cycle (Kirk and Kirk, 1976). Light intensity was maintained at 1400 foot-candles, as measured at culture level, and a constant temperature of 32°C maintained by immersing the cultures in a temperature-controlled water bath receiving illumination from the bottom and two sides (see Figure 2). After several generations, when the concentration of spheroids had reached about 50-100 spheroids/ml in a given flask, continuous synchrony and population density were maintained by regular transfers of aliquots of cultures to fresh flasks of SVM on alternate days, 24 hr after the start of the illumination period. At the time of transfer culture flasks were removed from their growth chamber and placed near and slightly below a small lamp. Those spheroids which aggregated at the point in the culture flask closest to the light source were transferred via a sterile Pasteur pipette to a fresh flask of media.

Culture Conditions for Radioisotopic Labelling

Radioisotopic labeling of spheroids was usually carried out in either 20 x 150 mm glass culture tubes aerated by gentle mechanical shaking or in 250 ml flasks containing 100 ml of medium aerated by cotton-filtered "house" air. Material was prepared for incubation with label by concentrating the contents of an experimental culture on Miracloth or a Nitex filter (Yates et al., 1975) and resuspending in the appropriate volume of sterile SVM as described in the figure legends. Details of incubation conditions are also given in the figure legends.

Radioisotopic Labeling

The labeled precursor primarily used in these studies was 2-³H-adenine (NEN, 27 Ci/mmol). Other labeled precursors used were 5-³H-uridine (26.7 Ci/mmol), (5,6)-³H-uracil (40 mCi/mmol), and K₂ ³³PO₄ (500 uCi/ml) but, for reasons detailed in the 'Results', 2-³H-adenine was routinely used in most experiments. All radioisotopic labeling was carried out in SVM except where otherwise noted.

Uptake and incorporation of labeled precursor was found to be most efficient when the concentration of spheroids did not exceed 700-800 spheroids/ml, thus labeling was normally carried out on cultures containing about 500 spheroids/ml.

Uptake and incorporation of radioisotope by whole spheroids was measured in 5 or 10 ml cultures in disposable tubes, aerated by shaking. Immediately upon addition of label ("time 0"), and at regular intervals thereafter, duplicate 1 ml aliquots were removed from the incubating culture. Spheroids were separated from culture medium by filtration through 20 μ Nitex screens, and the uptake assessed by measuring the depletion of radioactive label from the medium. Ten or 20 μ l aliquots of this medium were spotted, in duplicate, on Whatman No. 3 (2.4 cm) filter circles. After drying under a sunlamp, the circles were transferred to scintillation vials, 4 ml of a standard toluene-based scintillant added, and capped vials counted in a Nuclear Chicago Mark II liquid scintillation spectrometer. Incorporation of label into RNA was determined by measuring the amount of cold trichloroacetic acid (TCA) precipitable, alkali labile radioactive material found in experimental spheroids after incubation with radioactive precursor. The spheroids, collected by filtration, were resuspended in 1 ml of a homogenizing solution containing 0.01 M Tris, 0.001 M EDTA, pH 7.5 and 0.5% sodium dodecyl sulfate (SDS) and the suspension incubated for 5 min at 55°C. Each homogenate was then divided into two 0.5 ml portions, one of which was immediately treated with an equal volume of cold 10% TCA for 1 hr. The other portion

was incubated with an equal volume of 1 N NaOH for 2 hr at 65°C to hydrolyze RNA, and after cooling it was similarly precipitated with Whatman GF/A glass fiber filters under mild vacuum, washed with 5% TCA, 95% ethanol, dried, placed in scintillation vials with 4 ml of scintillant, and counted.

Preparation of RNA Extracts

For bulk isolation of non-radioactive RNA, 300 or 500 ml stock cultures of spheroids were harvested on Miracloth and resuspended in 100 ml of fresh medium. Protease (Sigma, Type VI) was added to a concentration of 500 or 1000 ug/ml and the culture incubated with light and bubbling at room temperature until gonidia and somatic cells were essentially free of parental spheroid matrix as determined by microscopic examination. The two types of cells were separated at this point by the method described below and either RNA was extracted from each individual cell type or prepared from total cell suspensions. Separation of gonidia and somatic cells was accomplished using the method of Yates et al. (1975). The method consists of passing the cell suspension sequentially through a series of 40 μ and 20 μ mesh Nitex filters. The larger gonidia were trapped on the 40 μ filter while the smaller somatic cells passed through the 20 μ filter and were collected essentially free of gonidia.

For RNA extraction, cells were washed with SVM, collected by centrifugation at 4000 x G for 5 min, and resuspended in 5 vol of ice cold homogenizing solution (0.1 M Tris, 0.05 M NaCl, 0.001 M EDTA, pH 7.5 or pH 9.0, 0.5% SDS, 0.5% diethylpyrocarbonate (DEPC)). The suspension was vigorously agitated for 3-4 min at 4°C on a Vortex Genie Mixer. An equal volume of a solution of buffer-saturated phenol, chloroform, and isoamyl alcohol (25:24:1) was mixed with the homogenate for 1 min and the phases separated by centrifugation at 10,000 x G for 10 min in a Sorvall HB-4 swinging-bucket rotor. Following removal of the upper aqueous phase, the interphase gel and organic phase were reextracted with fresh homogenizing solution. The first and second aqueous phases were combined and deproteinized repeatedly with phenol-chloroform-isoamyl alcohol until there was no precipitated material visible at the interface between the aqueous and organic phases. Nucleic acids were then precipitated from the final aqueous in the presence of 0.1 vol of 20% KCl and 2 vol of absolute ethanol for at least 15 hr at -20°C. Purified nucleic acids were stored under ethanol at -20°C until needed.

Final purification of RNA

When the elimination of DNA was required from a particular sample, the nucleic acid precipitate was collected by centrifugation, washed with 70% ethanol, dissolved in a

buffer containing 0.1 M Tris, 0.05 M NaCl, 0.01 M MgCl₂, pH 7.5 and incubated with 20 ug/ml of DNase (Worthington, EP, RNase-free) for 30 min at room temperature. The treated sample was then deproteinized and precipitated as previously described.

Isolation of Polyadenylated RNA

RNA containing tracts of polyadenylate was isolated from total cellular RNA using either unsubstituted cellulose (Sullivan and Roberts, 1971) or oligo (dT)-cellulose chromatography (Aviv and Leder, 1972).

For chromatography on unsubstituted cellulose, purified RNA precipitates were dissolved in binding buffer (TSE, 0.1 M Tris, pH 7.5, 0.5 M NaCl, 0.001 M EDTA). Columns were prepared by loading a 4% suspension of cellulose (Sigmacel) in TSE into a 1 cm diameter glass tube with a layer of glass wool at the bottom. The cellulose was packed under atmospheric pressure to form a column 7 x 1 cm; this was then equilibrated with TSE. Samples, usually containing 5-10 A₂₆₀ units of RNA, were layered on top of the column in 2 ml of TSE Buffer and allowed to run onto the cellulose by gravity. Five 3 ml fractions were eluted with TSE and then 5 x 3 ml fractions were collected following elution with sterile distilled water (DW), pH 7.0. UV absorbing material was continuously monitored by an ISCO Model UA-2 ultraviolet analyzer.

The distribution of radioactivity in the samples was determined by spotting a 20 μ l aliquot of each fraction on a Whatman No. 3 filter disc and measuring cpm in a liquid scintillation spectrometer. For subsequent analysis by polyacrylamide gel electrophoresis, peak fractions in the DW eluate were pooled, the ionic strength rendered 0.3 M with 0.1 vol of 20% KCl, and the pooled sample precipitated with 2 vol of absolute ethanol in the presence of 25 μ g/ml of cold carrier rRNA isolated from yeast ribosomes.

Columns for oligo (dT)-cellulose chromatography were prepared by suspending 100 mg of oligo (dT)-cellulose in binding buffer (0.01 M Tris, pH 7.5, 0.4 M KCl, 0.001 M EDTA) and loading this suspension into a small, water-jacketed column made from a Pasteur-pipette (0.4 cm diameter) plugged with glass wool at the bottom. The oligo (dT)-cellulose was allowed to pack under gravity. After equilibration with binding buffer, the column was loaded with a sample of purified RNA in 0.5 ml of binding buffer at 12°C and washed with 3-4 ml of elution buffer (0.01 M Tris, pH 7.5, 0.001 M EDTA). 0.1 vol of 20% KCl was added to the eluted material and both unbound and bound RNA were precipitated separately with 2 vol of ethanol in the presence of 50 μ g/ml of unlabeled carrier yeast rRNA.

Isolation of Poly(A) Segments

Samples of total cellular RNA, which had been DNase-treated, were collected and dissolved in a buffer containing 0.1 M Tris, pH 7.5, and 0.3 M KCl. RNA was digested by incubating samples with 10 $\mu\text{g}/\text{ml}$ of pancreatic RNase A and 20 units/ml of RNase T₁ for 1 hr at 37°C. The digest was deproteinized once with phenol-chloroform-isoamyl alcohol and RNase-resistant material precipitated with ethanol in the presence of yeast rRNA carrier. After collection of the precipitate and dissolution in binding buffer the sample was applied to an oligo (dT)-cellulose column at 12°C. The column was washed with 3-4 ml of binding buffer at 12°C and then with 3-4 ml of elution buffer at 37°C, which were collected in 1 ml fractions. Peak fractions were pooled and ethanol precipitated in the presence of 50 $\mu\text{g}/\text{ml}$ of yeast 4 and 5S RNA carrier.

Electrophoretic Analysis of RNA and Poly(A) Segments

RNA samples were analyzed on composite polyacrylamide-agarose slab gels essentially according to the procedure of Peacock and Dingman (1968). Gels consisting of 2.0% polyacrylamide (Acrylamide, Eastman No. 5512) 0.5% bis-acrylamide, and 0.5% agarose were prepared in Peacock's Buffer (0.09M Tris, 0.09M Boric acid, 0.003 M EDTA, pH 8.3) and

polymerized with TEMED (N,N,N',N'-tetramethylethylenediamine) and ammonium persulfate. Ten μ l to 50 μ l samples of RNA, dissolved in Peacock's Buffer containing 20% glycerol, were layered onto gels which had been pre-electrophoresed for at least 50 min. The running buffer was Peacock's Buffer containing 0.1% SDS. Electrophoresis was carried out for between 1½ to 2 hr at 5-10°C with a constant voltage of 200 to 225 V. After completion of the run, sample lanes were cut from the gel slab, frozen, and then sliced transversely into 1.6 mm slices using a multiple razor blade device. Each slice was incubated with 0.2 ml of Protosol solution (9 parts Protosol, 1 part water, 10 parts toluene) at 45°C for 15 hr and then counted with a standard toluene-based scintillant in a liquid scintillation spectrometer.

Analysis of poly(A) segments was performed on 12% polyacrylamide gels in 5 mm ID acrylic plastic tubes. The gels were prepared in Loening "E" Buffer (0.036 M Tris, 0.030 M NaH_2PO_4 , 0.001 M EDTA, pH 7.8) and polymerized with TEMED and ammonium persulfate; gels were pre-electrophoresed for 1 hr. Radioactively-labeled samples in Loening Buffer containing 20% glycerol and cold yeast 4 and 5S RNA as a marker were applied to the gels and electrophoresed in Loening Buffer containing 0.1% SDS for about 4 hr at 4°C with a constant current of 4 ma per gel tube. After the run gels were sliced

without freezing; subsequent procedures were identical to those described earlier for slab gels.

RNA Rapid Microextraction Procedure for Gonidia and Embryos

This procedure was devised to permit the rapid isolation of radioactively labeled RNA from small numbers of gonidia or embryos. Two to 5 ml of synchronously growing spheroids, at a concentration of 400-500 spheroids/ml, were labeled in a culture tube with 50-100 $\mu\text{Ci/ml}$ of 2- ^3H -adenine for 5 hr as previously described. At the end of the incubation period the entire contents of the tube was poured into a chilled Dounce homogenizer and gonidia (embryos) released from parental spheroids by ten gentle strokes of the loose-fitting ("A") pestle. The "Dounced" material was passed through a tandem of 80 μ and 40 μ mesh Nitex filters under slight vacuum. After washing with 50 ml of SVM, the gonidia (embryos) trapped on the 40 μ mesh screen were suspended in 0.3 ml of a purified Ludox AM (Kirk and Kirk, 1976), SVM, polyvinylpyrrolidone stock solution (24 parts, 25 parts, 42 parts, specific gravity = 1.043 gm/cc) and diluted 1:3 with SVM to give a final specific gravity of 1.030 gm/cc. Specific gravity measurements were made by weighing accurately measured volumes of sample solution. A 20 μl aliquot of this sample was taken for a count of gonidia (embryos) present and

the remainder spun at top speed for 1 min in a microfuge at room temperature. The supernatant, containing unsolubilized cell sheets, was removed and the pelleted gonidia (embryos) resuspended in 0.1 ml of homogenizing solution (0.090 M Tris, 0.090 M boric acid, 0.003 M EDTA, pH 8.3, 20% glycerol, 0.5% SDS, 1.0% diethylpyrocarbonate). These were then homogenized in the microfuge tube using a home-made microhomogenizer. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added and, after mixing, the aqueous and organic layers were separated by centrifugation at top speed for 2.5 min in a microfuge at room temperature. The aqueous phase was removed and stored frozen at -40°C until it was required for electrophoretic analysis.

Measurement of ATP Pools and Pool Specific
Activity in Gonidia and Embryos

Synchronously grown 300 ml stock cultures at various stages of gonidial or embryonic development were harvested on Miracloth under sterile conditions and resuspended in 100 ml of fresh SVM in 250 ml flasks. Three hundred μCi ($3 \mu\text{Ci}/\text{ml}$) of 2- ^3H -adenine were added and the cultures incubated for 3 hr periods under standard light and aeration conditions. At the end of each incubation, cultures were harvested on Miracloth and resuspended in a small volume of fresh SVM. Gonidia (embryos) were separated from parent spheroids by

1-5 gentle strokes of the loose-fitting ("A") pestle of a Dounce homogenizer followed by filtration through a tandem of 80 μ and 40 μ Nitex filters as described previously. The highly enriched gonidial (embryo) fraction was further purified by 2 min of centrifugation in Ludox solution (purified Ludox AM diluted 1:11 with SVM - specific gravity 1.030 gm/cc). The supernatant was removed and the pellet containing gonidia washed with 1 ml of SVM. A 10 μ l aliquot of this suspension was taken and assayed for the number of gonidia (embryos); the gonidia were then repelleted by centrifugation.

Cold 0.4N perchloric acid (PCA) was added to bring the total volume of the sample to 0.3 ml and homogenized for 1 min in a motor-driven, teflon-pestled, pre-cooled Potter-Elvehjem homogenizer. One-tenth ml fractions of the sample were placed in plastic microfuge tubes and neutralized with 0.04 ml of 1 N KOH. Insoluble debris and acid-precipitable material were removed by microfugation for 2 min. The supernatants were transferred to new plastic microfuge tubes and frozen at -40°C until further use.

Samples were assayed for ATP by the luciferin-luciferase reaction as described by Stanley and Williams (1969) and as modified by Emerson and Humphreys (1971). A 50 μ l aliquot of a thawed PCA extract or 5-30 μ l sample of a 10^{-7}M ATP standard in 0.01 M potassium phosphate pH 7.4, was added to

1 ml of 0.05 M glycylglycine pH 7.4 in a glass scintillation vial insert. One hundred microliters of a solution containing a buffered extract of firefly lanterns (Sigma FLE-250 reconstituted in 25 ml of distilled water) were added and mixed thoroughly with the sample. This was then counted immediately for six consecutive 0.1 min intervals, separated by a six sec printout time in a Beckman LS 130 scintillation spectrometer using the standard tritium window setting. The fourth or fifth successive 0.1 min count was generally found to be proportional to the ATP content of the sample as determined by the measurement of a series of ATP (Sigma, A2383) standards.

The amount of radioactivity associated with ATP in each sample was established by chromatographically purifying ATP from samples and counting in a liquid scintillation spectrometer as described previously. Aliquots of KOH neutralized acid soluble extracts were chromatographed on pre-coated silica gel thin layer plates. Two microliters of a 2 mg/ml ATP standard solution were spotted at the origin of a 5 x 10 cm silica gel plate, and then 5 μ l of a neutralized acid extract were spotted precisely on top of the standard. Chromatography was performed for approx. 4 hr using a mixture of isobutyric acid, water, and NH_4OH (66:33:1) as the solvent. Spots were scraped from the regions corresponding to the

positions of the ATP standards, into scintillation vial inserts and incubated with Protosol solution for 4 hr at 45°C. Four ml of a standard toluene scintillant were then added and each sample counted in a liquid scintillation spectrometer.

Specific activity is calculated as cpm per pmol of ATP.

Electron Microscopy

Whole spheroids, gonidia, or embryos were fixed in 2% glutaraldehyde buffered with 0.02 M sodium veronal, 0.015 M sodium acetate, 0.001 M CaCl_2 , pH 7.2. All dilutions of the stock glutaraldehyde solution were made with SVM instead of distilled water (B. Hershenov, personal communication). One hour was sufficient time for fixation of all material.

Samples were post-fixed in veronal acetate-buffered OsO_4 (Palade's fluid, 0.2% OsO_4 , 0.03 M sodium veronal, 0.016 M sodium acetate, 0.001 M CaCl_2 , pH 7.2) (Palade, 1952) for 20 min, washed, and stained with aqueous uranyl acetate (1%) for 1 hr. After dehydration in a graded ethanol series (75%, 95%, 100%), and washes with 100% ethanol-propylene oxide, propylene oxide, and propylene oxide - Maraglas (1:1), the material was embedded in Maraglas (Freeman and Spurlock, 1962). Thin sections were cut on a Porter-Blum MT-2 ultra-microtome and post-stained with lead citrate (Reynolds, 1963). Observations and electronmicrographs were made with a Zeiss

EM9S-2 electron microscope. Negatives were developed for 4 min at 20°C and prints made on Ilford single weight glossy paper processed with the Ilford rapid print processing system.

Glassware and Solutions

All glassware used for RNA experiments was detergent-washed, distilled water-rinsed, autoclaved, and oven-baked for at least 3 hr. Buffer solutions were autoclaved.

RESULTS

Growth of Synchronous Cultures of
Volvox carteri f. nagariensis Strain HK-10

Success in biochemical studies of Volvox development depends, in the first place, on the establishment of substantial populations of synchronously growing spheroids with regular and predictable generation times. The synchrony method employed in this study is a modification of a system first developed in the laboratory of Richard Starr (personal communication; Kirk and Kirk, 1976). It is based on the ability of Volvox carteri, strain HK-10 to accelerate its rate of development in high pH media (Starr and Jaenicke, 1974). Under these conditions an entire life cycle is completed in 48 hr. Synchrony is achieved by growing populations of spheroids on a 32 hr light - 16 hr dark illumination cycle.

