DNA METABOLISM IN DEVELOPING VOLVOX

by

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Abstract

Various biophysical and biosynthetic characteristics of DNA from Volvox carteri are presented. The DNA from three strains (HK-10, NB-7 and KA-1) is compared, and all strains are shown to contain at least two distinct DNA species which band at densities of 1.714-1.715 and 1.704-1.705 g/cm^3 in neutral CsCl and correspond to nuclear and "cytoplasmic" DNA, respectively. Base compositions calculated from these densities, 55-56% G+C for nuclear DNA and 45-46% G+C for cytoplasmic DNA, are in close agreement with % G+C values estimated from thermal denaturation data. Total DNA from strain KA-1 has a third component with a buoyant density of 1.693 g/cm^3 (34% G+C).

DNA synthesis was analyzed using radioactively labeled heterogeneously grown strains of Volvox carteri and profiles obtained following preparative CsCl density gradient centrifugation are presented. In addition, profiles obtained using synchronous cultures of strain HK-10 demonstrate that patterns of nuclear and cytoplasmic DNA synthesis are different during the various stages of the asexual life cycle. These differences in temporal patterns of DNA synthesis indicate that while nuclear DNA is made to some degree throughout the life cycle, cytoplasmic DNA synthesis appears to occur only at discrete intervals and mainly during the postinversion, prerelease expansion phase.

Alterations of these natural patterns were also studied. Treatment of HK-10 spheroids with delta-9-tetrahydrocannabinol (THC)

or ethidium bromide (EB) produced effects on patterns of development and DNA metabolism characteristic to each drug. It was demonstrated that, during THC (300-400 ug/ml) treatment the reproductive cells of the spheroids become arrested at the life cycle stage prior to cleavage, and those of EB (25 ug/ml) treated organisms begin cleavage but arrest in the process after several divisions. EB treatment (2.5-25 ug/ml) drastically depresses the synthesis of both nuclear and cytoplasmic DNA and residual synthesis is mainly cytoplasmic; THC treatment (300-400 ug/ml), however, specifically inhibits cytoplasmic DNA synthesis while that of nuclear DNA continues. Both drugs were demonstrated to mediate in the selective degradation of existing cytoplasmic DNA.

Studies were also performed using a developmental mutant of strain HK-10, "somatic cell regenerator" strain HMK-01. It was demonstrated that the cells of HMK-01 contain higher proportions of cytoplasmic DNA to total DNA than those of HK-10 and that the effects of THC treatment on the DNA metabolism and gonidial morphology of this mutant are similar to those described for wild-type spheroids.

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Opening Statement

The development of a multicellular organism from a single cell is a complex process involving the coordinated phenomena of cell division and differentiation. These events lead to the formation of a highly organized pattern of two major classes of cells - reproductive, which will contribute to the formation of future generations, and somatic, which are specialized and generally present in many differentiated forms.

The fate of a cell is usually determined long before the phenotypic specializations of its differentiated state are expressed. Under normal conditions, most forms of differentiation, once determined, are terminal and there are numerous examples where patterns of later differentiation may be traced to the localization of possible "determinative factors" in the cytoplasm of the original cell itself. One of the fundamental objectives of developmental biology is, therefore, to elucidate the mechanism of how these events are programmed into this cell, a developmental program which determines not only which cells will differentiate into particular specialized forms but also the temporal sequence in which such differentiation will occur.

It was my interest to study aspects of the regulation of development at the molecular level. Most current research of this type employs fairly complex multicellular organisms (e.g. sea urchin,

amphibian and mammalian embryos), in which the differentiated state is manifested by many varied cell types. In addition to their inherent complexity, numerous external experimental complications may arise during the study of such organisms, and I therefore thought that the control mechanisms of development might be more readily elucidated by studies employing a much simpler system.

The organism Volvox was chosen for this research because of the simplicity with which it expresses true differentiation. It has a simple organization and contains only two cell types. At the time this study was undertaken much information was available about the morphological development of Volvox and evidence of its applicability to biochemical studies was beginning to appear. Volvox was, however, a new organism to the field of molecular biology so the feasibility of its use in this type of research had to first be established by preliminary studies of a basic nature.

Since little was known about the nucleic acid metabolism of Volvox, I chose to study the biosynthesis of DNA in Volvox carteri f. nagariensis and its regulatory relationship to the asexual development of the organism. I began this work by characterizing the DNA species present in this as yet uncharacterized organism.

The total genome of an organism encompasses organelle DNA as well as nuclear DNA and there is increasing evidence for the involvement of organelle genomes in aspects of cellular development. In most organisms, the coordinated interaction of these DNA classes is necessary for the continuation of normal life cycle and/or cell cycle synthetic events. I therefore decided to

investigate the temporal patterns of nuclear and organelle DNA synthesis which occur during the asexual life cycle of Volvox and to examine the developmental consequences caused by the disruption of these patterns.