A diagram of the life cycle illustrating the key stages of development and the corresponding time in the light-- dark illumination cycle is shown in Figure 1. During the first 26-27 hr of the light period (stage 1, see Margolis-Kazan and Blamire, 1976) there is an expansion of young newly-released spheroids as the gonidia within them are enlarging and becoming highly vacuolated. At 28-29 hr condensation occurs (stage 2), a stage marked by the apparent disintegration of

Figure 1. Life Cycle of Volvox. Diagrammatic representation of the asexual life cycle. For fuller description see text.

Stage 1: Post-release expansion

Stage 2: Gonidial "condensation"

Stage 3: First cleavage

Stage 4: Cleavage

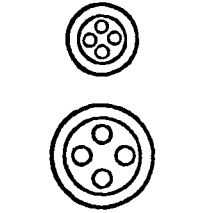
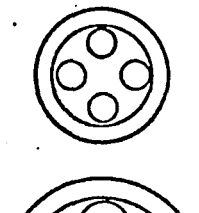
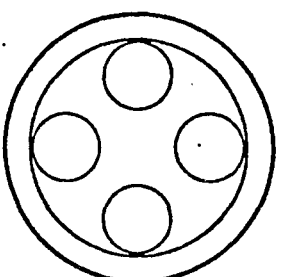
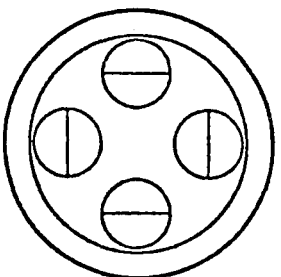
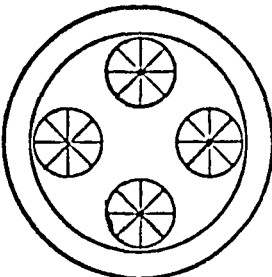
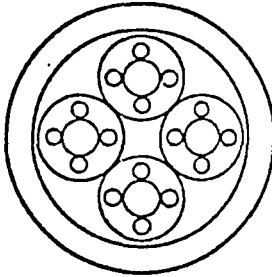
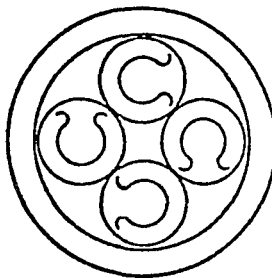
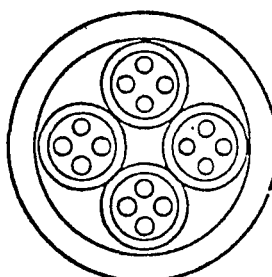
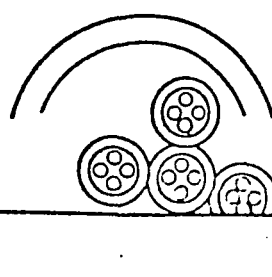
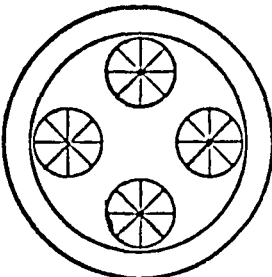
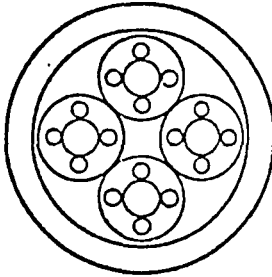
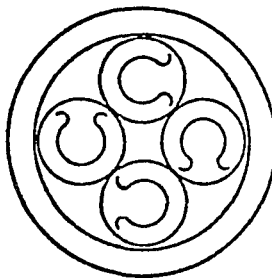
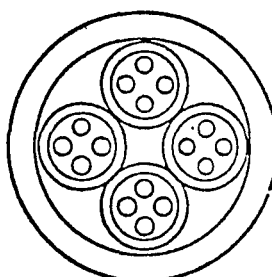
Stage 5: Post-cleavage, pre-inversion

Stage 6: Inversion

Stage 7: Pre-release expansion

Stage 8: Release

The relative lengths of the light and dark periods in the diagram are not to scale.

		LIGHT	
			
			
			
			
1		2	
		DARK	
			
			
			
			
4		5	
		6	
		7	
		8	
			

the large vacuoles. The cytoplasm of the condensed gonidium assumes a dark green, coarsely granular appearance under the light microscope. Embryogenesis, which includes an initial period of rapid cleavage (stages 3-5) and inversion (stage 6), begins at approximately the 29th - 30th hr and continues for 6-7 hours. The newly-developed young spheroids begin their expansion within the parent (stage 7) and are released during the final hours of the dark period.

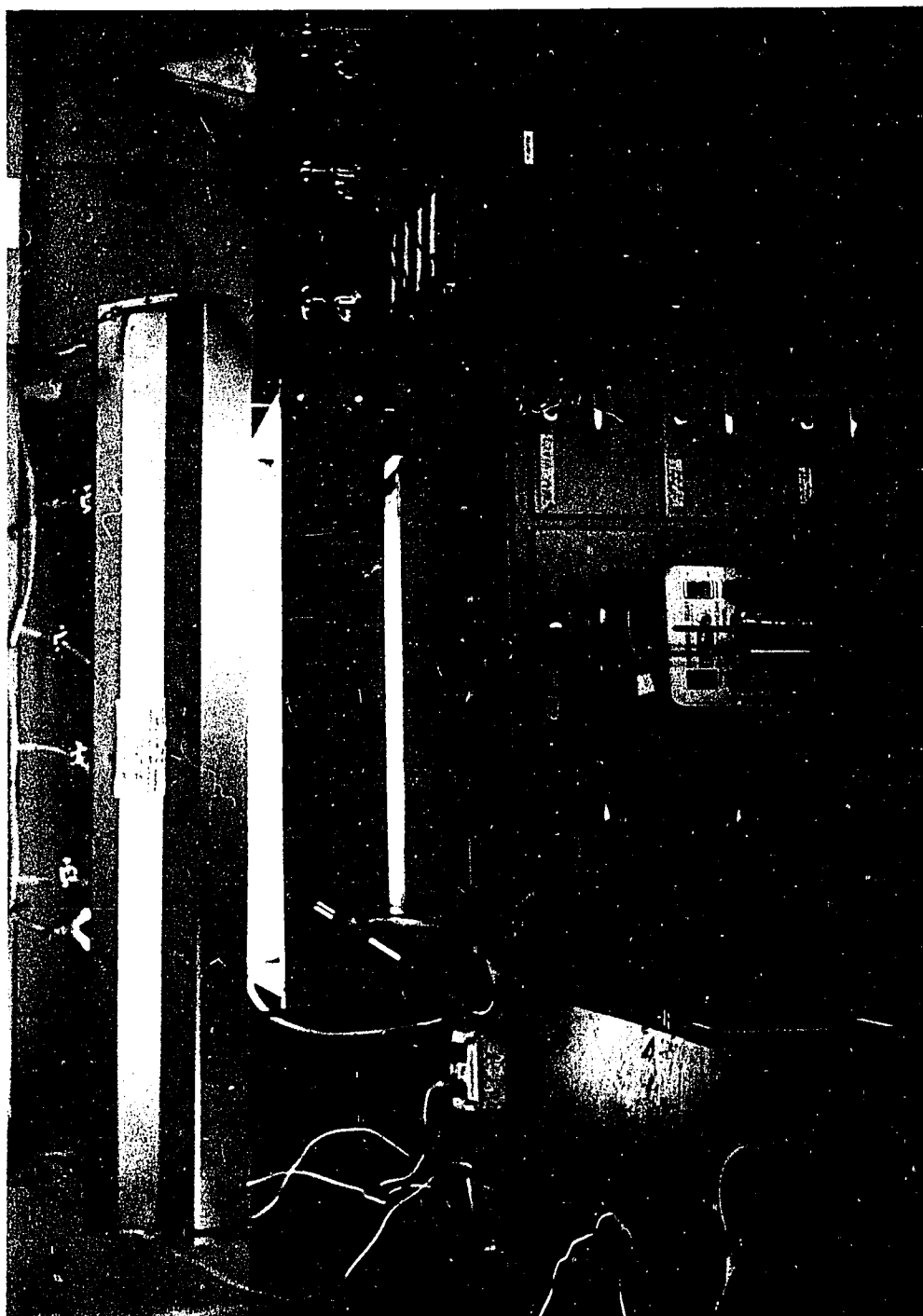
The degree of synchrony is assessed by microscopic inspection of samples taken from a culture. If at least 90% of the gonidia in the culture have entered embryogenesis, the culture is then used for experimental analysis. Cultures not meeting this criterion for synchrony are discarded.

Figure 2 displays the constant temperature water bath and automatically programmed light system that were employed in all of the synchrony studies reported here.

Radioactive Precursor Labeling Kinetics

The second requirement for this study involving the biochemical analysis of RNA metabolism in development, was a suitable radioactive precursor. Ideally, the precursor should be reasonably specific in the labeling of RNA, should be incorporated to high specific activity, and should label all species of RNA uniformly.

Figure 2. Water Bath - Growth Chamber used in experiments requiring synchronous populations of Volvox spheroids.



Four radioactive precursors were examined with the above characteristics in mind - 2-³H-adenine, 5-³H-uridine, (5,6)-³H-uracil, and K₂³³PO₄. Shaking tube cultures containing known quantities of actively growing heterogeneous spheroids were incubated individually with each of the labeled precursors (see Materials and Methods and appropriate figure legends). At "zero time" and at regular intervals thereafter aliquots of each culture were taken and assayed for uptake of radioactive label from the medium and incorporation into RNA as described in Materials and Methods.

The uptake and incorporation of 2-³H-adenine and (5,6)-³H-uracil in heterogeneous populations of spheroids were compared as shown in Figure 3. Although identical amounts of radioactive label were used (10 µCi/ml), 2-³H-adenine was incorporated into RNA to an approximately 10-fold greater extent (Figure 3B). The specific activity of 2-³H-adenine is several orders of magnitude greater than the specific activity of (5,6)-³H-uracil and this is certainly a major factor in the more efficient utilization. However, this phenomenon could not be attributed to preferential uptake of 2-³H-adenine by the spheroids as is shown in Figure 3A. Both precursors were taken up to about the same extent after a period of 7-8 hr. In fact, (5,6)-³H-uracil exhibited a greater rate of uptake by spheroids during the first 3 hr, exceeding that of

2-³H-adenine by as much as 100%.

A similar experiment was carried out with 5-³H-uridine and 2-³H-adenine (data not presented) and it was shown that, once again, 2-³H-adenine was incorporated into RNA with an approximately 5-10 fold greater efficiency.

The uptake and incorporation kinetics of ³³P were compared to those of 2-³H-adenine using heterogeneous cultures growing in standard SVM, and in SVM from which the sources of phosphate had been deleted. In standard SVM (Figure 4), while ³H-adenine showed essentially linear uptake and incorporation kinetics for the first 4-6 hr, the uptake of ³³P was, at best, negligible and incorporation did not appear to be linear. When the experiment was repeated using phosphate-free SVM the results shown in Figure 5 were obtained. The uptake and incorporation rates of 2-³H-adenine were approximately constant for the entire labeling period, but the incorporation rate of ³³P was obviously increasing over the same period.

Because 2-³H-adenine was shown to exhibit linear uptake and incorporation kinetics over the first 4 or 5 hours of the labeling period and appeared to be utilized the most efficiently by the spheroids, it was the radioactive precursor used in all of the subsequent labeling experiments to be reported in this study.

Following these initial studies, the uptake and incorporation behavior of ^3H -adenine was characterized more carefully. When 10 ml shaking cultures of spheroids (500/ml) were incubated with 50 μCi of label (10 $\mu\text{Ci}/\text{ml}$) the results shown in Figure 6 were obtained. Under these conditions both uptake and incorporation were linear for at least the first 4 hr (compare Figure 6 with the previous Figures showing 2- ^3H -adenine incorporation and uptake). A decrease in the rate of uptake, and an actual decrease in the amount of label incorporated in RNA, after approximately 5-6 hr incubation were consistently observed. (The experiment represented in Figure 5 shows atypical patterns, but it should be noted that labeling was not performed in the standard medium (SVM) in this case.) Since the percentage of label taken up never exceeded 90% it was assumed that the spheroids accumulated all of the available 2- ^3H -adenine in the culture medium but did not accumulate another tritiated impurity, which was present as a contaminant in the commercial 2- ^3H -adenine solution. Alternatively, the leveling-off of the uptake curve may have reflected an equilibrium between 2- ^3H -adenine being taken up and tritiated metabolic waste products being excreted by the cells.

If the plateaus that were observed both in the uptake and incorporation of 2- ^3H -adenine under the labeling condi-

tions described in the legend to Figure 6, were the result of depletion of labeled precursor from the culture medium, an identical culture incubated with a higher concentration of 2-³H-adenine should show continued linear uptake and incorporation beyond the time point where pleateauing occurred in a culture labeled with a lower concentration of 2-³H-adenine. The results of this experiment, in which cultures were labeled with 10 uCi/ml and 50 uCi/ml of 2-³H-adenine, are shown in Figure 7. Up to approximately 2 hr, the uptake and incorporation kinetics for both concentrations of 2-³H-adenine were very similar. At 3 hr and beyond, the characteristic leveling-off of uptake and incorporation became apparent in the culture labeled with 10 uCi/ml of 2-³H-adenine, while the culture labeled with 50 uCi/ml of 2-³H-adenine maintained approximately linear uptake and incorporation rates throughout this period. The almost identical initial rates of uptake and incorporation despite a five-fold difference in the concentration of exogenous label suggests that adenine may be transported into the cell by means of a saturable carrier-mediated system in the cell membrane. Using the stated specific activity of 27 Ci/mmol (supplied by the manufacturer), the calculated exogeneous concentrations of radioisotope at time "0" are $3.7 \times 10^{-7}M$ and $1.8 \times 10^{-6}M$ for the cultures containing 10 uCi/ml and 50 uCi/ml of 2-³H-adenine, respectively. These concentrations are similar to those which were found

Figure 3. Uptake and Incorporation of 2-³H-adenine and [5, 6]-³H-uracil into Volvox and RNA.

Duplicate 10 ml shaking tube cultures containing 400 asyn-
chronous spheroids/ml were incubated with 5 μ Ci/ml of 2-³H-
adenine and [5,6]-³H-uracil respectively in continuous light
at 25°C. At the times indicated, duplicate 1 ml aliquots of
culture were taken from each tube. Spheroids were separated
from culture medium. Uptake was measured by assaying the
supernatant for radioactivity and incorporation was measured
by determining cold TCA precipitable hot alkali labile radio-
activity in the spheroids (see Materials and Methods) % up-
take represents the average of cpms at time "0" minus average
cpms at each time point divided by time "0" cpms.

- A. % uptake = 2-³H-adenine (solid line)
[5,6]-³H-uracil (dashed line)
- B. Incorporation = 2-³H-adenine (solid line)
5,6-³H-uracil (dashed line)

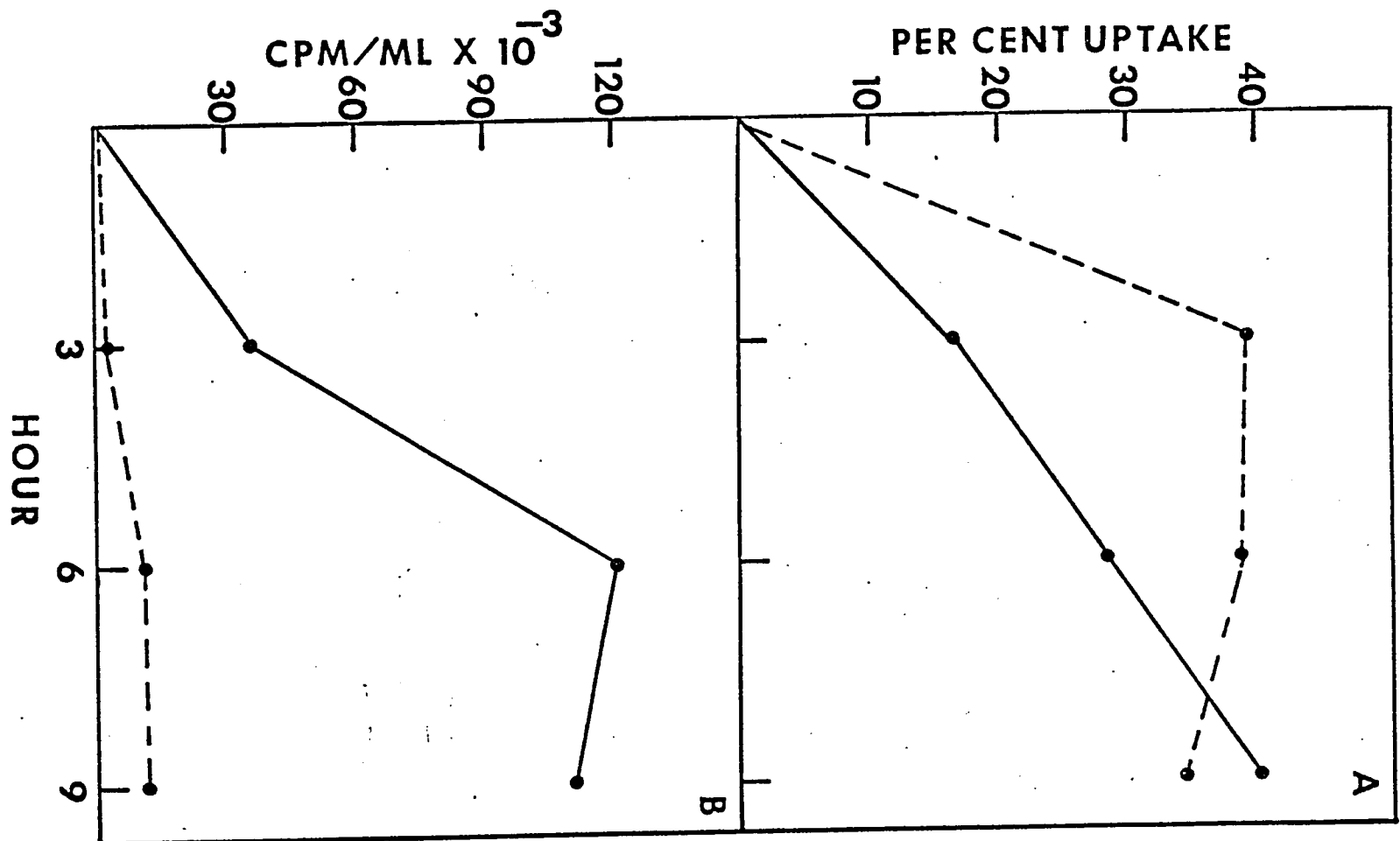


Figure 4. Uptake and incorporation of 2-³H-adenine and ³³PO₄ into Volvox growing in SVM.