Differentiation and Development

Background

The mysteries concerning the mechanism of determination and differentiation have intrigued scientists for centuries. In the 1800's most theories explaining differentiation involved the qualitative distribution of diverse genetic determinants into the different cells of an organism. Thus, a differential loss of genetic material would lead to a narrowing of capabilities and subsequently be expressed as the differing functional activities of somatic cells. Such losses of genetic material are rare however, and even in the few nematodes and insects where actual loss of chromosomes or parts of chromosomes does occur during somatic cell formation (Boveri, 1889; Geyer-Duszynska, 1966), a qualitative distribution of genetic material to somatic cells has not been demonstrated.

Most evidence supports an equivalence in the genetic make-up of each cell and therefore indicates that differentiation between somatic and germ cells and between functionally differing somatic cells, does not involve the selective loss of genetic material. A quantitative constancy of DNA content among differentiated cell nuclei has been observed for most organisms (Mirsky and Ris, 1949) and more recently, the concept of genomic equivalence has been demonstrated at the molecular level. DNA-DNA reassociation studies have indicated that

the same complements of DNA sequences are usually present in embryonic, tissue culture and adult organ cells from the same organism (first demonstrated by McCarthy and Hoyer, 1964).

Another form of qualitative distribution of the genetic material could conceivably be achieved by a selective, permanent inactivation of portions of the DNA during development. This does not seem to occur, however, and evidence for the absence of such irreversible changes during differentiation comes from observations that the full genetic potential is retained by specialized cells. Gurdon (1963) achieved a dramatic demonstration of this phenomenon when he showed that nucleic from differentiated somatic cells of Xenopus are able to direct the formation of a complete new organism upon transplantation into enucleated oocytes.

Yet another form of qualitative distribution of DNA could involve the selective amplification of various portions of the genome in different cell types. Instances of this phenomenon are rare, however, and restricted to unusual situations (as in the amplification of ribosomal DNA in developing oocytes discussed below). Hybridization experiments show that most transcribed DNA is present in low copy number and that whether cells are active or inactive in the production of a particular gene product, they do not differ in the number of DNA sequence copies present which specify that product (Harrison et al., 1974).

Molecular patterns accompanying differentiation have been elucidated and most evidence points to the fact that differential gene activity, regulated on the level of controlled transcription from the same DNA complement, is the way functional specialization is

expressed in most cell types. Cytological manifestations of variable gene activity in RNA production have been demonstrated (e.g. Littau et al, 1964; Grossbach, 1974) and sequence homology comparisons of RNAs isolated from various differentiated tissues demonstrate that different mRNA populations are present during various developmental stages or in different tissues from the same organism (reviewed by Davidson and Britten, 1973).

The basis of this transcriptional level regulation in higher eukaryotes is not yet understood but models have been presented (e.g. Georgiev, 1972; Britten and Davidson, 1969; Crick, 1971) which are dependent, to various extents, upon the organization of DNA sequences in the genome. One of the most popular current models, that of Britten and Davidson (1969, modified and supported, Davidson and Britten, 1973), is based on the ordered patterns of interspersion of repetitive and non-repetitive sequences that have been demonstrated in the genomes of metazoa, and theorizes that the interspersed repetitive sequences play a role in structural gene function leading to differential transcription. They suggest that particular "batteries" of genes are activated together, during specific periods of development or various states of differentiation because they share homologous repetitive sequences or "receptors" which serve as binding sites for diffusible sequence-specific "activators." The functionally associated genes of a battery are thus defined by the particular sequence repetitions present in the receptors which will recognize the same activator molecules. Davidson et al. (1976) have recently demonstrated that the set of genes complementary to sea urchin gastrula mRNA tends to share

a particular group of repetitive sequences, thus supporting the idea that special subsets of repetitive sequences are contiguous to those structural genes expressed in a given state of differentiation.

Current knowledge therefore indicates that large-scale regulation of structural gene transcription underlies the processes of differentiation and development. The detailed molecular events involved in this phenomenon are not yet known however, and even if they were, the fundamental question as to how this information is programmed into the original cell from which an organism is derived would remain unanswered. Knowledge of the contents and organization of this cell is therefore critical for the understanding of subsequent developmental events.

Cytoplasmic Localization

The localization of "factors" in the egg cytoplasm which influence later development and differentiation has long been postulated. Most early concepts regarding the distinction between somatic and germ cells were influenced by the writings of August Weismann (1885) who theorized the localization of a "germ plasm" providing continuity between successive generations. Although Weismann's concept that the germ plasm alone contains the information necessary for the formation of an entire organism has since been negated, a modified form of his theory still survives.

Germ cell determinants are now known to exist in the egg cytoplasm of many organisms and the region containing them is often marked by easily visualized inclusions. These "polar granules," which are distributed only to primordial germ cells, have been identified in

many animals including the human (see Eddy, 1975).

Germ plasm localization has been studied most extensively in amphibian and insect eggs and the presence of germ cell determinant cytoplasm in the vegetal pole of uncleaved oocytes of Rana pipiens has been convincingly demonstrated (Smith, 1966). Ultraviolet irradiation of this area always leads to complete sterility, but, if cytoplasm from the vegetal pole of an unirradiated egg is injected, the recipient eggs give rise to embryos which contain functional germ cells. Similar results have since been obtained for Drosophila as well (Okada et al., 1974).

Such results indicate that the germ plasm causes nuclei with which it comes in contact, to initiate a developmental pattern that leads to differentiation involving gamete formation; however the mechanism causing this phenomenon is unknown.

Many other forms of localization are evident in various oocytes and early embryos. Classical biologists conducted cell lineage studies in numerous organisms which demonstrated that particular blastomeres give rise only to specific differentiated cell types, and other studies involving localization within the polar lobes of certain molluscan eggs showed that determinants necessary for normal differentiation are present in these non-nucleated lobes, (see Wilson, 1925). Recent molecular studies imply that, because some of the effects of lobe removal on protein synthesis are insensitive to actinomycin, it is possible that the lobe cytoplasm houses a qualitatively different set of messenger RNAs which was donated by the uncleaved oocyte (see below).

These phenomena of localization suggest that specific developmen-

tal programs are somehow sequestered in the egg cytoplasm, so that as the egg divides, cells appear to inherit instructions which prepare them for their various future differentiated functions. Although the nature of these morphogenetic determinants is unknown, most evidence implies that they are synthesized during oogenesis.

Maternal Messenger RNA

The first demonstrations that information localized in the unfertilized egg is indeed utilized during early development were made in the late nineteenth century. Echinoderm species-hybrid experiments in which species exhibiting distinct early developmental morphogenetic processes were crossed, showed that early developmental patterns exhibited only maternal characteristics. Later studies on numerous organisms have further demonstrated that no paternal traits are evident at least until gastrulation, and biochemical studies, confirming these observations, indicate the absence of most paternal type proteins before gastrulation. (Reviewed by Chen, 1967.)

Physically enucleated eggs have been shown to undergo cleavage, and in some cases even blastula formation, further substantiating the idea that such processes require only maternal constituents and that these are located in the egg cytoplasm. More recently, "chemical enucleation" has been achieved by preventing transcription from DNA through the use of actinomycin D. Gross and Cousineau (1963, 1964) first used this technique on sea urchin eggs and showed that cleavage can continue in the virtual absence of RNA synthesis whereas gastrulation never occurs in the drug's presence. Later experiments on numerous other organisms have

yielded similar results. (See below.)

Actinomycin studies on early embryos have also demonstrated the transcription of material necessary for differentiation prior to the onset of its expression. In Illyanassa embryos, for example, there are many cases of actinomycin sensitive activity required for particular early events of embryonic differentiation which occur long before these differentiations are expressed (Collier, 1966). It is therefore likely that new transcripts concerning events of later differentiation are precociously synthesized during the period when the early morphogenetic events programmed in the egg cytoplasm are occuring, and that this may be the molecular mechanism of determination.

The oocyte actinomycin D experiments of Gross and Cousineau (1964) also demonstrated that during the period of continued cleavage, the total rate of protein synthesis was unaffected as well. Since actinomycin blocks mRNA transcription, such results imply that protein synthesis in early embryos occurs via preformed templates of RNA derived from the oocyte (termed "maternal messenger RNA").

Other actinomycin experiments, conducted on various organisms, have yielded essentially similar results. Actinomycin does block cleavage in mammalian oocytes, however this phenomenon is now believed to be due to other toxic side effects of the drug since similar evidence of continued cleavage and maternal programming has been demonstrated in mouse embryos treated with another inhibitor of RNA synthesis, alpha-amanitin (Golbus et al., 1973).

The existence of maternal messenger RNA in the mature oocyte and the suggestion that it carries programs for most pregastrular morphogenesis is thus implied. Since protein synthesis is required for early cytodifferentiation and cell division, the presence of maternal messenger RNA best explains the results of species hybrid experiments as well as the complex patterns of protein synthesis which continue in actinomycin treated and enucleated eggs.

The fact that organelle genomes present in the cytoplasm are not the source of most of these proteins is also known. The non-mitochondrial nature of most protein synthesized in enucleated sea urchins was demonstrated by Craig and Piatigorsky (1971) who used ethidium bromide to block organelle biosynthesis and found no change in the proteins produced.

Direct molecular evidence for the existence of maternal messenger RNA, its quantitative contribution to the synthesis of embryonic proteins, its possible storage form, and theories on the means of its mobilization are reviewed extensively by Davidson (1976).

The fact that messages transcribed in the oocytes are stored to be used later in development shows that levels of control other than transcriptional, are operative, at least in early development. Although the mechanism of their post-transcriptional regulation is not known, the complexity and persistence throughout early development of these transcripts has been studied in numerous renaturation and hybridization experiments. The single copy sequence complexity of oocyte RNA from all organisms studied is

similar, which suggests that a general set of transcripts, in the form of maternal mRNA, is required for early development. The high complexity values obtained for oocyte structural gene transcripts, 2×10^7 to 4×10^7 nucleotides (Davidson, 1976), is indicative of their tremendous diversity, and complex sets of repetitive sequence transcripts are present in the mature oocyte as well.