Duplicate 10 ml shaking tube cultures containing 500 spheroids/ml in SVM were incubated with 5μCi/ml of 2-³H-adenine and ³³PO₄ respectively, in continuous light. At the times indicated 1 ml aliquots were withdrawn from each tube; uptake and incorporation were measured as described in the legend to Figure 3 and Materials and Methods.

- A. % uptake = 2-³H-adenine (solid circles)
 ³³PO₄ (X's)
- B. Incorporation = 2-³H-adenine (solid circles)
 ³³PO₄ (X's)

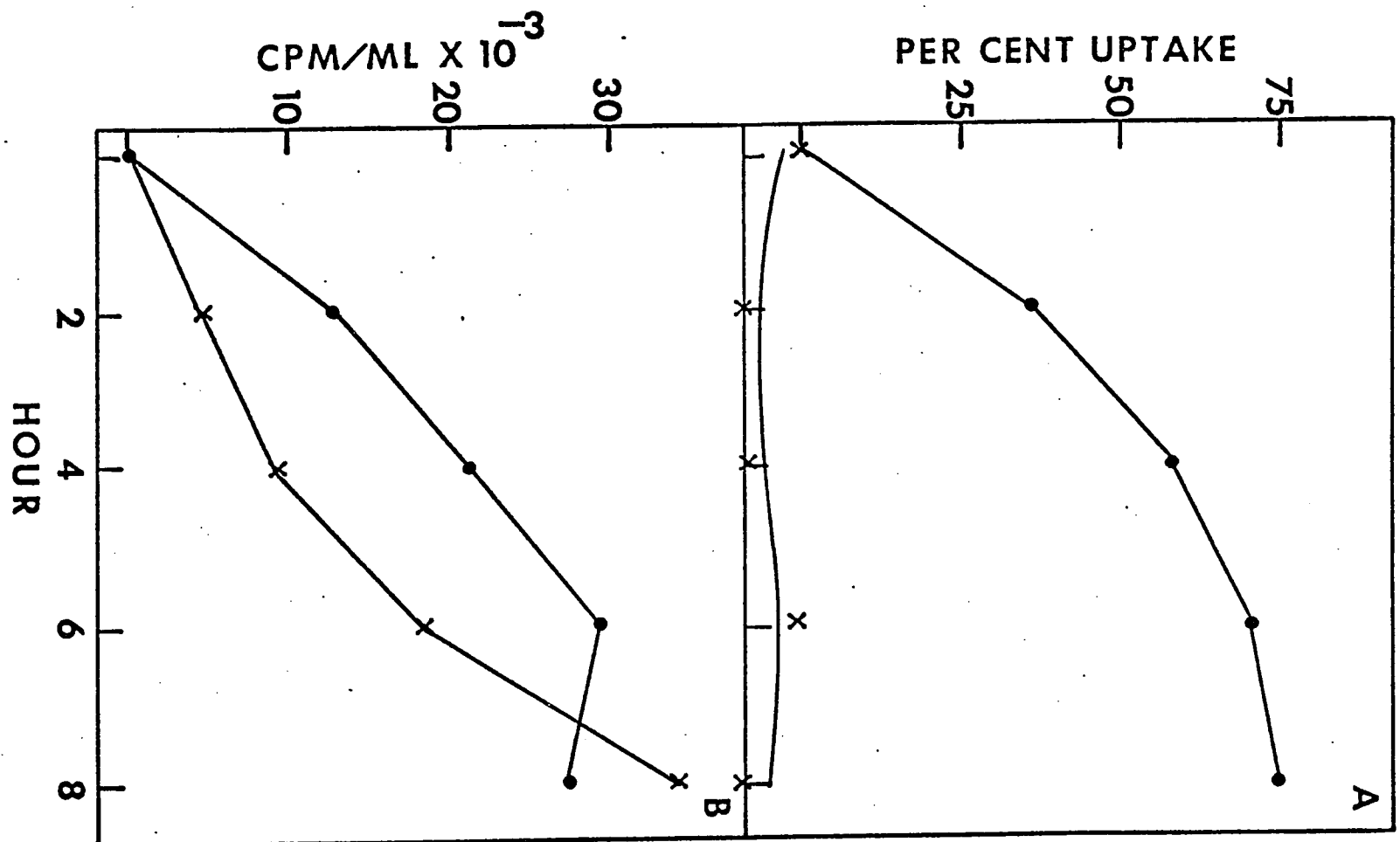


Figure 5. Uptake and Incorporation of 2-³H-adenine and ³³PO₄ into Volvox growing in phosphate-free SVM.

Duplicate 10 ml shaking tube cultures containing 500 spheroids/ml in SVM without phosphate (alpha-glycerophosphate was not included in the medium) were incubated with 5μCi/ml of 2-³H-adenine and ³³PO₄, respectively in continuous light. The procedures for measuring % uptake and incorporation were as described in the legend to Figure 3 and Materials and Methods.

- A. % Uptake = 2-³H-adenine (solid line)
 ³³PO₄ (dashed line)
- B. Incorporation = 2-³H-adenine (solid line)
 ³³PO₄ (dashed line)

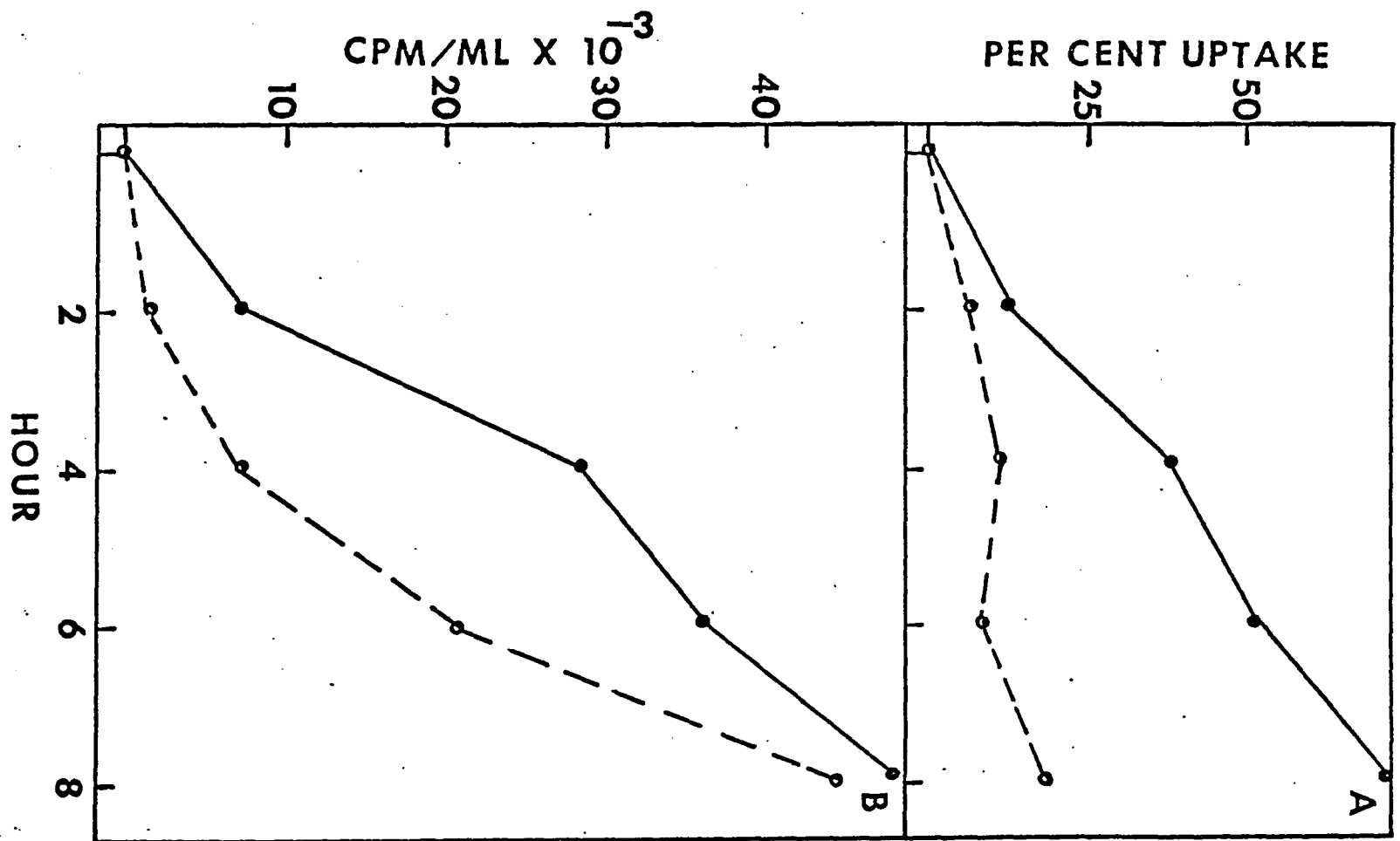


Figure 6. Uptake and Incorporation of 2-³H-adenine

A 10 ml shaking tube culture containing 500 spheroids/ml was incubated with 5uCi/ml of 2-³H-adenine in continuous light. Procedures for measuring % uptake and incorporation were as described in the legend to Figure 3 and Materials and Methods.

A. Uptake

B. Incorporation

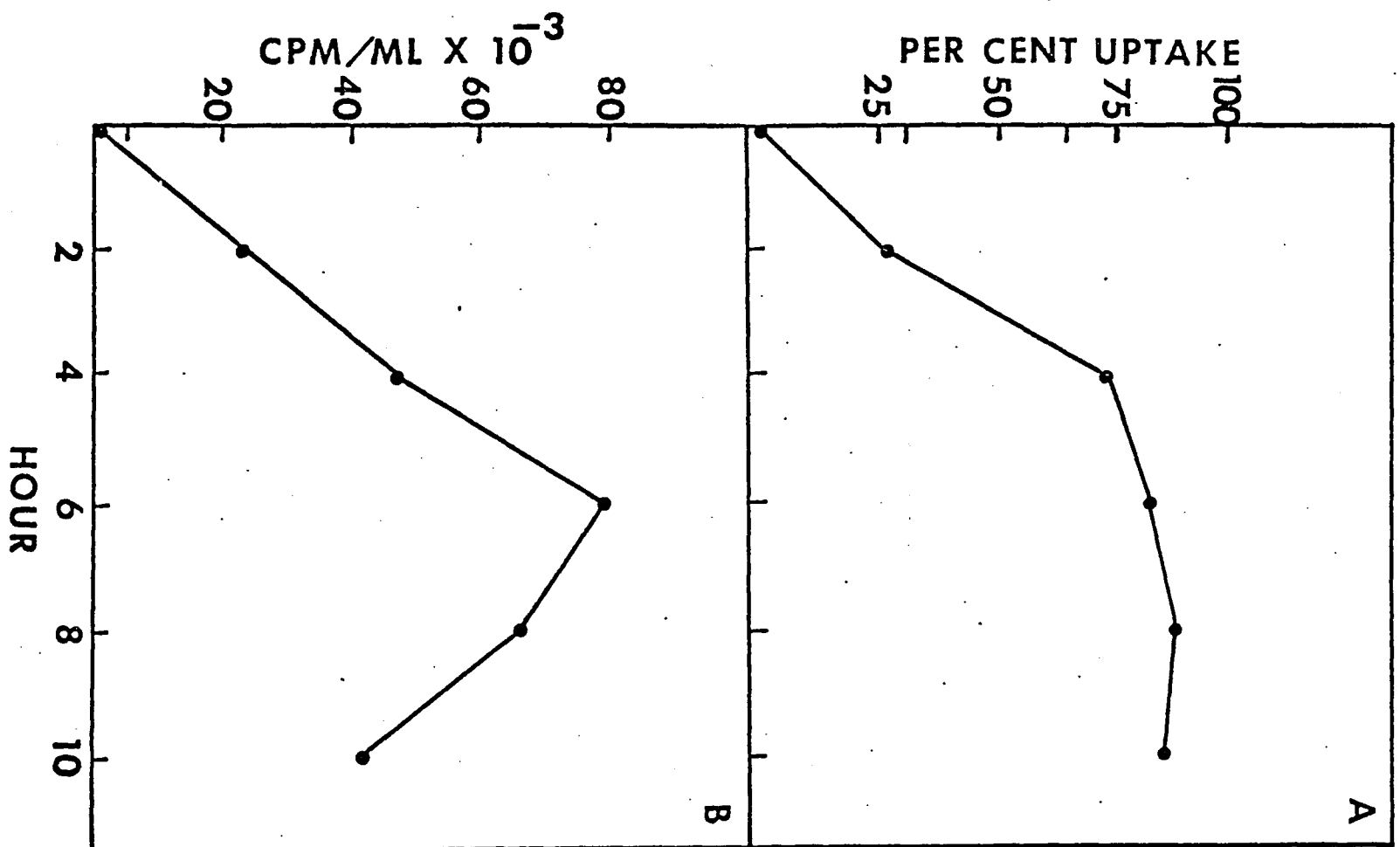
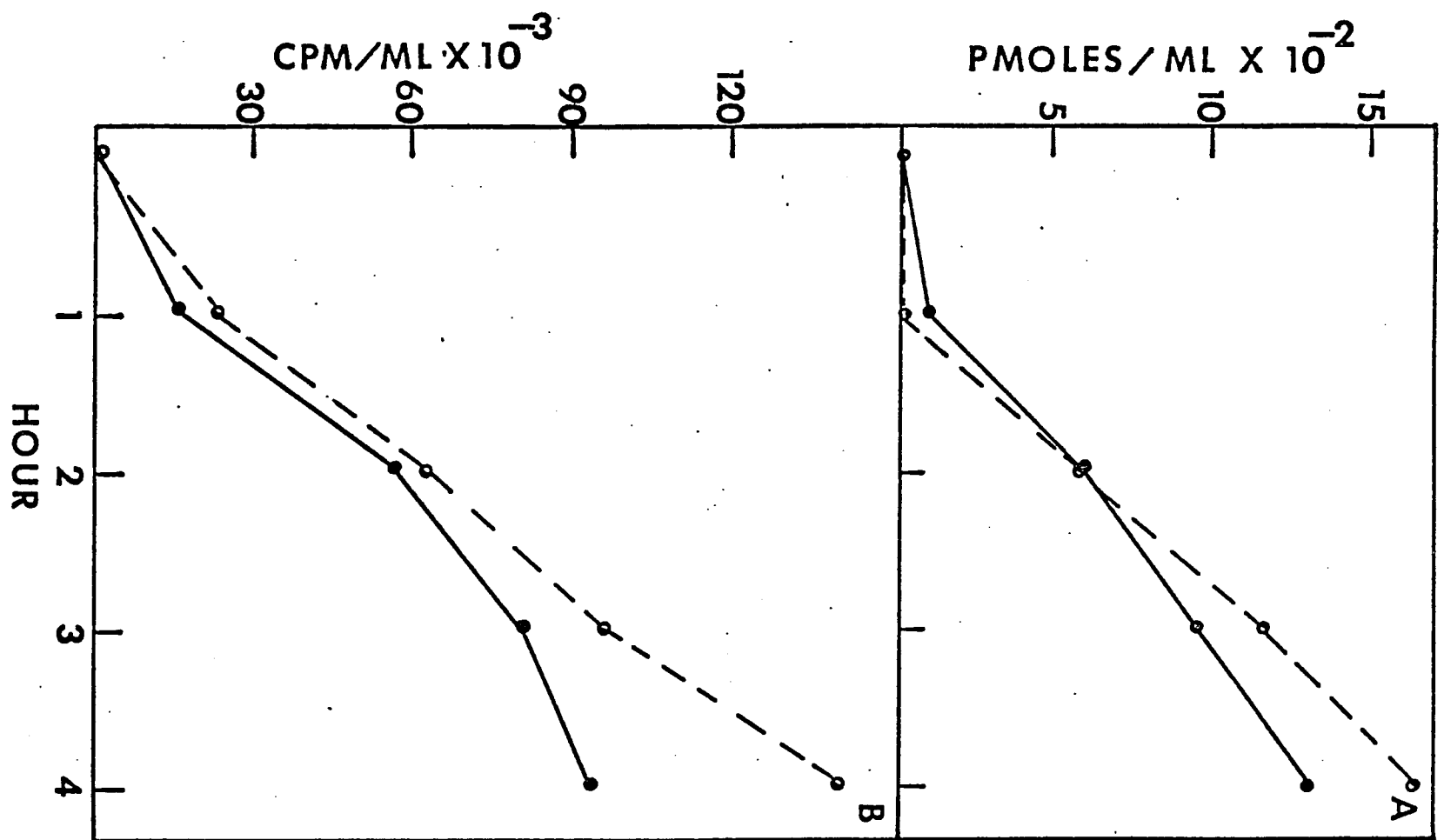


Figure 7. Kinetics of Uptake and Incorporation of 2-³H-adenine into Volvox using different concentrations of 2-³H-adenine.

Duplicate 10 ml shaking tube cultures containing 500 spheroids/ml were incubated with 5 μ Ci/ml and 25 μ Ci/ml of 2-³H-adenine, respectively in continuous light. Procedures for measuring uptake and incorporation were as described in the legend to Figure 3 and Materials and Methods. Uptake, expressed as p moles of adenine taken up per ml of culture, was calculated using specific activity information supplied with the radioactive label and the average cpm/ml taken up over each time interval.

- A. Uptake = 5 μ Ci/ml of 2-³H-adenine (solid line)
 25 μ Ci/ml of 2-³H-adenine (dashed line)
- B. Incorporation = 5 μ Ci/ml of 2-³H-adenine (solid line)
 25 μ Ci/ml of 2-³H-adenine (dashed line)



by Kirk and Kirk (1978) to give saturation kinetics for both arginine and urea (see Discussion).