Much of this RNA is known to persist through embryogenesis and its extreme stability has been demonstrated by Gurdon et al. (1974). There is however, still much mystery concerning the functional value of many of these transcripts to the early embryo, such as that of the 200,000 copies of adult globin RNA sequences recently demonstrated to be present in mature oocytes of Xenopus laevis (Perlman et al, 1977).

Oocyte Genome

The presence of structural gene transcripts of such high quantity and diversity, part of which must serve as maternal messenger, indicates that the genome of the oocyte is the site of unusually active RNA synthesis. Thus, the intense activity involved in oocyte maturation is generally accompanied by transcription from greater than the diploid (2C) complement of DNA.

The amount of nuclear DNA in meiotic prophase oocyte nuclei has been measured spectrophotometrically in many organisms, and results indicate the presence of at least the 4C nuclear DNA content, (e.g. mouse, rabbit, insects; see Davidson, 1976, for references).

Lampbrush chromosomes are a common structural form of the 4C nuclear genome observed in oocytes during their usual arrest in meiotic prophase. These chromosome forms contain lateral loops of DNA inserted into condensed chromomeric regions. Autoradiographic studies have demonstrated that the loops are involved in transcription and that their RNA synthesis is sensitive to actinomycin D (Izawa et al., 1963; Snow and Callan, 1969). The RNA synthesized on these loops in amphibian oocytes is large, heterogeneous in size and reflects the base composition of total chromosomal DNA (Sommerville, 1973; Sommerville and Molcolm, 1976). Hybridization with complementary DNA derived from oocyte poly-A containing mRNA, has demonstrated the presence of mRNA like molecules in the loop transcrips (Sommerville and Malcolm, 1976). Autoradiographic evidence and light and electron microscopic observations all indicate that initiation and termination sites for RNA transcription are located close to insertion sites at the chromomere and that the transcriptional unit extends over the entire loop length. The resulting primary transcript is therefore believed to consist of a single copy of "informative" mRNA attached to a "non-informative" or regulatory portion containing moderately repetitive sequences (reviewed by Sommerville and Malcolm, 1976).

Lampbrush chromosome forms, which are also present during spermatogenesis, are almost ubiquitous in oocytes. They are, however, absent in certain insect groups (meroistic) where excess DNA for transcription is supplied by nurse cells which play a dominant role in oogenesis. Nurse cell nuclei are usually highly polytene and contain several hundred times the DNA quantity of the oocyte nucleus (where the 4C complement is present, but in highly condensed form).

Autoradiographic evidence indicates that oocyte RNA is synthesized in nurse cells and then transported to the egg through cytoplasmic connecting bridges and that much of this RNA is ribosomal (Hughes and Berry, 1970).

In addition to the generally increased DNA quantity which accompanies the high transcriptional activity observed during oocyte maturation, differential amplification of specific genome portions, commonly occurs as well.

It is now well known that in many organisms the genes coding for the precursors of ribosomal RNAs are selectively amplified during early oogenesis (see Tobler, 1975 for review). In Xenopus, this ribosomal DNA (rDNA) amplification results in the production of about 1,500 extrachromosomal nucleoli, each containing several sets of rDNA genes. Brown and Dawid (1968) demonstrated that while the somatic cells of Xenopus laevis contain about 450 repeated genes for rRNA, the mature oocyte possesses about 4,000 times this amount.

While selective DNA amplification has been demonstrated for rDNA in most animal systems, and for the genes coding for histones in developing sea urchins (Kedes and Birnstiel, 1971), there is no definitive evidence for differential DNA amplification during the development of higher plants (Ingle and Sinclair, 1972).

The DNA of mitochondria is also found to be amplified in mature oocytes of numerous organisms. In Xenopus and Rana pipiens, for example, oocytes contain amounts of mitochondrial DNA sufficient to provide 1.8×10^8 mitochondrial genomes, and new mitochondrial DNA synthesis has not been detected before the tadpole stage in these organisms (Chase, 1970; Chase and Dawid, 1972). The fact that

mitochondrial DNA is probably not present in amplified form to provide excess gene products (Craig and Piatigorsky, 1971, see above), suggests that its function may be one of supplying mitochondrial genomes to the cells of the embryo under conditions where rapid cleavages do not allow sufficient time for their synthesis. The apparent necessity of sufficient and constant quantities of organelle DNA per cell, as indicated by the otherwise usual tight coupling of its synthesis to the cell cycle, is described in detail below.

Cytoplasmic DNA

Background

Cytoplasmic organelles contribute to the development of an organism through genetic control outside that of the nuclear chromosomes. The existence of cytoplasmic genes was first reported in the early 1900's by Erwin Baur and Carl Correns in their description of the non-mendelian inheritance of a factor which influences chloroplast development and causes leaf variegation, and this type of cytoplasmic or maternal inheritance has since become an accepted phenomenon (see Sager, 1972).