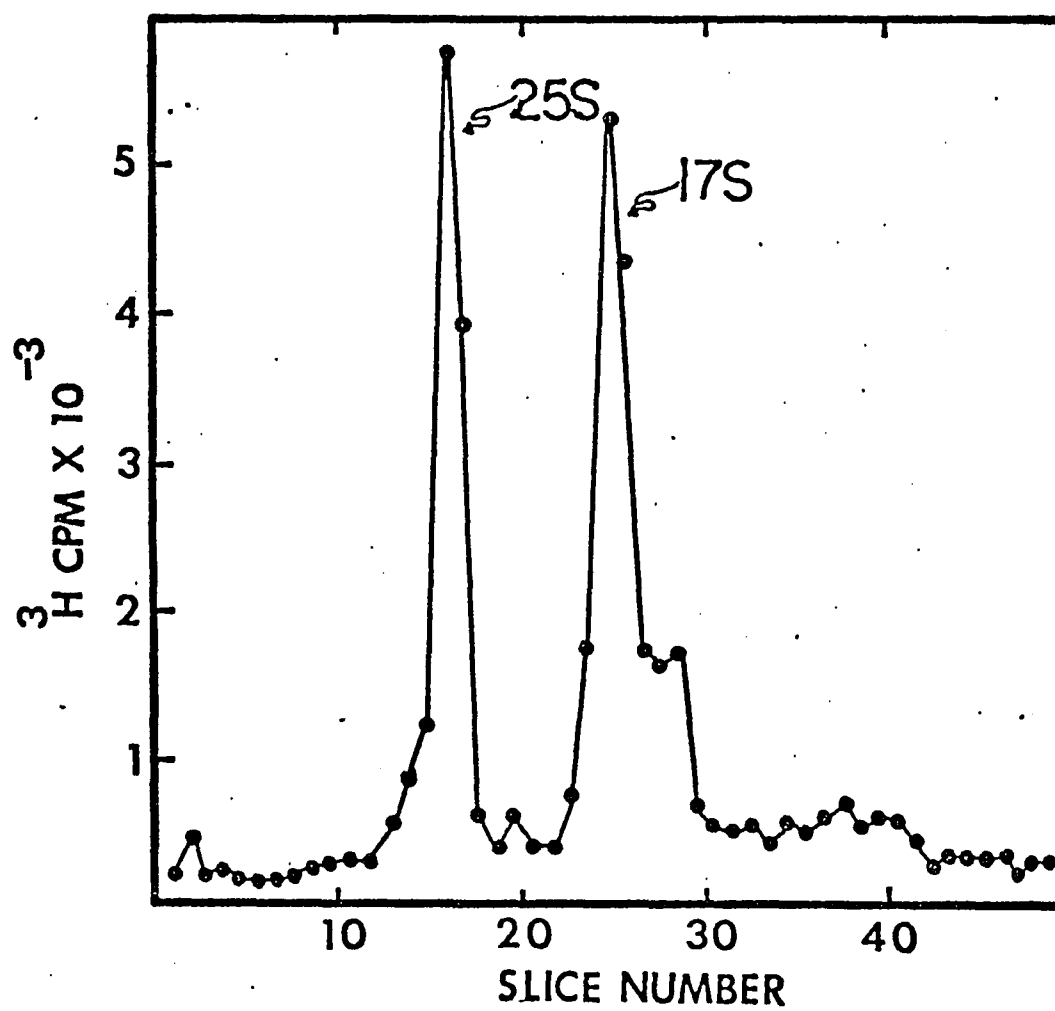
Isolation and Electrophoresis of RNA

RNA, isolated and purified by the procedure described in Materials and Methods from a radioactively labeled heterogeneous population of spheroids, was electrophoresed in composite 2.0% polyacrylamide-0.5% agarose slab gels according to the method of Peacock and Dingman (1968). Figure 8 shows an electrophoretic profile of total RNA labeled for 15-20 hr with 7 μ Ci/ml 2-³H-adenine. Two major species of RNA are observed. The faster electrophoresing material at slice 24 corresponds to the 17S RNA of the small ribosomal subunit, while the slower moving material at slice 16 corresponds to the 25S RNA of the large ribosomal subunit (Kochert and Sansing, 1971). The small peak in slice 20 represents chloroplast large ribosomal subunit (23S) RNA (Kochert and Sansing, 1971) while the peak on the leading edge of the 17S RNA (slice 28) represents chloroplast small ribosomal subunit (16S) RNA.

As has been reported previously (Kochert and Sansing, 1971) for other closely related strains of Volvox carteri, even the most effective measures taken to inhibit ribonucleolytic activity during the extraction procedure do not prevent a selective degradation of 23S RNA. The breakdown products

Figure 8. Electrophoretic pattern of total RNA.

A shaking, 5 ml tube culture of spheroids (500/ml) was incubated with 10 μ Ci/ml of 2-³H-adenine for 15 hr at 25°C with constant illumination. One hr prior to RNA extraction 500 ug/ml of protease was added to the culture to solubilize intercellular matrix material. RNA was extracted from the cell suspension and the precipitated, purified RNA sample, dissolved in Peacock Buffer/20% glycerol, was electrophoresed on a composite 2% polyacrylamide-0.5% agarose slab gel for 1 hr 50 min at 8°C. After electrophoresis gels were analyzed for the localization of radioactivity as described in Materials and Methods.



may be seen as a small peak at slice 36 and as a shoulder on the leading edge of the 17S RNA (slice 27). Degradation during RNA isolation may be the result of an in vivo break in the primary structure of the 23S RNA (see Introduction).

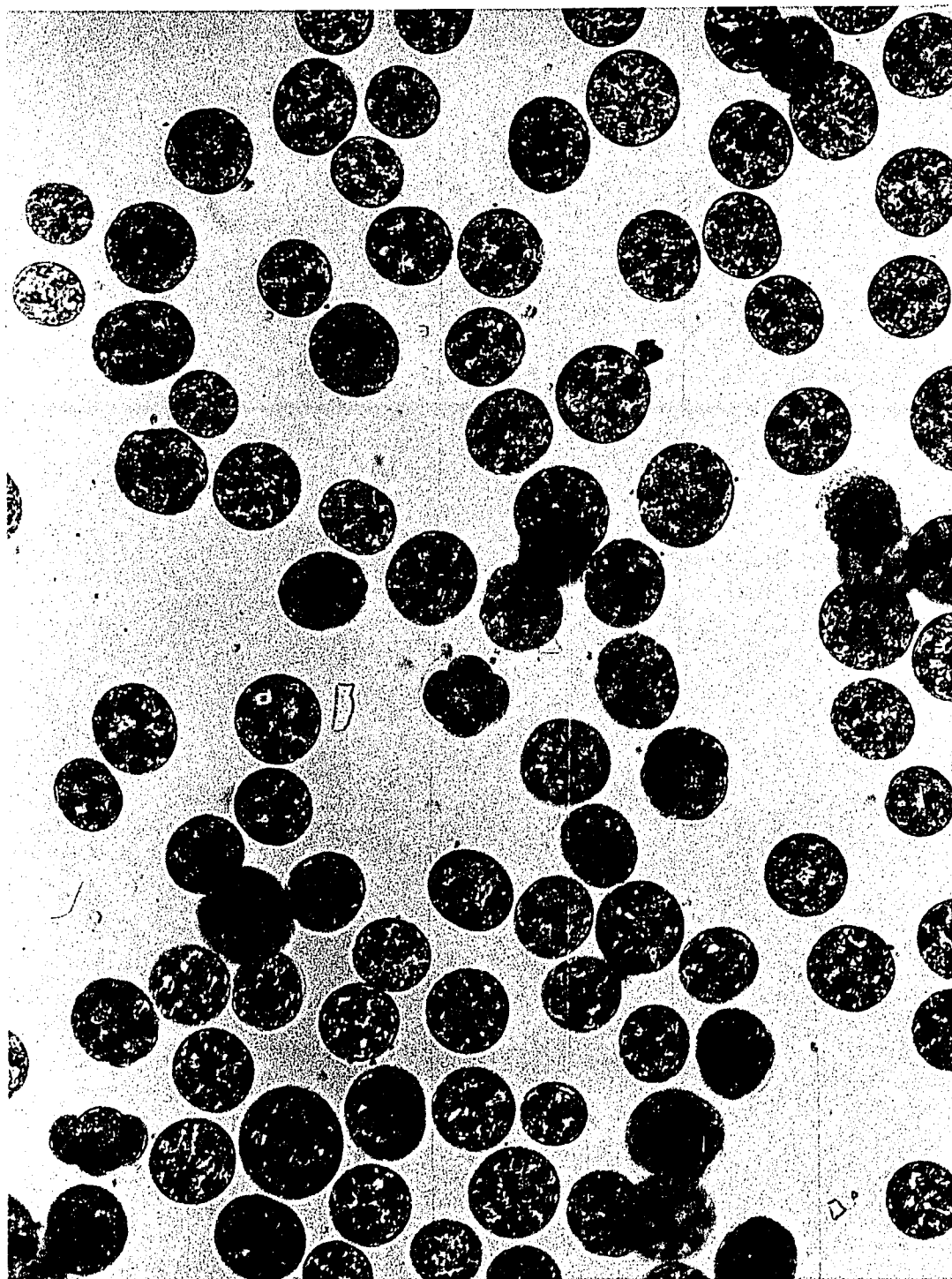
RNA Synthesis in Development

This section presents an electrophoretic analysis of RNA synthesis during the asexual development of Volvox carteri f. naqariensis. It also establishes a generally applicable procedure for the rapid micro-scale preparation and biochemical analysis of nucleic acids.

The developmental interval encompassed by this study includes the entire maturation period of the gonidium, cleavage, inversion, and the post-inversion period prior to release. The protocol used for the isolation and analysis of pulse-labeled RNA for these stages is described in Materials and Methods (see "Rapid Microscale Isolation of RNA"). The cell separation procedure involving selective filtration with "Nitex" screens and buoyant density separation with Ludox gives virtually pure preparations of gonidia and embryos as is shown in Figure 9. The entire procedure requires 15-20 min and the resultant nucleic acid isolate is sufficiently pure to be directly fractionated by electrophoresis.

The efficiency of this rapid isolation procedure was determined by measuring the percentage of total nucleic acids

Figure 9. Light Micrograph of Purified Gonidia. Gonidia were isolated from spheroids as described in "RNA Rapid Microextraction Procedure for Gonidia and Embryos" in Materials and Methods.



recovered in the deproteinized extract. Following phenol-chloroform deproteinization of the cell homogenate (see Materials and Methods), 5 μ l aliquots of the separated aqueous and combined inter- and organic phases were each treated with an equal volume of cold 10% TCA. The resulting precipitates were collected on glass fiber filters and the amount of radioactivity on them measured (see Materials and Methods). Percent recovery was calculated as the cpm measured in the aqueous phase divided by the total cpm in the aqueous phase, interphase, and organic phase. An average of 75-80% of the nucleic acids in the homogenate were recovered in the aqueous phase (data not presented), which was used for the subsequent electrophoretic analysis.

RNA synthesis during development was studied by following the electrophoretic separation of pulse-labeled RNA from six consecutive developmental stages on composite polyacrylamide-agarose gels. These stages were 1) immature gonidia from young spheroids, 2) enlarged, vacuolated gonidia, 3) precleavage gonidia undergoing condensation, 4) cleaving embryos, 5) pre-inversion embryos, and 6) post-inversion but pre-release spheroids. The results of one such analysis are displayed in Figure 10. It should be emphasized that, whereas in the normal illumination program for synchronous cultures the lights go off soon after the gonidia begin cleavage, in

these experiments all of the pulse-labeled incubations were carried out in the presence of continuous light (see Discussion).

When the percentages of total RNA radioactivity as ribosomal or transfer RNA radioactivity are calculated from the electrophoretic data, for each stage of development, differences in the specific accumulation of radioactivity in ribosomal RNA (only cytoplasmic rRNA is considered here; see Discussion) and transfer RNA are apparent (Figure 11).

These results indicate that very immature gonidia (Figure 10A) do not synthesize ribosomal RNA to an appreciable extent. During the subsequent stage of gonidial maturation, however, there is a dramatic change in the pattern (Figure 10B) with fully 50% of RNA radioactivity accumulated during this period found in ribosomal RNA. There is a decrease in the proportion of radioactivity in ribosomal RNA in the period of gonidia development leading up to the onset of cleavage (Figure 10C). Throughout the cleavage stages of embryogenesis specific ribosomal RNA synthesis appears to be extremely low (Figure 10D). As the inversion stage approaches ribosomal RNA shows an appreciable increase in the amount of accumulated radioactivity (Figure 10E). Post-inversion, pre-release juvenile spheroids give evidence of high ribosomal RNA synthetic activity. It may be significant that during

Figure 10. Electrophoretic Patterns of Radioactively labeled RNA extracted during different stages of the asexual life cycle of Volvox carteri f. nagariensis strain HK-10.

(A.-E.) The young spheroids from a synchronously grown 500 ml culture (31 hr light - 17 hr dark cycle) were collected on Miracloth 24 hr after the onset of the illumination period and resuspended in 100 ml of sterile SVM in a 250 ml flask fitted with a bubbler. The concentrated culture was returned to the normal synchronous growth, light and temperature conditions. At time "0" and at the intervals specified below, a 2 ml aliquot of the culture was removed to a sterile cotton plugged 13x100mm test tube and incubated with 100 μ Ci/ml of 2-³H-adenine for 5 hr (except "C" and "F"). At the end of the labeling period, gonidia (embryos) were isolated and RNA extracted as described (see RNA Rapid Microextraction Procedure for Gonidia and Embryos in Materials and Methods).

(F.) All procedures were the same as outlined above except that 3 ml of culture were labeled with 100 μ Ci/ml of 2-³H-adenine.

Note that the ordinate represents cpm per 1000 gonidia (embryos). The two vertical bars represent the positions of 25S (left) and 17S ribosomal RNA.

- A. Immature gonidia from newly released spheroids
- B. Enlarging vacuolated gonidia
- C. Enlarged, pre-"condensation" and "condensed" gonidia labeled for 12 hr.
- D. Cleaving embryos - early through late cleavage stages

E. Pre- and post-inversion embryos

F. Post-inversion and pre-release fully developed embryos
and young spheroids.

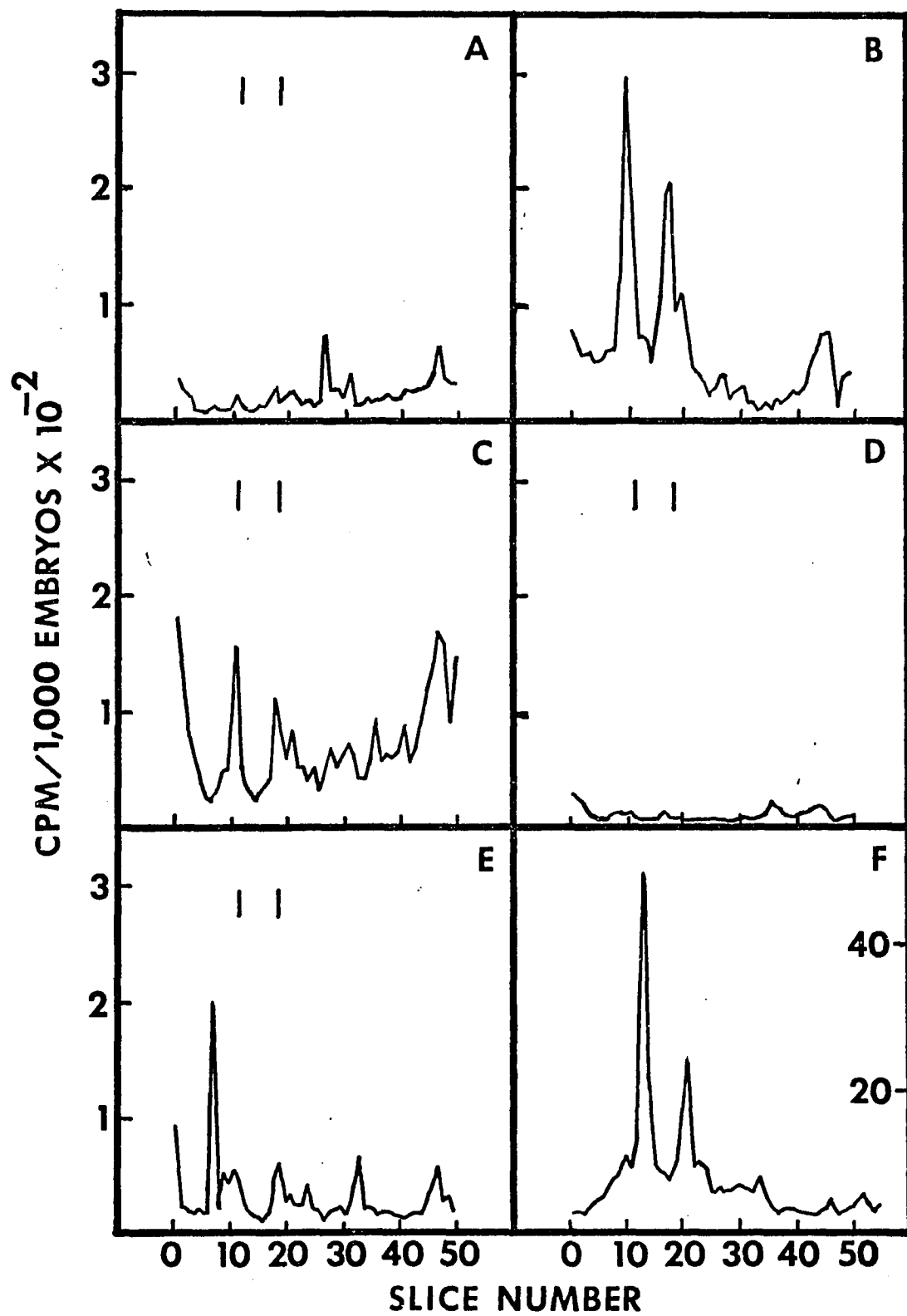
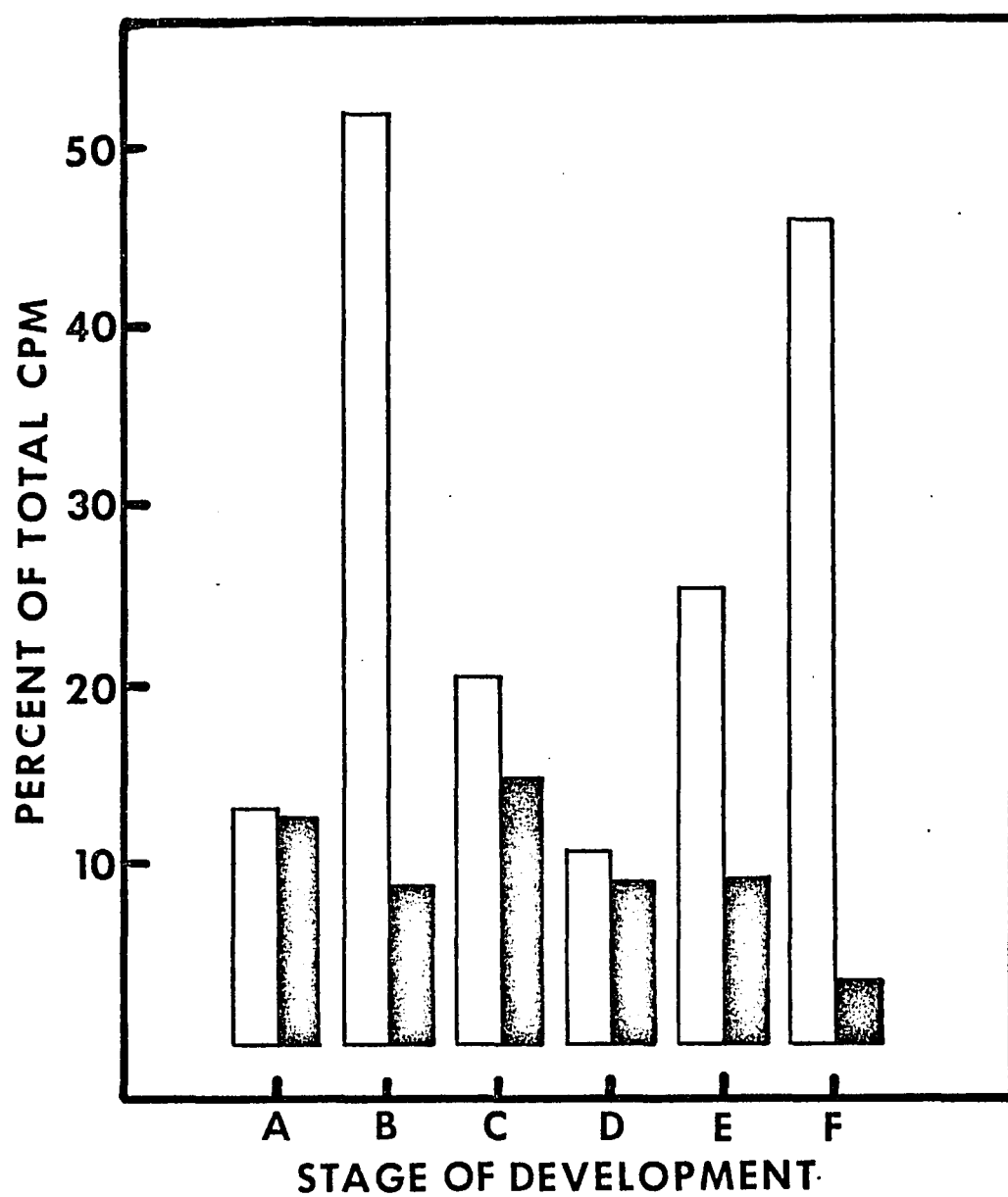