Chloroplast DNA (ctDNA) was first detected by Chiba in 1951 and this discovery was later confirmed by ultrastructural (Ris and Plaut, 1962) and biochemical (Chun et al., 1963) evidence. Cytochemical and electron microscopic studies by Nass and Nass (1963) indicated the presence of DNA in mitochondria, but the unequivocal identification of mitochondrial DNA (mtDNA) was subsequently made by means of CsCl density gradient centrifugation (Luck and Reich, 1964, see below). The distinct base compositions and buoyant densities of many organelle DNAs has facilitated their isolation and characterization by this

method, although positive identification may only come by agreement of such values with those obtained from DNA extracted from isolated organelles.

Reich and Luck (1966) extracted DNA from isolated mitochondria of Neurospora crassa and found its buoyant density value, 1.702 g/cm^3, comparable to the "presumed" mtDNA seen as a shoulder (ρ=1.701 g/cm^3) on nuclear DNA which bands at 1.712 g/cm^3 in neutral CsCl.

Most mammalian mtDNAs band at densities of 1.701-1.704 g/cm^3 and those of birds at 1.707-1.711 g/cm^3. Higher plants contain mtDNAs with buoyant densities of 1.706 g/cm^3 while those from Acetabularia and Chlorella band at 1.714 and 1.712 g/cm^3 respectively. The average of the buoyant densities reported for yeast (S. cerevisiae) mtDNA is 1.684 g/cm^3 (see Sager, 1972).

In many algae, the DNA of the chloroplast has a buoyant density value in neutral CsCl that is clearly distinguishable from that of the nucleus. Buoyant densities of ctDNAs are 1.696 g/cm^3 from Acetabularia mediterranea (Gibor, 1967), 1.685 g/cm^3 from Euglena gracilis (Ray and Hanawalt, 1964), 1.687 g/cm^3 from Chlorella pyrenoidosa (Bayen and Rode, 1973), and 1.695 g/cm^3 from Chlamydomonas reinhardi (Sager and Ishida, 1963), while corresponding nuclear DNAs have buoyant densities ranging from 1.702 g/cm^3 for Acetabularia to 1.724 g/cm^3 for Chlamydomonas. The buoyant densities of ctDNAs found in higher plants (1.694-1.698 g/cm^3), however, are close to and therefore usually indistinguishable from those of corresponding nuclear DNAs (Kung, 1977).

The distinction between the organelle DNAs of chloroplasts and mitochondria has been established for Euglena. Studies employing enriched chloroplast fractions (Brawerman and Eisenstadt, 1964), showed that the DNA species banding at 1.686 g/cm^3 in CsCl is indeed of chloroplast origin. The isolation of DNA from Euglena mutants which lack ctDNA facilitated the identification of mtDNA, a small DNA fraction banding at 1.691 g/cm^3, which was later extracted from isolated mitochondria of these mutants as well (Ray and Hanawalt, 1965; Edelman et al., 1966).

DNA Synthesis in Mitochondria and Chloroplasts

Evidence for the semiconservative replication of ctDNA comes from the work of Chiang and Sueoka (1967) who examined synchronous Chlamydomonas cultures by the ^{15}N-^{14}N density transfer method of Meselson and Stahl (1958). The replication of mtDNA from Saccharomyces lactis (Smith et al., 1968) and Tetrahymena pyriformis (Parsons and Rustad, 1968) appears to be semiconservative as well.

DNA synthesis in isolated chloroplasts was first reported for Euglena (Scott et al., 1968) and replication of mtDNA was shown to occur in mitochondria isolated from rat liver cells (Karol and Simpson, 1968).

Patterns of Nuclear and Organelle DNA Synthesis During the Cell Cycle

Nuclear DNA synthesis in eukaryotes is generally periodic and does not occupy all of the cell cycle. It occurs during a restricted period of interphase (S) which is generally preceeded (G1) and followed (G2) by periods of no DNA replication.

Temporal patterns of the cell cycle and DNA synthesis differ among cells from different organisms, different cell types from the same organism, and may even vary in the same cell type during different stages of development. Early embryos, which divide without growth, usually show the greatest deviations from the "normal" DNA cycle patterns. Cell cycle times are much shorter, and amphibian embryos even lack a G1 phase in these early cycles (see Mitchison, 1971 for review of eukaryotic and embryonic DNA cycles).

The patterns of synthesis of the DNA in chloroplasts and mitochondria are different from those of nuclear DNA, and the number of periods of this cytoplasmic DNA synthesis, and the timing of its replication with respect to the cell cycle, varies among organisms. Chlamydomonas reinhardi has been shown to have two periods of ctDNA replication, which are out of sequence with each other and with the period of nuclear DNA synthesis (Chiang and Sueoka, 1967). The ctDNA of Euglena gracilis is replicated 1.5 times during a period partially out of phase with nuclear DNA synthesis (Manning and Richards, 1972). Satellite DNA of Chlorella pyrenoidosa (presumed to be of chloroplast and mitochondrial origin) is synthesized at a low rate throughout the cell cycle, while nuclear DNA synthesis occurs only in the period just prior to and during cell division (Wanka et al., 1970).