Figure 11. Percentage of Total RNA Radioactivity Incorporated in Ribosomal RNA and Transfer RNA.

The cpm in each ribosomal RNA and tRNA peak were determined by summing the cpm in individual fractions included in each peak. The total cpm on each gel were determined by summing the cpm in the first 50 slices. Percent of total cpm represents the ratio of pooled cpm for all rRNA peaks and of tRNA peaks to total cpm for each panel in Figure 10. The letters A - F correspond to panels A - F in Figure 10. White bars represent rRNA; shaded bars represent tRNA.



this stage the young spheroids begin to expand as a result of the synthesis and secretion of matrix material (mainly glycoprotein) by the somatic cells.

Changes in radioactivity incorporated into transfer RNA from stage to stage are particularly difficult to interpret for two reasons. First, the "transfer RNA peak" is composed partly of 5S ribosomal RNA (seen in some instances as a trailing shoulder on the tRNA peak). Second, radioactive adenine can be incorporated into the CCA 3' end-terminal nucleotide of transfer RNA by a cytoplasmic reaction (Hecht, et al., 1958); such incorporation, does not, of course, reflect de novo RNA synthesis.

In order to compare relative rates of RNA synthesis from stage to stage using radioactive nucleotide incorporation data, information regarding the specific radioactivity of intracellular nucleotide precursor pools is required. In this study (with 2-³H-adenine being used as a radioisotope label) the specific activity of the intra-cellular ATP pool was measured for two important periods of development, the maturing gonidial (stage B) and cleaving embryo (stage D) stages, since these showed strongly contrasting electrophoretic profiles.

The ATP content per gonidium and embryo was measured by the luciferin-luciferase assay as described in Materials

and Methods. A standard curve was generated using a series of known ATP concentrations ranging from 5 to 30 picomoles, for each experiment. A composite curve consisting of standard determinations from several experiments is shown in Figure 12. The sample analyses from four experiments is shown in Table 1. An average gonidium contains $9.93 \times 10^{-4} \pm 0.43 \times 10^{-4}$ picomoles of ATP while a cleaving embryo contains $13.5 \times 10^{-4} \pm 2.45 \times 10^{-4}$ picomoles of ATP. These values for the acid soluble ATP pools, in either case, are very small when compared with those of sea urchin eggs, which are roughly equal in size to a gonidium (see Discussion). The apparent increase in pool size seen in the cleaving embryos is statistically insignificant ($p > 0.10$, 6 d.f.).

The specific activity of the ATP pools was determined by measuring the quantity of radioactive ATP in a known acid-soluble pool. Since 2-³H-adenine, upon entering the cell, can be converted into a variety of ribose and deoxyribose purine nucleotide derivatives, it is necessary to distinguish radioactive ATP from all other labeled derivatives. Aliquots of KOH-neutralized acid soluble extracts were, therefore, chromatographed on silica gel thin layer plates, with ATP standards, in order to isolate the radioactive ATP free from other nucleotides. Samples were scraped from the regions containing the ATP standards and the radioactivity in the

spots was measured as described previously. When the specific activities, calculated as cpm per picomole of ATP, of gonidia and cleaving embryos are compared, as is shown in Table 2, a significant decrease in ATP pool specific activity in embryos is apparent. However, this difference of almost 50% cannot, by itself, account for a 20-fold decrease in the amount of radioactivity incorporated into ribosomal RNA (from column 3, lines 2 and 4, Table 3). These results strongly indicate (but do not prove) that the rate of ribosomal RNA synthesis in mature, precondensation gonidia is high compared to its rate in early cleavage embryos.

Polyadenylated RNA in *Volvox carteri*

The universal occurrence of polyadenylated RNA in eucaryotic cells is generally acknowledged; yet there is almost total ignorance of the function of the poly(A) segment (see Introduction). While early statements that the 3'-terminal polyadenylate segment typifies virtually all messenger RNAs (e.g. Adesnik, et al., 1972) have had to be qualified, the results of poly(A) analyses have been taken as measures of the extent and nature (e.g. size, proteins coded for) of messenger RNAs present in different cell types and at different stages of development. This study is a partial characterization of the polyadenylated RNAs of *V. carteri*.

Figure 12. Standard Curve for the Luciferin-Luciferase Assay.
Constructed as described in Materials and Methods.

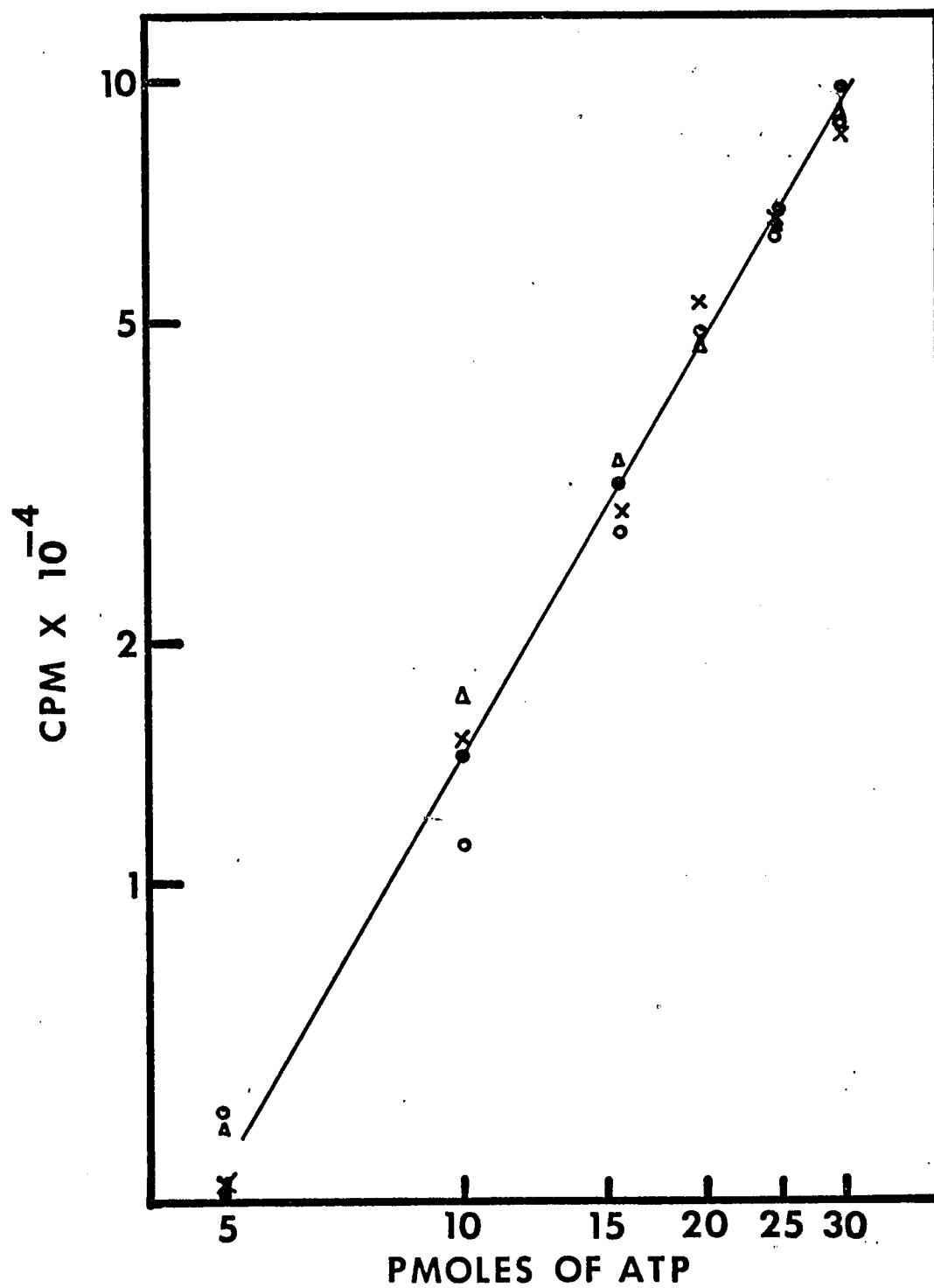


TABLE 1

ATP POOL SIZE OF GONIDIA AND EMBRYOS

Expt. No.	Pmoles ATP/Gonidium	Pmoles ATP/Embryo
1	9.4×10^{-4}	19.0×10^{-4}
2	9.5×10^{-4}	15.0×10^{-4}
3	13.0×10^{-4}	13.0×10^{-4}
4	7.8×10^{-4}	7.0×10^{-4}
Mean	9.93×10^{-4}	13.5×10^{-4}
S.E.	$\pm 0.43 \times 10^{-4}$	$\pm 2.45 \times 10^{-4}$

$t = 1.412$; $p = 0.10$; 6 d.f.

See Results for explanation.

TABLE 2

SPECIFIC ACTIVITY OF ATP POOLS IN GONIDIA AND EMBRYOS

Expt. No.	CPM/pmole ATP/ Gonidium	CPM/pmole ATP/ Embryo
1	20,562	11,121
2	34,054	17,610
Mean	27,308	14,366

See Results for explanation.

TABLE 3

PERCENTAGE OF TOTAL CPM AS rRNA AND tRNA CPM FROM VARIOUS DEVELOPMENTAL STAGES (See Figures 10 and 11).

Stage	Total CPM ^a	rRNA CPM ^b	% $\frac{\text{rRNA}}{\text{Total}}$	tRNA CPM	% $\frac{\text{tRNA}}{\text{Total}}$
A	1143	150	13.1	145	12.7
B	3140	1635	52.0	273	8.7
C	3914	794	20.3	588	15
D	480	51	10.6	44	9.1
E	1331	354	26.6	125	9.3
F	39543	18500	46.0	1402	3.5

See Results for explanation.

^aTotal CPM on gel.

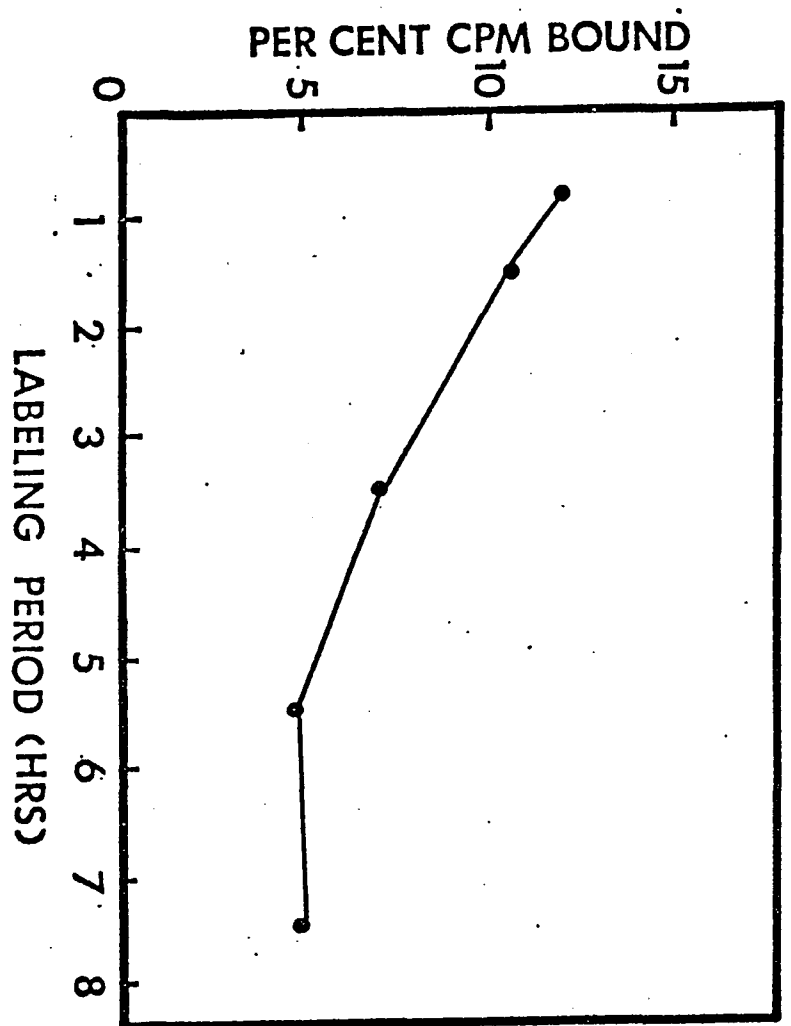
^bSum of all CPM in rRNA peaks.

RNA, extracted from spheroids labeled for 15-20 hr, was fractionated on oligo (dT)-cellulose columns and poly(A)-containing RNA isolated. The proportion of RNA retained by the column varied when the binding procedure was performed at different temperatures (results not shown). When binding and elution were carried out at room temperature (22-25°C) 2-3% of RNA-associated 2-³H-adenine activity was recovered in the bound fraction. Under much less stringent binding conditions (column temperature 0°C) as much as 10% of the total activity of a long-term labeled sample was retained by the column but the subsequent electrophoretic analysis revealed significant contamination with rRNA (results not shown). I have adopted 12°C as the standard binding temperature and 37°C as the elution temperature because these conditions give more quantitative binding of polyadenylated RNA with acceptably low levels of contamination by ribosomal RNA. The use of an unlabeled yeast rRNA carrier in the column fractionation procedure has also been effective in reducing non-specific binding of labeled rRNA to the column, presumably by competing with it for binding sites.

When the percentage of total labeled RNA as labeled poly(A) RNA was measured as a function of pulse-labeling time, the highest percentages were obtained from the shortest labeling periods (Figure 13). This would be expected if

Figure 13. Percent Poly(A)-associated RNA in total RNA as a function of length of labeling period.

Five ml shaking tube cultures containing 1000 spheroids/ml were each incubated with 100 μ Ci/ml of ^{3} H-adenine for varying time periods. At each time point RNA was extracted from a culture and purified. Each RNA sample was treated with DNase and deproteinized as described in Materials and Methods. After precipitation in ethanol with "cold" carrier rRNA samples were dissolved in binding buffer and fractionated by oligo(dT)-cellulose chromatography. Aliquots of the pooled bound and unbound material were treated with RNase A and T_1 and described in Materials and Methods, to correct for the radioactivity contributed by the poly(A) tracts; the difference in cpm between RNase treated and untreated aliquots of each fraction is presumed to represent radioactivity associated with transcribed RNA. Percentages reflect the ratio of bound to total bound plus unbound material.



polyadenylation was associated with rapidly turning over RNA species.

Isolation of polyadenylated RNA was achieved by chromatography on unsubstituted cellulose as well as oligo (dT)-cellulose columns. Although the basis of the binding interaction between poly(A) tracts and the ligand in either system differs (Sullivan and Roberts, 1973; Kitos et al., 1974), the electrophoretic profiles of pulse-labeled material isolated by each method were very similar (Figure 14). The distribution of RNA on gels was broad with the major portion migrating more slowly than the 17S rRNA marker and a significant portion of the material more slowly than 25S rRNA. Since these electropherograms represent total cellular RNA extracts, some of the material may be nuclear in origin (heterogeneous nuclear RNA) but the length of the labeling period should minimize the contribution of those species with very short intranuclear half lives. These results suggest that the molecular weight of poly(A) containing RNA is variable and on average high in comparison with that of some other lower eucaryotes that have been examined (Firtel and Lodish, 1973; Groner, et al., 1974; Groner and Phillips, 1975; Rodriguez-Pousada and Hayes, 1976; Timberlake, et al., 1977; Lucas et al., 1977).

The electrophoretic data reported above represents the pooled poly(A) RNA of, at least two different cell types and a spectrum of developmental stages. To determine whether the poly(A) populations extracted from differentiated somatic cells, gonidia, and cleaving embryos differed in their general electrophoretic behavior, I pulse labeled synchronously growing populations of spheroids at various developmental stages for 6-12 hr. At the end of the labeling period populations were fractionated into their constituent somatic cells, and gonidia or cleaving embryos. The electrophoretic analysis of poly(A) RNA isolated from each of these sources indicates detectable and reproducible differences. Although it is impossible to make a peak for peak comparison between the electrophoretic profiles of gonidial and early embryonic poly(A) RNA isolates, (Figures 15A,B), there is an overall similarity in the modal distribution of radioactivity. It appears to be at, or just less than 25S. The electrophoretic profiles of somatic cells isolated from young spheroids (containing gonidia) and those of older spheroids (containing cleaving embryos) (Figure 15C,D) both display a greater proportion of RNA species migrating considerably slower than 25S.

These differences in size distributions could be due to variations in the average lengths of transcribed sequences attached to poly(A) tracts or to variations in the lengths of

Figure 14. Electrophoretic patterns of poly(A) RNA isolated by chromatography on unsubstituted cellulose and oligo(dT)-cellulose.

Two 100 ml aerated cultures (500 spheroids/ml) were labeled with 200 μ Ci of ^{3} H-adenine for 8 hr. RNA was extracted from each culture and fractionated either by unsubstituted cellulose or oligo(dT)-cellulose chromatography. Poly(A) RNA, isolated by each method, was electrophoresed on composite 2.0% polyacrylamide - 0.5% agarose slab gels and analyzed as described in Materials and Methods. A) Poly(A) RNA isolated on unsubstituted cellulose, electrophoresed for 2 hr at 200V. B) Poly(A) RNA isolated on oligo(dT)-cellulose, electrophoresed for 1 hr 45 min at 225V. Note different positions of 25S and 17S rRNA markers in each panel.

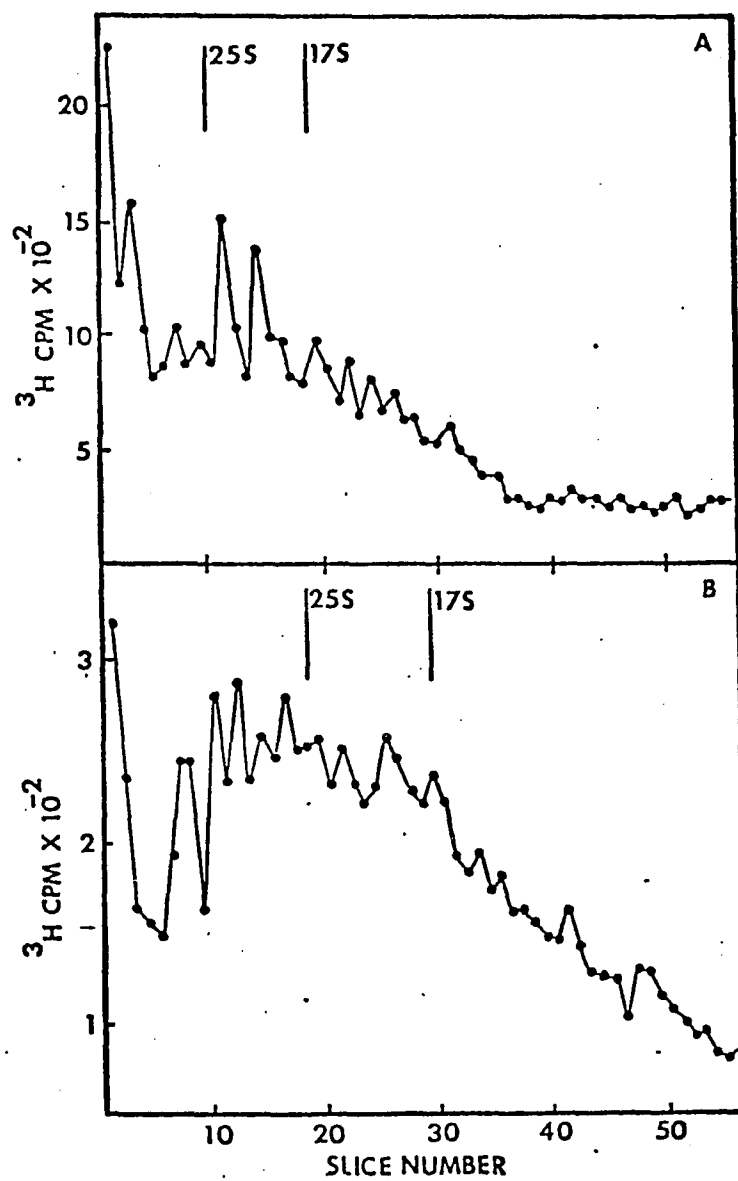
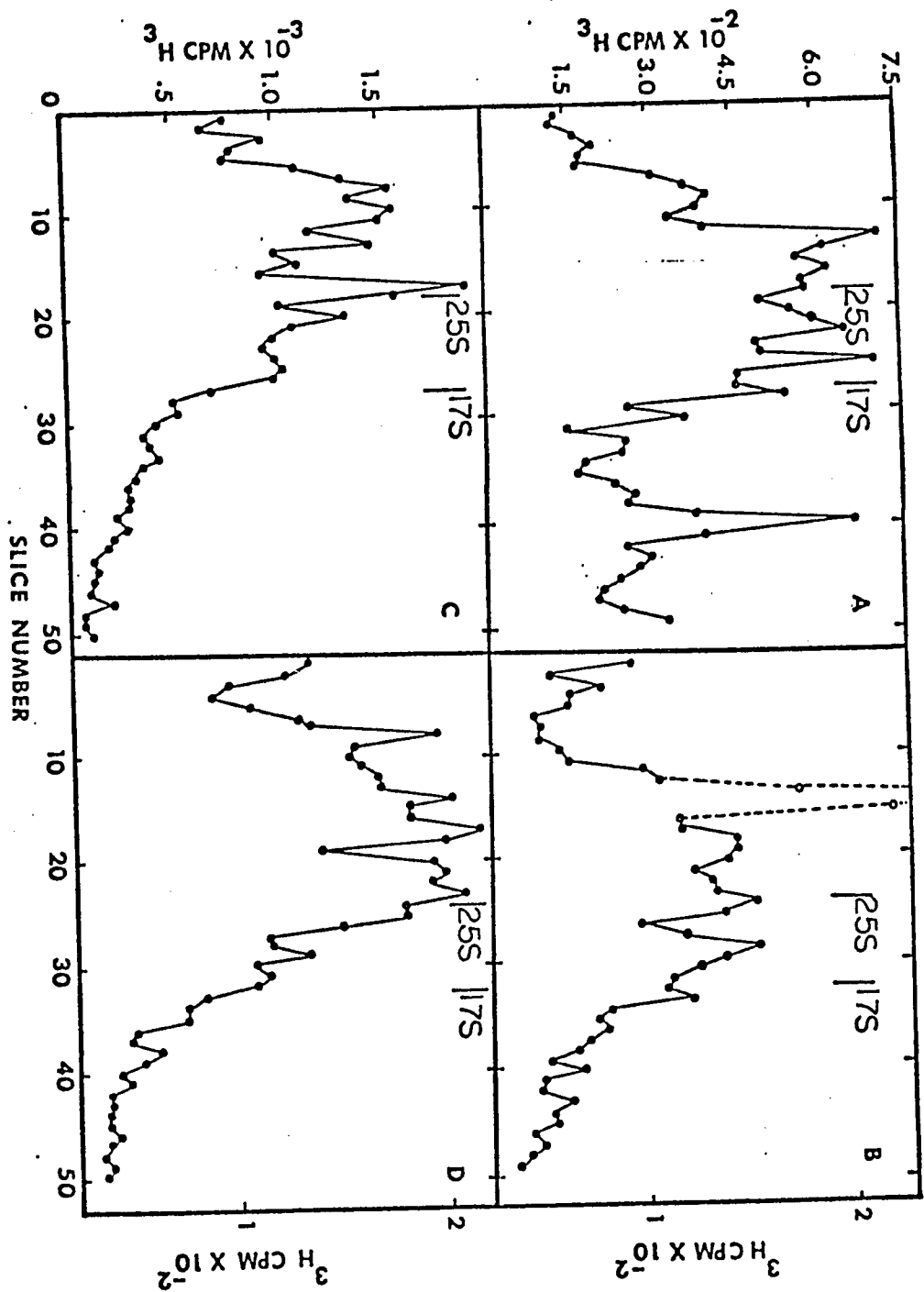


Figure 15. Electrophoretic patterns of poly(A) RNA from gonidia, embryos, and somatic cells.

Two 100 ml synchronous cultures (400 spheroids/ml) containing spheroids with maturing gonidia and spheroids with cleaving embryos, respectively, were incubated with 300 μ Ci of ^{3} H-adenine for 6 hr. Cell types were separated as described in Materials and Methods. RNA isolation, fractionation, and electrophoresis were as described in the legend to Figure 3.

A) Poly(A) RNA from maturing gonidia. B) Poly(A) RNA from embryos. C) Poly(A) RNA from young (6-12 hr) somatic cells, and D) Poly(A) RNA from older (12-20 hr) somatic cells.



the poly(A) tracts themselves. In the latter case, extremely short poly(A) tracts and consequently the RNA transcripts attached to them might not efficiently bind to the oligo (dT)-cellulose columns, resulting in the apparent changes in size distribution of poly(A) RNA. Evidence in favor of one possibility or the other will be obtained when the average lengths of the poly(A) tracts attached to unbound and bound RNA molecules from different cell types at different stages are compared.

Measurements of poly(A) tract lengths were made by analyzing the behavior of poly(A) tracts obtained by selective RNase digestion of total RNA in a high salt buffer. RNase resistant material in the digests, presumed to be mainly poly(A) material, was further purified by passage through oligo (dT)-cellulose columns as described in Materials and Methods. The bound material was collected and electrophoresed on 12% polyacrylamide gels with unlabeled yeast 4 and 5S RNA standards. It has been demonstrated that these RNA molecules migrate more rapidly than poly(A) homopolymers containing equal numbers of nucleotides (Burness, et al., 1976; Nokin, et al., 1976). Four S RNA normally co-migrates with poly(A) standards containing 25-30 nucleotides and 5S RNA runs with poly(A) standards of 50-55 nucleotides. Using these calibrations for our markers I have estimated the longest and most pre-

Figure 16. Electrophoretic pattern of poly(A) tracts from total Volvox RNA.

A 100 ml culture (500 spheroids/ml) was labeled with 300 μ Ci of ^{3} H-adenine for 20 hr. RNA was extracted and DNase treated as described in Materials and Methods. Purified RNA was treated with 10 μ g/ml RNase A and 20 units/ml RNase T₁ for 1 hr at 37°C. After deproteinization and ethanol precipitation RNase resistant material, chromatographed on oligo(dT)-cellulose was electrophoresed on a 12% polyacrylamide gel at 4°C for 4 hr with 4S and 5S RNA as markers. Positions of markers were determined by UV absorbance scans of gels.

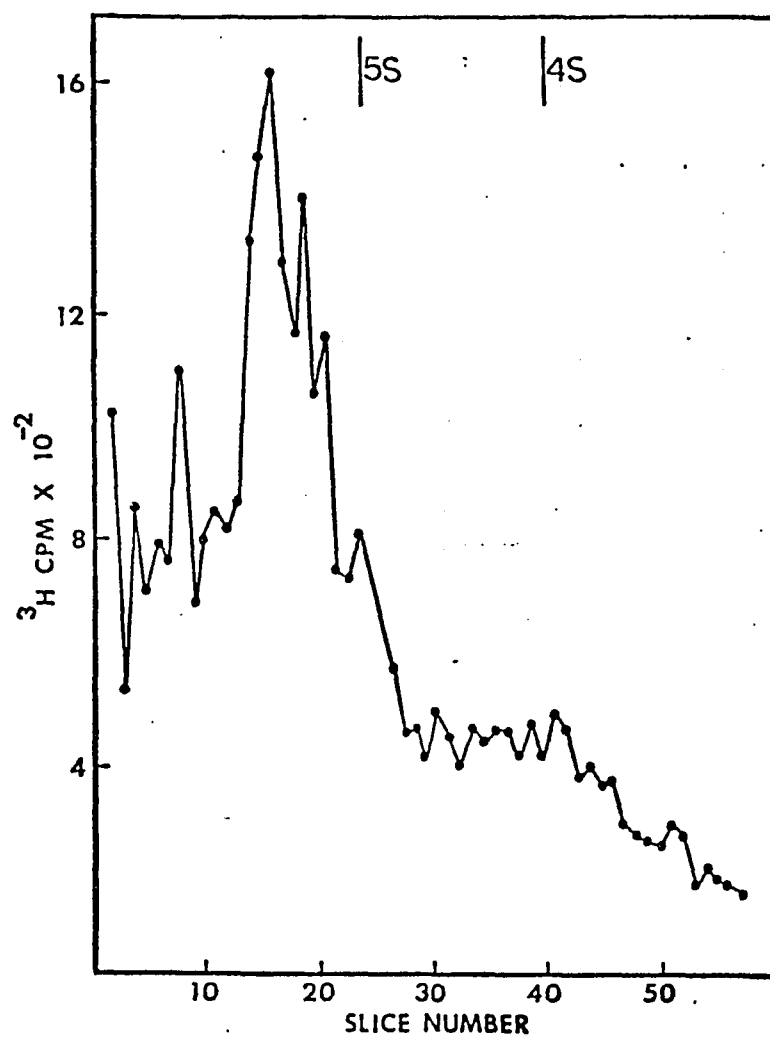
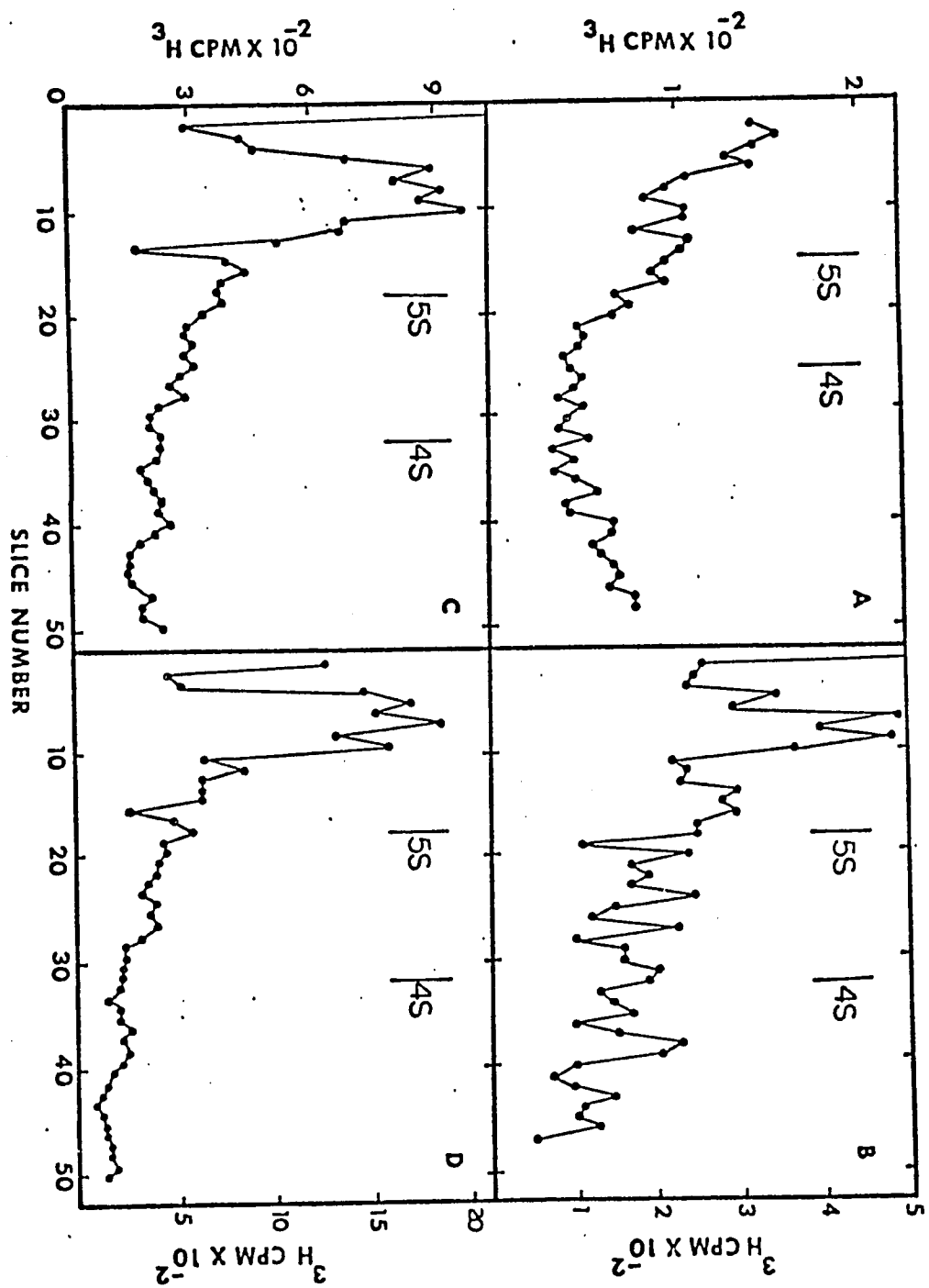


Figure 17. Electrophoretic patterns of poly(A) tracts isolated from gonidia, embryos, and somatic cells.

Two 100 ml synchronous cultures were labeled as described in the legend to Figure 4. Cell types were fractionated as described in Materials and Methods. RNA was extracted as described in Materials and Methods and all subsequent procedures were as described in the legend to Figure 5. A) Poly(A) tracts isolated from gonidia, B) Poly(A) tracts isolated from embryos, C) Poly(A) tracts isolated from young somatic cells (4-12 hr), and D) Poly(A) tracts isolated from older somatic cells (12-20 hr).



dominant size class of poly(A) tract in Volvox to be 75-80 nucleotides in number with several distinct smaller species (Figure 16).

A comparison of the gel profiles of poly(A) tract isolates of gonidia, cleaving embryos, the somatic cells of young spheroids (4-12 hr post-release), and the somatic cells of older spheroids (12-20 hr post-release), reveals changes in proportions of the various size classes of poly(A) tracts. In gonidia (Figure 17A), there are several peaks of activity discernible between the first major peak, representing the longest poly(A) tracts, and the 4S marker. In cleaving embryos there is a greater proportion of activity in the major peak (Figure 17B) than in this peak from gonidia. Somatic cells, of either stage examined, (Figure 17C,D) show the greatest proportion of the largest poly(A) tracts relative to the smaller poly(A) tract size classes. Thus, it appears that there is, at least, some difference in poly(A) metabolism in gonidia, embryos, and somatic cells.