Patterns of mtDNA synthesis have also been found to differ greatly from those of nuclear DNA. MtDNA is synthesized at a relatively constant rate during the entire cell cycle in Physarum (Braun and Evans, 1969) and Tetrahymena (Cameron, 1966). By monitoring DNA replication in Saccharomyces cerevisiae by ^{14}N

incorporation into ^{15}N labeled DNA, Sena et al. (1975) showed that mtDNA is replicated continuously during the cell cycle while nuclear DNA synthesis is discontinuous. This type of continuous mtDNA synthesis was demonstrated in chick fibroblasts using autoradiography (Meyer and Ris, 1966), and recent biochemical studies have shown that the synthesis of mtDNA also continues throughout the cell cycle in mouse L cells (Bogenhagen and Clayton, 1977).

Nuclear and Organelle Interaction in DNA Replication

The very existence of temporal differences in the synthesis of nuclear and organelle DNA during the cell cycle, implies at least some separation in the control of their replication. The main supporting evidence for this view comes from instances of organisms surviving irreversible losses of organelle DNA. Strains of Euglena lacking ctDNA can grow well heterotrophically (Edelman et al., 1965) and yeast mutants which have irreversibly lost mtDNA can grow anerobically (Goldring et al., 1970). In these cases, nuclear DNA synthesis continues indefinitely in the absence of organelle DNA replication.

Most evidence, however, suggests that genomic interaction is involved in the regulation of DNA synthesis. Irreversible losses of DNA are rare and the maintenance of constant proportions of nuclear and organelle DNA in cells grown under different physiological regimes also implies that these DNA replication cycles are tightly coupled (see Sager, 1972). In cases where the replication of one class of DNA is inhibited while synthesis of the other continues, the uninhibited DNA species usually does not exceed one round of replication and the organism subsequently dies (e.g. Flechtner and Sager, 1973; Grossman et al., 1969, see below).

The naturally occurring patterns of DNA syntheis have been disrupted by direct or indirect interference with the replication of either nuclear or organelle DNA. Agents which selectively alter the metabolism of one class of DNA, or mutants (usually temperature sensitive) which are defective in some aspect of DNA replication, have been employed for studies of direct synthesis inhibition. The use of agents which specifically inhibit nuclear or organelle transcription or translation has, in many instances, prevented the synthesis of a particular class of DNA, and thereby provided information on the genomic origin of factors involved in the regulation of its replication.

Preferential synthesis of mtDNA in the absence of protein synthesis on cytoplasmic ribosomes has been demonstrated in the yeast Saccharomyces cerevisiae, where the antibiotic cycloheximide prevents nuclear DNA replication while allowing mtDNA synthesis to continue temporarily (Grossman et al., 1969). Yeast mtDNA replication also persists in cases where cell division cycle mutations prevent the initiation of nuclear DNA replication (Hartwell et al., 1973), however mutations which block the elongation of nuclear DNA being synthesized, block mtDNA synthesis as well (Newlon and Fangman, 1975). This suggests that there exist certain gene products at least, which are necessary for both nuclear and mtDNA replication in yeast.

The uncoupling of mitochondrial and nuclear DNA synthesis has been demonstrated in mouse L cells as well. Treatment with 5-fluoro-deoxyuridine or methotrexate allows mtDNA synthesis to continue in the absence of replication of nuclear DNA (Bogenhagen and Clayton, 1976), whereas nuclear DNA replication persists in the absence of

mitochondrial, when mouse L cells are treated with ethidium bromide (Smith et al., 1971). (See section on ethidium bromide below.)

Studies involving the uncoupling of nuclear and chloroplast DNA replication have also been conducted. Treatment of Chlorella with cycloheximide leads to consequences similar to those exhibited by yeast. Organelle DNA is synthesized in the absence of nuclear DNA replication upon exposure to this protein synthesis inhibitor (Wanka and Moors, 1970), implying that the organelle DNA polymerases in Chlorella are not made on cytoplasmic ribosomes. Although the activity of the nuclear DNA polymerase is unaffected by cycloheximide treatment (Wanka and Moors, 1970), Wanka et al., (1972) found that there is another specific protein, synthesized during the S phase, that is required for nuclear DNA replication in this organism.

In Euglena, results of studies with cycloheximide are not as sharply defined. Nuclear DNA synthesis is strongly inhibited by the drug, but that of organelle (mt and ct) DNA is somewhat inhibited as well (Richards et al., 1971). However, since chloramphenicol, which is specific for organelle protein synthesis, does not have a preferential inhibitory effect on either type of DNA replication in this organism (Smillie et al., 1967), the proteins necessary for organelle DNA synthesis in Euglena are probably made on cytoplasmic ribosomes (Richards et al., 1971).