Ultrastructure of Development

General features of the ultrastructure of Volvox carteri have been previously described (Kochert and Olson, 1970). This study compares cytoplasmic organization and the specific distribution of subcellular components in maturing gonidia, cleaving embryos, and somatic cells. In the metazoa, it has

been demonstrated that the distribution of certain subcellular components to definite regions within the egg cytoplasm prior to fertilization determines the developmental fate of the cells derived from these regions during embryogenesis. Among the phenomena which reflect the asymmetric distribution of cytoplasmic material are the establishment of animal-vegetal polarity and the localization of the germ plasm. It was hoped that because observable cellular differentiation (marking the somatic and germ cell lines) occurs so early in development in Volvox, we would be more likely to see some visible evidence of cytoplasmic localization.

A comparison of vacuolated gonidia and somatic cells (Figures 18 and 25) reveals the extensive modifications to intracellular architecture connected with the asexual reproductive cells. The gonidium, five to ten times the diameter of a somatic cell, contains a centrally located nucleus surrounded by several very large vacuoles. The dense-staining, amorphous material seen in some vacuoles appears to be aggregates of polyphosphate (Brachet, 1957); the ground substance in these vacuoles has not been characterized. Most of the chloroplast membranes are located at the perimeter of the cell, perhaps because they are excluded from the interior by the vacuoles. It is not known whether this extensive membrane system comprises a single highly convoluted chloroplast or a

Figure 18. Developing Gonidium. The centrally located nucleus is not visible in this section. There are a few scattered polyphosphate (volutin) granules (arrows). CH, chloroplast membranes; VA, vacuole.



number of smaller ones. During cleavage it appears that this material is distributed to the cleaving embryonic cells with no net synthesis of new membrane during that period.

The large nucleolus usually seen in the nuclei of gonidia at this stage is not seen on the section in Figure 18. In Figure 19 the nucleus of a gonidium is shown with its characteristic nucleolus. The substantial granular component of this structure (seen in close-up in Figure 20) indicates that it is an active center of ribosome synthesis. The fibrillar zone is, likewise, rather extensive and is apparently permeated by channels as evidenced by the numerous electron-lucent regions. There is a small isolated fibrillar structure also visible, which is commonly seen in association with nucleoli in vacuolated gonidia. On the basis of similarity of appearance to the fibrillar region of the nucleolus, it is identified as an accessory nucleolus although its function remains a matter for speculation (see Discussion). Fully differentiated somatic cells, in contrast, (Figure 25) are never seen with morphologically normal nucleoli; only a clump of dense-staining material, always eccentrically situated, can be tentatively identified as a nucleolus but its appearance offers no clue to the true nature or extent of its activity.

During the later stages of maturation of the gonidium the cytoplasm undergoes further extensive restructuring in apparent anticipation of the onset of embryogenesis. An hour

Figure 19. Nucleus of Developing Gonidium. NO, nucleolus; FI, fibrillar region of nucleolus; GR, granular region of nucleolus; AN, accessory nucleolus.



Figure 20. Accessory nucleolus. The fine structure of this body resembles that of the fibrillar region of the nucleolus. AN, accessory nucleolus; NO, nucleolus.



or so prior to the first cleavage division, the large vacuoles begin to break down and as seen under the light microscope the cytoplasm becomes dark green and coarsely granular. This sequence is commonly known as condensation. A flattening on one side of the condensed gonidium signals the beginning of the first cleavage division; it also marks the anterior pole of the young embryo.

In the condensed gonidium (Figure 21), the large vacuoles, the most conspicuous feature of maturing gonidia, break up into much smaller vesicles which are scattered throughout the cytoplasm. Some of these vesicles may be seen to contain small electron dense granules which are presumed to be the polyphosphate-containing volutin granules (see Discussion) found in many algae.

Chloroplast membranes are scattered throughout the gonidial cytoplasm and appear more fragmented than in the vacuolated gonidium. It is still not possible however to identify individual chloroplasts. Other features of note in the precleavage gonidium are the scattered oil droplets, low ribosome density, and the mitochondria with undeveloped internal structure (see Discussion).

As early cleavage divisions proceed a central hollow analogous to the blastocoel in many metazoan embryos, is generated. Transverse sections of embryos at this stage

Figure 21. Cytoplasm of "Condensed" Gonidium. VA, vacuole; VG, volutin granule; MI, mitochondrion; CH, chloroplast membrane; OD, oil droplet.



reveal a stratification of organelles and cytoplasmic inclusions within the syncytial blastomeres. The nuclei are all located at the ends of the cells adjacent to the internal cavity; nucleoli are seen in some blastomeres but not in others. Chloroplast membranes and associated structures are generally absent from the areas surrounding the nuclei and tend to predominate at the centrifugal ends of the cells. Mitochondria with electron-lucent matrices (as seen in pre-cleavage gonidia) are found in all areas of the cytoplasm. Ribosomes are more in evidence here than they were in pre-cleavage gonidia and a small portion of these are associated with polysomes and endoplasmic reticulum. Golgi bodies (dictyosomes) are present in the perinuclear regions and are often seen closely apposed to elements of smooth endoplasmic reticulum. Oil droplets and the volutin granule-containing vacuoles are the most prominent inclusions in the cytoplasm between the nucleus and the chloroplast membrane. Flagella are not found in early cleavage blastomeres although centrioles or basal bodies are occasionally observed.

Figure 22. Cleaving Embryo. NU, nucleus; CH, chloroplast membrane (the extremely electron-lucent material within is starch); OD, oil droplets. Note that the cells of the cleaving embryo are highly syncytial. None of the nuclei in this section appear to contain nucleoli although they are usually present .



Figure 23. Detail of Embryonic Cell. Part of a nucleolus is visible in this section. NO, nucleolus; NU, nucleus; MI, mitochondria; VG, volutin granules; CH, chloroplast membranes; OD, oil droplets. A cytoplasmic bridge between adjacent cells is indicated by the arrow.



Figure 24. Post-inversion Embryo. GI, gonidial initial;
SI, somatic cell initial; SO, somatic cell of parental
spheroid.



Figure 25. Mature somatic cell. Note the great number of polysomes and membrane-bound ribosomes. NU, nucleus; GO, Golgi apparatus; CH, chloroplast membrane; MI, mitochondrion; ES, eyespot; ER, endoplasmic reticulum.



DISCUSSION

Volvox has been the subject of a comparatively small number of studies of nucleic acid and protein metabolism in the past several years (see Introduction). This dissertation is to a certain degree an extension of earlier studies of RNA synthesis in Volvox carteri (Kochert, 1971; Kochert, and Sansing, 1971; Yates, 1974). It includes an initial characterization of uptake and incorporation kinetics of several radioisotopically labeled compounds which were considered for use in RNA synthesis experiments. The best of these RNA precursors, based on these studies, was 2-³H-adenine, which was then used to examine the physical nature and synthesis of various RNA species. The amount of ribosomal RNA synthesized was shown to vary through the course of asexual development. ATP pool size and specific activity studies supported the fact that differential incorporation of labeled precursor truly reflected differences in rates of synthesis. Polyadenylated RNA was isolated and partially characterized; somatic cells, gonidia, and embryos all exhibited differences with respect to the average size of poly(A) RNA tracts and the metabolism of poly(A) tracts. An electron microscopic study of development was performed to try and relate biochemical events to visible changes in cyto-

plasmic organization occurring in maturing gonidia and developing embryos.

Radioactive Precursor Labeling Kinetics

When the RNA synthesis studies reported here, were initiated, 2-³H-adenine was used as a radio-label because it was the most readily available isotope in our laboratory at the time. Since they are presumed to give greater specificity of labeling of RNA, (5,6)-³H-uracil and 5-³H-uridine were tried as possible radioactively-labeled precursors. It was found that neither of them was accumulated by the cells or incorporated into RNA nearly as efficiently as 2-³H-adenine. (While 5-³H-uridine was available with approximately the same specific activity as 2-³H-adenine, 5-³H-uracil could only be obtained at reasonable cost with very much lower specific activity.)

Radioactively-labeled phosphate ($K_2(^{33}P)O_4$) was also investigated as a labeled RNA precursor. Several earlier studies on RNA metabolism in Volvox carteri had employed $H_3(^{32}P)O_4$ (Kochert and Sansing, 1971; Kochert, 1971; Yates, 1974) for pulse-labeling. These precursors are comparatively inexpensive, present no cell membrane permeability problems, and uniformly label all nucleic acids without regard to differences in base composition or intracellular location. How-

ever, inorganic phosphate is also incorporated into the large intracellular pools of polyphosphate found in these organisms. These latter molecules exhibit some of the physical properties of nucleic acids such as ethanol and trichloroacetic acid insolubility. Contamination of nucleic acid preparations and consequent interference with the interpretation of experimental results can present problems (Kochert, personal communication). Despite this shortcoming, ^{33}P was examined as a radioactive label for use in certain studies of polyadenylate metabolism.

The incorporation kinetics of ^{33}P , in contrast to those of 2- ^3H -adenine, were non-linear. This is especially obvious in phosphate-free Volvox medium, where the rate of ^{33}P incorporation into RNA appears to be increasing exponentially with time. No explanation can be offered for these results and further analysis of this question is indicated.

Of the radioisotopes that were compared, 2- ^3H -adenine was the most satisfactory and was chosen for all studies requiring labeling of RNA. Uptake and incorporation of 2- ^3H -adenine was essentially linear over a wide range of concentrations for time periods up to, at least, 3 hr. The similarity in the rate of uptake and incorporation up to 2 hr in the two cultures which contained a five-fold difference in the amount of label added, indicates that uptake may be by

some mechanism other than simple passive diffusion. A possible alternative may be that uptake of adenine is by means of saturable carrier-mediated membrane transport. Although no such system has been reported for purine assimilation in any close relative of Volvox, specific membrane transport systems for urea and arginine have been identified and characterized in V. carteri f. nagariensis (Kirk and Kirk, 1976; Kirk and Kirk, 1978). Another explanation for the results reported here is that the rate of uptake of adenine is governed by its rate of incorporation into nucleic acids. The circumstantial evidence for this is provided by the data which indicate that when uptake and incorporation were followed over a period of time both parameters showed the same overall patterns of change, even where exogenous label had not yet become limiting (see Figures 6 & 7). This hypothesis would remain tenable only if the precursor (ATP) pool size were not independently changing. The data regarding ATP intracellular pools in Volvox are very incomplete but the two developmental stages that have been examined in this study, do not show any significant difference in ATP pool size.

Isolation and Electrophoresis of Volvox RNA

The isolation of pure intact nucleic acids, and especially RNA, from Volvox presents several difficulties. The

first and major one encountered, is associated with the presence of the matrix material in which the cells of the spheroid are embedded. This material, if not removed prior to nucleic acid extraction, will reduce the final recovery of nucleic acids and contaminate the isolated nucleic acids sufficiently to interfere with further analysis. When the nucleic acids of gonidia or embryos alone are all that is desired, this problem is avoided by gently mechanically disrupting the spheroids and separating the gonidia or embryos from somatic cells and matrix by filtration and buoyant density centrifugation (see Materials and Methods). However, extraction of RNA from somatic cells or whole spheroids requires a different approach; the matrix material is solubilized with protease or pronase (Kochert and Sansing, 1971). The sensitivity of the spheroids, especially of strain HK-10, to this treatment has been found to vary, depending to a large extent on the stage of the life cycle. Large, mature spheroids respond most rapidly.

With the matrix removed, RNA may be extracted from the cells using the protocol described in Materials and Methods. Several alternative extraction buffers were tried in the procedure before the one employed throughout this study was chosen. The extraction buffer, consisting of 0.1 M Tris, 0.05 M NaCl, 0.001 M EDTA, 0.5% SDS, and 0.5% diethylpyrocarbonate was used either at pH 7.5 or 9.0. (Diethylpyro-

carbonate was used in the extraction buffer because it has been demonstrated to be an effective inhibitor of ribonuclease activity (Fedorcsak et al., 1966).) The pH 9.0 buffer is lower in ionic strength than the pH 7.5 buffer since Tris occurs primarily in its non-ionized form at the higher pH. This greatly improves the recovery of polyadenylated RNA during purification (Brawerman, 1976).

RNA isolated and purified by the procedure described in Materials and Methods was electrophoresed in composite 2.0% polyacrylamide - 0.5% agarose slab gels according to the method of Peacock and Dingman (1968). Figure 8 shows an electrophoretic profile of total RNA extracted from Volvox labeled for 15-20 hr with 2-³H-adenine. As has been reported previously (Kochert and Sansing, 1971) for other closely related strains of Volvox carteri, even the most effective measures taken to inhibit ribonuclease activity during the extraction procedure do not prevent a selective degradation of 23S rRNA. Degradation during RNA isolation may be the result of an in vivo break in the primary structure of the 23S rRNA in the ribosome (see Introduction - RNA Synthesis and Processing and Regulation of Gene Expression). The breakdown products may be seen as a small peak at approximately 13S (slice 36) and as a shoulder on the leading edge of the 17S rRNA (slice 27). A greater proportion of the 23S rRNA

may be recovered intact on polyacrylamide gels by adding a divalent cation, such as Mg^{++} , to the gel running buffer (Kochert and Sansing, 1971). This modification to the running buffer, however, was often found to cause aggregation of RNA at the origin and otherwise inconsistent gel patterns and so was not routinely employed in this study. Cattolico and Jones (1972) used a high salt extraction buffer containing Mg^{++} to isolate intact 23S rRNA from Chlamydomonas reinhardtii. Electrophoresis of RNA showed good recovery of intact 23S rRNA despite the fact that the gel running buffer contained no Mg^{++} . Using this same extraction buffer for the isolation of RNA from Volvox, I was unable to significantly increase the amount of 23S rRNA recovered, although it should be noted that there are additional preliminary steps required in the Volvox RNA extraction procedure which make it impossible to duplicate exactly the published conditions for RNA isolation for Chlamydomonas.

RNA in Development

Electrophoretic patterns of RNA synthesis at sequential stages of development demonstrate that transcriptional control, whether it is a primary or secondary effect, play a role in the synthesis of high molecular weight ribosomal and perhaps tRNA. During one period of the early maturation of

the gonidium, cytoplasmic ribosomal RNA is synthesized in relative abundance (see Figures 10 and 11); immediately before and after this its synthesis is much depressed. Through early and middle cleavage, incorporation of label into cytoplasmic rRNA is barely detectable but as the inversion stage is reached, incorporation of label into cytoplasmic rRNA increases significantly. In post-inversion embryos there is a further dramatic increase in the quantity of rRNA synthesized per embryo over the quantities seen in earlier stages of embryogenesis.

Under our routine light synchrony conditions gonidial cleavage occurs in the dark. Since it was conceivable that the depressed rate of RNA synthesis observed in cleavage was the result of a light dependency of RNA synthesis (Yates, 1974), the experimental results shown were obtained with all experimental cultures grown and labeled in continuous light. This change in program did not cause any apparent change in length of life cycle or degree of synchrony over the course of the experiment.

The results are essentially in accord with a similar study made by Yates (1974) with V. carteri f. weismannia. In both cases the rate of cytoplasmic rRNA synthesis appears to be much diminished during gonidial cleavage while there are "bursts" of cytoplasmic rRNA synthesis during gonidial

maturation and again as the embryos (i.e. daughter colonies) attain full morphological development within the parent. Agreement in this instance is especially significant because similar results were obtained using different experimental procedures and methods of analysis. The earlier study on V. carteri f. weismannia was based on an electrophoretic analysis of RNA extracted from whole spheroids that had been pulse-labeled with ^{32}P . Evaluation of relative synthesis rates of rRNA were made by ascertaining the specific radioactivity of the rRNA peaks fractionated on polyacrylamide gels. In the present study, pulse-labeled RNA from isolated gonidia (or embryos) was analyzed by calculating the ratio of percentage of incorporated label in rRNA to that in total RNA (see Figure 11). Furthermore, RNA precursor pool sizes and pool specific activities were measured for vacuolated gonidia and cleaving embryos, the two developmental stages showing the greatest difference in these ratios. Assuming rRNA is not synthesized utilizing a separate intracellular ATP pool, the latter study strongly supports the view that the absolute rate of rRNA synthesis in the maturing gonidium is much greater than that in the cleaving embryos. Although the ATP pool specific activity in the embryo was calculated to be approximately half that in the gonidium, this difference alone cannot account for the differences in incorporation of

label into gonidial and cleavage stage rRNA. Based on the combined results of two independent studies it must be concluded, that the rate of accumulation of mature cytoplasmic ribosomal RNA is subject to programmed changes during development in Volvox carteri. Such control may be considered to be exercised at both the levels of transcription and processing based on the available evidence.

This conclusion cannot be extended to all members of the genus, however. In V. carteri, the period of greatest DNA synthesis is during cleavage (Yates, 1974; Margolis-Kazan and Blamire, 1976), the very stage at which rRNA synthesis per embryo appears to be most depressed. Tucker and Darden (1972) reported that in V. aureus, both DNA and RNA synthesis increase in parallel during cleavage. Volvox carteri and V. aureus belong to different subdivisions of the genus and, as Yates has observed, differ fundamentally in their developmental programs. Cleavage in V. carteri follows an extensive period of gonidial maturation. It progresses rapidly with no cell growth between divisions and there is little time for rRNA synthesis. In V. aureus gonidia begin dividing when they are still small and there is a substantial interphase period between each cell division during which cell growth and nucleic acid synthesis (DNA and RNA) occur.

That this temporal programming of rRNA synthesis is related to the evolution of the multicellular condition and development of successive generations from germ cells is suggested by comparing regulation of rRNA synthesis in Volvox and Chlamydomonas, a unicellular member of the Volvocine series. Ribosomal RNA synthesis in Chlamydomonas reinhardtii is cell cycle-dependent. Most of the cytoplasmic ribosomal and chloroplast ribosomal RNA synthesized during the cell cycle is made in G_1 (Cattolico et al., 1973; Wilson and Chiang, 1977) and there is no further net synthesis of any rRNA during S phase or the remainder of the cell cycle. Ribosomal RNA synthesis in V. carteri occurs during the period of gonidial maturation - in a sense an extended G_1 . The cells in the rapidly cleaving embryos have a much abbreviated G_1 or perhaps none at all. Only at or after inversion is rRNA synthesis vigorously resumed in embryonic cells; the rate of cell division is severely reduced and stops completely as somatic cells attain terminal differentiation. These cells have dropped out of the mitotic cycle and are said to be in G_0 - a sort of permanent G_1 . Thus, if any conclusion may be drawn based on phylogenetic relationship between Volvox and Chlamydomonas, it is that both cytoplasmic and organelle rRNA synthesis occur almost exclusively in cells in G_1 . In Volvox carteri this phase (or its equiva-

lent) occurs in pre-cleavage gonidia and post-inversion spheroids, but not during cleavage.

As has been suggested previously by Yates (1974) and Kochert (1975) maturing gonidia synthesize and stockpile sufficient rRNA and ribosomes to supply the embryo through the entire cleavage period. There is, thus, little or no need for concomitant rRNA synthesis during cleavage. Only with morphogenesis (inversion), differentiation, of the specialized somatic cells, and the resumption of growth is synthesis of nascent rRNA once again detectable. This closely parallels the apparent series of events related to rRNA synthesis in the early development of many of the metazoa (see Introduction and Davidson, 1976). During oogenesis in all organisms which have been examined, the predominant type of RNA synthesized is ribosomal. In many oocytes, the capacity to synthesize high molecular weight rRNA rapidly is augmented by the selective endoreplication of the ribosomal RNA genes (gene amplification). There is no direct evidence for the occurrence of this phenomenon in Volvox, but there are indications that the nucleus of the mature gonidium contains more than a single genomic equivalent of DNA. Kochert (1975) reported that nearly mature gonidia contain 25 times as much DNA as somatic cells. Since only 30% of this DNA was found to be of cytoplasmic origin there must be 15 to

20 times the haploid amount of nuclear DNA. Margolis-Kazan (1978) has similarly inferred from the relative amounts of radioisotope incorporated into the nuclear DNA of expanding spheroids (containing maturing gonidia) and cleaving embryos that there is a great excess of nuclear DNA in the mature gonidium.

Could the surplus DNA in the nucleus of the gonidium be, wholly or in part, amplified ribosomal cistrons? For the sake of comparison, when the ribosomal DNA of Xenopus oocytes is replicated during the pachytene stage of meiosis I, the germinal vesicle contains 7 times the amount of DNA found in a diploid cell. Part of this extra DNA is accounted for by the 4C quantity of DNA in the premeiotic oocyte; however, there is still approximately two and a half times as much rDNA in the germinal vesicle as all of the other DNA combined. To maintain that all of the excess DNA in the nucleus of a gonidium is amplified rDNA is without additional evidence, unwarranted but the possibility of selective gene amplification should be considered. The nucleus of a vacuolated gonidium is much larger than a somatic cell nucleus and contains a very large nucleolus (see Results - Ultrastructure of Development and Discussion, below). That there is only a single nucleolus and not many as is found in the Xenopus oocyte does not preclude the existence of supernumary

copies of the nucleolar organizer region. For example, the amplified rDNA in the amphibians Ambystoma (Mexican axolotl) and Plethodon sp. (salamanders) remain attached to the nucleolar organizer region of the chromosome (Peacock, 1965; Callan, 1966). Further, reversible amplification of rDNA cistrons has been reported to occur during the vegetative cell cycle of Chlamydomonas reinhardtii (Howell, 1972). Work in this area should be pursued vigorously to establish the number of tandem repeats of the rDNA cistron and the possible presence of gene amplification.

Little detailed analysis can be given concerning the synthesis of transfer (4S) and low molecular weight (5S) ribosomal RNA in Volvox development except to say that, taken together, they appear to constitute an almost constant fraction of RNA made at each stage of development (see Figure 11). The difficulty with end-group turnover in previously synthesized tRNA and the near confluence of the 4S and 5S peaks on the low percentage polyacrylamide gels has already been alluded to (see Results). It is presumed that these molecules as well as the high molecular weight cytoplasmic ribosomal RNAs are stockpiled during the period of gonidial maturation for later use in embryogenic cleavage. That the 5S and high molecular weight ribosomal RNAs must (for efficiency sake) be made in stoichiometric amounts and yet the 5S RNA has never been reported to be amplified offers, no

dilemma. The 5S rRNA cistron is probably highly reiterated in the genome (see Introduction).

Polyadenylated RNA

The results reported here establish the presence of poly(A) tracts associated with total cellular RNA in Volvox. That the percentage of polyadenylated RNA in total cellular RNA extracts decreases with the length of the labeling period up to approximately 5 hr suggests the association of poly(A) with the rapidly turning over species of cellular RNA - heterogeneous nuclear and messenger RNA. The length of the labeling periods and the fact that polyadenylated RNA has been generally reported to have a short half-life in the nucleus and is either degraded or transported to the cytoplasm within a hour leads me to suppose that I am analyzing mainly cytoplasmic poly(A) RNA.

The degree of heterogeneity and size distribution of Volvox polyadenylated RNA populations fractionated electrophoretically are essentially in accord with those seen in RNA extracted from some other lower eucaryotes (Firtel and Lodish, 1973; Groner et al., 1974; Fraser, 1975; Rodrigues-Pousada and Hayes, 1976; Lucas et al., 1977; Timberlake et al., 1977) although there is a fraction of RNA migrating significantly more slowly than is commonly reported for lower

eucaryotes. While there may be some contamination from rRNA precursors or, in some instances, DNA, I do not believe that these factors significantly affect the results for the following reasons: First, while I have consistently observed DNase sensitive material at a characteristic position in electropherograms of RNA from cell types in which DNA synthesis is presumably occurring (Margolis-Kazan and Blamire, 1976), the slowly migrating material is, in fact, most prominent in the terminally differentiated somatic cells which should not be carrying on any significant amount of DNA synthesis. Second, it is unlikely that rRNA precursors could contribute significantly to the observed pattern because the RNA fractionation conditions give minimal non-specific binding by the mature rRNA species.

As noted, there is clearly a difference in the average migration rates of polyadenylated RNAs fractionated from gonidia, embryos, and somatic cells. The poly(A) RNA extracted from somatic cells has a higher apparent molecular weight than the poly(A) RNAs of gonidia or embryos. Developmental stage-specific differences between average sedimentation rates of total cellular poly(A) RNA from unfertilized eggs and zygotes of sea urchins have been reported (Slater et al., 1974; Wilt, 1977) but the significance of these differences in this organism is unknown. If we assume the

electrophoretic profiles to be a fair reflection of the total polyadenylated RNA species of the cellular material, then it could mean that a greater proportion of larger RNA is being synthesized and polyadenylated or that a greater proportion of larger preexisting RNA is being polyadenylated during the pulse labeling period. While I do not know the significance of the changes I observe in the average migration rates of the poly(A) RNAs in Volvox, the reproducibility of the general features of the electrophoretic patterns indicates that they represent real physiological phenomena.

The largest poly(A) tracts obtained by selective digestion of total RNA are estimated to be about 75-80 nucleotides. It is difficult to make a meaningful comparison between the lengths we have established for Volvox poly(A) tracts and the reported average tract lengths from other organisms because of the complications introduced by using transfer (4S) and 5S ribosomal RNAs as markers in electrophoretic analysis (see Results). When the average poly(A) tract length found in Volvox is compared to those reported for other representative lower eucaryotes such as yeast (20-60 nucleotides, Groner, et al., 1974), Neurospora (30, 55, 70 nucleotides, Lucas, et al., 1977), Tetrahymena (100 nucleotides, Rodriguez-Pousada and Hayes, 1976), and Dictyostelium (100 nucleotides, Firtel, et al., 1973) it

appears to be of an intermediate size with respect to the other tract lengths in this group. However, in all of the above cases, it was assumed that both 4S (70-80 nucleotides) and 5S (approx. 120 nucleotides) RNAs displayed the same electrophoretic behavior as poly(A) homopolymers containing respectively equal numbers of nucleotides. When the electropherograms of Volvox poly(A) tracts and those of the organisms cited above are compared, the Volvox poly(A) tracts show the lowest migration rate, which indicates that they are longer than any of the others from several lower eucaryotes. In fact, the largest Volvox poly(A) tracts are comparable in size to tracts from invertebrates (Lytechinus, Wilt, 1977; Ilyanassa, Clark and Kidder, 1977) and vertebrates (Xenopus, Rosbash and Ford, 1974; rabbit globin messenger, Nokin et al , 1976) using essentially identical calibrations for the 4 and 5S RNA electrophoretic markers that I have used. Apparently, there is little relationship between organismic complexity and average size of the poly(A) tract.

The observed heterogeneity of poly(A) tracts in gonidia and embryos and a trend toward uniformly larger poly(A) tracts in the terminally differentiated somatic cells suggests either a lengthening of existing poly(A) sequences by the terminal addition of adenylate residues or the de novo

synthesis of RNA molecules during differentiation containing longer terminal poly(A) segments. Another possibility is that the nucleotide residues in the terminal poly(A) tracts are constantly turning over in the cytoplasm as has been reported in other organisms (Diez and Brawerman, 1974; Dolecki et al., 1977). An increase in average poly(A) tract length would reflect a decrease in the ratio of exonucleolytic to adenylating enzyme activities.

The goal of this work is to characterize the metabolism of polyadenylated RNA in Volvox at different stages of development and in different cell types to determine its role in cellular differentiation. My work shows that there are differences between the poly(A) RNAs of the gonidia, embryos and somatic cells. The significance of these early results is, as yet, not understood but continued work involving fractionation of intracellular compartments (nucleus, cytosol, polysomes) should provide further information regarding poly(A) metabolism in Volvox.

ATP Pool Analysis

An analysis of intracellular ATP pools was performed on vacuolated gonidia and cleaving embryos. These two stages were chosen for comparison because they display the greatest apparent difference in the rate of ribosomal RNA synthesis (see Figures 10 and 11). The values obtained in

this study, along with data on the incorporation of radioactive label into the ATP pools, were used to calculate precursor pool specific activities for measurements of rates of RNA synthesis (see Discussion - RNA Synthesis in Development).

The number of pmoles of ATP per gonidium or embryo was measured using the very sensitive luciferin-luciferase assay as described in Materials and Methods. The high specificity of this chemical assay makes separation of ATP from all other soluble nucleotides and nucleotide precursors, unnecessary; but to measure radioactive label in ATP, it must be separated from all of the other labeled components. This was accomplished chromatographically as described in Materials and Methods.

The values of 9.93×10^{-4} picomoles of ATP per gonidium and 13.5×10^{-4} picomoles of ATP per cleaving embryo are small when compared to the ATP content of the egg of the sea urchin, Strongylocentrotus purpuratus. This egg, equal in diameter to a mature gonidium, contains about 1.0 picomole of ATP (Epel, 1963). Why should the gonidium have three orders of magnitude less ATP than the sea urchin egg? The energy demands of gonidial maturation and embryogenesis are expected to be great; the ATP content of the gonidium should be larger than it is. It is possible that the gonidium and cells of the embryo are capable of rapidly utilizing the "high energy"

inorganic phosphate stored in large intracellular pools of polyphosphate (see Kuhl, 1974 for a review). The synthesis of this material, of general occurrence in algae, is promoted by the light reactions of photosynthesis. There is no evidence in algae that polyphosphate can be or is used as a reserve of inorganic phosphate for regenerating ATP, but some heterotrophic microorganisms, e.g., E. coli and Saccharomyces cerevisiae, possess enzymes which can catalyze the reversible transfer of phosphate from ATP to polyphosphate (Ebel and Dirheimer, 1957; Kornberg, 1957; Hoffman-Ostenhof and Slechta, 1958). Polyphosphate granules first become apparent in the large vacuoles of maturing gonidia; they are seen in greatest abundance in the cytoplasm of cleaving embryonic cells and persist in fully differentiated somatic cells (see Results - Ultrastructure of Development) some unicellular green algae use polyphosphates at certain developmental stages as a source of phosphorous for intermediate metabolism and nucleic acid biosynthesis (see Kuhl, 1968). Whether polyphosphates have any special role in Volvox development as a source of energy or inorganic phosphorous, remains to be established.

Ultrastructure of Development

A comparison of the precleavage gonidium and cells of the early cleavage embryos with fully differentiated somatic

cells indicates the extent of modification of cellular organization related to embryogenesis.

The protein synthetic machinery in the condensed gonidium would appear to be dormant as judged by the lack of polysomes. The cells of the cleaving embryo contain considerably more of the cytoplasmic structures associated with protein synthesis (polysomes and some rough endoplasmic reticulum). This implies an increased requirement for de novo protein synthesis in the developing embryo. The lack of substantial amounts of rough endoplasmic reticulum (ER) and Golgi bodies suggests that the proteins being synthesized at this time are not destined for export from the cell. In contrast, the mature somatic cells abound in rough ER and Golgi complexes which are probably associated with the synthesis and assembly of the glycoproteins which constitute the extracellular matrix of the Volvox spheroid (Bisalputra and Stein, 1966).

The energy requirements of rapidly cleaving embryos may be expected to be unusually high. Consistent with this notion, mitochondria are fairly plentiful in the cells of the cleaving embryos, but the ground substance within their matrices does not stain any more darkly than the surrounding cytoplasm. This contrasts with the appearance of similarly fixed and stained mitochondria observed in gonidia and mature

somatic cells. It is not known whether this morphological dissimilarity reflects either a difference in age of the organelle or in metabolic state.

One source of an oxidizable substrate for the energy transformations in the mitochondria of cleaving embryos appears to be a substantial reserve of starch associated with the chloroplast material. However, it is not clear whether the lipid droplets so numerous throughout the cytoplasm, actively contribute to the food reserve of the developing embryo. Cleavage and the subsequent stages of embryogenesis must rely on internal food reserves since they can proceed quite normally in the dark without the concomitant activity of the energy-producing, light-requiring systems of photosynthesis. In further support of this notion, there is the occurrence of numerous polyphosphate containing volutin granules. The possible role of polyphosphate as a depot for high energy inorganic phosphate capable of generating ATP from ADP has been discussed in connection with intracellular ATP pools (see Discussion - ATP Pools).

The significance of certain observations made regarding the nucleolus in the vacuolated gonidium was discussed earlier (see Discussion - RNA in Development). It is certainly active in ribosomal RNA synthesis and ribosome assembly as evidenced by the large granular region. The granules

found here are most probably pre-ribosomal particles and their appearance has been correlated with an increase in rRNA synthesis (Nemer, 1963; Giudice et al., 1968; Botte et al., 1977). The ribosomal RNA cistrons have been found to be repeated in the genome (G. Kochert, personal communication) but the question of their selective amplification remains open.

A small fibrillar structure often seen in proximity to the nucleolus in vacuolated gonidia has provisionally been designated an accessory nucleolus although its true nature is not known. It resembles in fine structure the fibrillar component of the main nucleolus. Accessory nucleoli are found in higher plants where they are closely associated with the main nucleolus (La Cour and Wells, 1975). Swift (1959) has described similar bodies in certain dipterans ("Nebennucleoli") either bound to polytene chromosomes or floating free in the nucleoplasm. In no case has a function been ascribed to them.

Nucleoli are often seen in the cells of cleaving embryos although even when the cells are all sectioned in the same plane as they apparently are in Figure 22 many lack visible nucleoli. This may be a "sectioning artifact" or there may be only a specific group of cleavage cells which are endowed with nucleoli. These might have a particular developmental

fate different from the others, such as the formation of gonidial initials. The observation of active nucleoli in cleaving embryos is in apparent contradiction to the biochemical evidence which indicates that the rate of rRNA synthesis during cleavage is very low. Of course, the presence of these nucleoli suggests nothing of the level of their synthetic activity. The sum total of all of the nucleolar activity in the embryo may still be low. With so much of the time occupied with DNA replication and mitosis there would seem to be relatively little available for synthetic activity in the nucleus. Indeed, nucleoli are observed in the blastomeres of cleaving sea urchin embryos, but these so-called cleavage nucleoli large lack the granular component and are believed to function only minimally (Brachet, 1974).

One final observation should be made regarding nucleoli. No fully differentiated somatic cell has been observed to contain a morphologically normal nucleolus (see Results). The absence of a functional nucleolus in these cells (if such is the case) may be either a factor in or a manifestation of terminal differentiation and/or senescence. This matter should be considered in future aging studies in Volvox.

The general features of organization in gonidia and embryonic cells have been described in the Results. This

preliminary study did not yield any visible evidence of the cytoplasmic localization phenomenon whose presence has been ascertained by experimental means (Kochert and Yates, 1970). It has, however, shown the extent of intracellular reorganization which accompanies gonidial maturation and embryogenesis.

Future Research

It was emphasized from the beginning that it is the simple multicellular organization of Volvox which makes it an object of interest to the molecular biologist. Future research along the lines initiated or continued in this study should concentrate on answering questions that have long challenged embryologists. The first of these concerns the precise localization and nature of germ cell determinants in the gonidial cytoplasm. The second concerns the role of selective gene expression in establishing and maintaining the differentiated state.

The evidence for the involvement of RNA in the cytoplasmic localization phenomenon in the embryos of many organisms is convincing (see Introduction). How the determinants reach their positions in the cytoplasm is unknown. This question should be investigated in Volvox. Perhaps, the most fruitful approach will combine cytology and genetics. In many organisms that have been examined there is visible

evidence of cytoplasmic localization (e.g. polar granules); such cytological evidence should be sought in Volvox. If it can be found (even if it cannot be seen), its time of appearance in gonidial development should be determined. Cytochemical methods or methods making use of specific synthesis inhibitors might be employed. It would then be of interest to examine the gonidia of the somatic regenerator mutants (see Introduction) to see if this determinant or evidence of it was localized in regions of the gonidium other than where it was normally found.

The second problem follows directly from the first: how do the cytoplasmic determinants exert their effect to establish the germ cell line and what factors contribute to the differentiation of somatic cells after a predetermined number of cleavage divisions? In either case control of genomic expression is implied. The first aspect of this problem that should next be confronted is the organization of the genome. This can be done using the methods of DNA renaturation and DNA-RNA hybridization which have been successfully applied in organisms having genomes similar in complexity to that of Volvox (see Introduction). These types of analyses have yielded useful results in many higher organisms (reviewed by Davidson, et al., 1975) and have led to interesting speculations concerning the relationship

between genomic structure and function (e.g. Davidson et al., 1977). As far as is technically feasible differences in gene products (RNA and protein) in different cell types should be investigated. Ultimately it would be of value to isolate the specific messenger RNA for a given protein and follow the pattern of its synthesis and utilization in the course of development. This approach usually requires a system in which a differentiated cell is producing predominantly one type of mRNA (e.g. chicken oviduct cells producing ovalbumin mRNA). Such a situation may exist in the somatic cells of Volvox which are presumed to synthesize very large amounts of the glycoproteins that constitute the intercellular matrix. Mutants, defective in matrix formation have already been isolated (dissolver; Sessoms and Huskey, 1973). Research along these lines has already begun in this and other laboratories.

The kinds of experiments and analyses proposed here may be considered conventional; they have been carried out on a number of different "systems" with reasonably good success. But none of these "systems" has so far presented a complete story concerning the molecular mechanisms underlying development and cellular differentiation; perhaps Volvox will.

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