Results of studies on Chlamydomonas reinhardi suggest that the genetic information for its ctDNA polymerase is located in the nucleus and that the enzyme is synthesized on cytoplasmic ribosomes. Treatment with rifampicin leads to the specific inhibition of ct RNA polymerase (Surzycki, 1969) and to the loss of chloroplast ribosomes

(Goodenough, 1971); however, ctDNA synthesis continues even when cells are kept on the drug for several generations. Synthesis of ct DNA persists in the presence of spectinomycin, as well, which specifically inhibits protein synthesis on chloroplast ribosomes (Surzycki et al., 1970), and uncoupling allowing the temporary continuation of only ctDNA replication, has been demonstrated using other antibiotics (Blamire et al., 1974, see below). Such evidence suggests that in Chlamydomonas, organelle DNA replication may continue in the absence of the transcription and translation of information from the chloroplast genome.

Most evidence of gene products from one genome influencing the replication of DNA in the other has implicated nuclear control in the regulation of organelle DNA synthesis. Although there have been reports that proteins of organelle origin act as regulators in the production of gene products made in the cytoplasm, these have only demonstrated the necessity of organelle regulation or intergenomic cooperation in further organelle biogenesis (e.g. Barath and Kuntzel, 1972, mitochondrial biogenesis in Neurospora; Hoober and Stegman, 1973, chloroplast biogenesis in Chlamydomonas).

There is evidence, however, that in Chlamydomonas reinhardi the chloroplast genome exerts control in the regulation of nuclear DNA replication (Blamire et al., 1974). Although Flechtner and Sager (1973) have shown that nuclear DNA synthesis in Chlamydomonas may continue in the absence of that of ctDNA (see ethidium bromide section below), Blamire et al. (1974) have demonstrated, that in this same organism, nuclear DNA synthesis is inhibited while that of the chloroplast continues in the presence of a series of antibiotics which

are known to specifically block only chloroplast macromolecular synthesis. Such evidence implicates chloroplast protein synthesis in the regulation of nuclear DNA replication and adds further support to the idea of interdependent genomic regulation in the tight coupling of organelle and nuclear DNA synthesis.

Ethidium Bromide Studies

Ethidium bromide (EB) has become a standard probe for analyzing nucleic acid structure. The intercalative binding of EB to DNA was developed into a test for superhelical winding by Vinograd and collaborators (Bauer and Vinograd, 1968), and the drug has been used extensively for DNA structural studies.

The *in vivo* biological and molecular effects of EB on DNA have been studied in numerous organisms and most extensively in yeast. In *S. cerevisiae*, the induction of respiratory deficient mutants that occurs upon treatment of growing cells with micromolar concentrations of EB (Slonimski *et al.*, 1968) is accompanied by the combined inhibition of mtDNA synthesis and the breakdown of mt DNA (Goldring *et al.*, 1970; Perlman and Mahler, 1971; Nagley and Linane, 1972). Mt DNA is completely lost from growing cells when EB treatment is extended; however treatment of cells under non-growing conditions causes only a limited breakage yielding large mt DNA fragments (Mahler and Bastos, 1974a; Mahler, 1973). If EB is present when growth is resumed, the ensuing inhibition of mt DNA synthesis and the further degradation of this DNA lead to its elimination; however, if growth is resumed in the absence of EB, mt DNA synthesis occurs but results in the formation of DNA with altered density (Perlman and Mahler, 1971).

Mechanisms involved in producing these effects have been studied using whole cells or isolated mitochondria and ^{3}H-labeled EB. It was found that there is an initial rapid binding of EB to mtDNA accompanied by a breakage of the DNA into larger fragments, which, in the presence of an energy source, is followed by the excision of bound EB and further degredation of mt DNA to oligonucleotides (Bastos and Mahler, 1974; Mahler and Bastos, 1974a & b). Criddle et al. (1976) have demonstrated that the initial limited fragmentation of mt DNA is followed by a reassembly of fragments (associated with an EB insensitive incorporation of ^{3}H-adenine into mtDNA) during the period corresponding to recovery of respiratory competence.

In cultured mouse and human cells EB rapidly inhibits mtDNA synthesis and induces a negative superhelix density in the pre-existing closed circular mt DNA (Smith et al., 1971). There have been reports (Koch, 1972, 1973) of EB induced nicking or degradation of mtDNA in cultured human cells where nuclear DNA contamination was removed by treatment of isolated mitochondria with DNase, but recent studies by Smith (1977) on cultured mouse L cells indicate that specifically labeled mtDNA from EB treated cells is not substantially nicked or degraded over the course of several days.

Studies demonstrating the effects of EB on ctDNA have also been conducted and Flechtner and Sager (1973) have shown that EB uncouples nuclear and ctDNA replication in Chlamydomonas by specifically inhibiting ctDNA synthesis. This study also demonstrates that pre-existing ctDNA is degraded in the presence of EB but that this loss is reversible when treatment does not exceed 12 hr or concentrations of 10 ug/ml.

Volvox: A Potential Model System for the Study of Development

Many of the basic principles underlying genetics and molecular biology have been elucidated through research employing prokaryotic and unicellular eukaryotic organisms. Their simplicity and ease of manipulation have aided greatly in accelerating the pace of research progress in these areas. Single-celled microorganisms have also been used extensively in cell cycle research (see above and Mitchison, 1971), but they have not been suitable systems for study in areas of developmental biology, since, by definition, they lack the ability to differentiate into organisms containing more than one cell type. Research of this nature has therefore been restricted to more complex multicellular organisms whose somatic cells differentiate into many specialized forms.

The recent advances in the elucidation of the molecular events accompanying development and differentiation have not yet provided answers as to how these processes are regulated. Various levels of molecular control appear to be operative during development and the further inherent and manipulative complications imposed by most of the complex multicellular organisms being studied may well be impeding progress in this research area. It would therefore be wise to utilize a more suitable model system, if one were available.

The ideal organism for such studies would have to meet certain criteria, the most fundamental of which is simplicity. Since the research objective is one of determining the molecular mechanisms responsible for coordinating the formation of organized patterns of various cell types from a single cell, the simplest system relevant

for such studies would be one in which cell differentiation is sharply defined, organized in a definite pattern, and yet manifested in the minimum number of different states. This organism should be easily manipulated as well, and in many respects exhibit similarities to microorganisms employed for biochemical research in other disciplines. The lack of extraneous complications involved in the study of such a model system, may make some of the "mysteries" surrounding the regulation of development and differentiation more readily decipherable.

A potential candidate for this "ideal organism" exists within the genus Volvox, and its applicability as a system for such studies is provided by the very simple terms in which it expresses true cellular differentiation. It has a simple organization and only two cell types, which are distinctive in both form and function.

The morphology of a mature asexual Volvox carteri individual is that of a spheroid with 3-5000 somatic cells on its surface and 10-16 reproductive cells within. The biflagellate somatic cells are formed during embryogenesis and do not divide again. During the asexual life cycle, the larger non-flagellate reproductive cells (gonidia) give rise to new individuals after a defined sequence of events which includes maturation, cleavage and inversion. The juvenile spheroids thus formed are subsequently released from the parent to begin the cycle anew. (See Starr, 1970 for life cycle details.)

Although Volvox is truly a differentiated multicellular organism, it is easily manipulated in the laboratory and therefore useful for biochemical studies. Large cultures of Volvox can be maintained axenically on a defined medium and external factors affecting its

growth can be controlled. (See Starr, 1970.) Established synchronous growth conditions can be used to provide large quantities of Volvox spheroids at the same developmental stage (Yates et al., 1975; Margolis-Kazan and Blamire, 1976), and the two cell types can be physically separated (Yates et al., 1975). Also, radioactively labeled macromolecular precursors are easily taken up by Volvox spheroids and incorporated into their nucleic acids and proteins (Kirk and Kirk, 1976; Margolis-Kazan and Blamire, 1976; Kochert, 1975 for review). The haploid Volvox genome, which facilitates the isolation of mutants, as well as the organism's existing sexual cycle, make genetic studies of developmental control mechanisms possible in this system as well (Sessoms and Huskey, 1973).

Thus, Volvox meets the criteria of ease and simplicity which may be necessary for a model system employed in studies of the molecular mechanisms of development and differentiation. The similarities of many of the fundamental principles of molecular biology in organisms as diverse as prokaryotes and multicellular eukaryotes makes it probable that the control mechanisms involved in Volvox development are operative in more complex multicellular organisms as well. The relevance of such research on Volvox to studies on animal development is further implied by the numerous similarities that exist between Volvox carteri embryogenesis and early development in more complex organisms. Rapid cleavage, with no growth between divisions, leads to the formation of a hollow sphere of cells, during Volvox embryogenesis and usually in blastula formation as well. Subsequently, morphogenetic movements follow--inversion in Volvox and gastrulation in animals embryos. Similarities even exist

between the cleavage patterns of a dividing gonidium and those of an amphibian or sea urchin egg (see Starr, 1970 for Volvox cleavage).

Numerous comparisons may also be drawn between the development of a Volvox carteri reproductive gonidium and the process of oocyte maturation. Unlike the gonidia of other Volvox species (e.g. V. aureus, Darden, 1966) and like most oocytes, V. carteri gonidia do not undergo periods of growth between divisions, and instead devote a larger proportion of their life cycle in preparation for cleavage. Gonidia increase greatly in size (Starr, 1970) and their synthesis of RNA and protein is highest during this period (Yates, 1974; Kochert, 1975). Yates (1974) has demonstrated that the 8-10 mature gonidia present in a precleavage spheroid of V. carteri f. weismannia, contain as much or more ribosomal RNA (rRNA) than do all 3,500 somatic cells. It is therefore probable that synthesis of this RNA species is not required until after cleavage. The synthesis of rRNA during oocyte maturation and early development has been studied extensively in many animal systems and most findings on rapidly dividing embryos indicate that embryonic rRNA synthesis does not begin before gastrulation. The rRNA present in cleaving cells is therefore contributed by the oocyte which synthesizes the necessary quantities during its development (see Davidson, 1976).

Weismann's "germ plasm" theory and the current concepts of cytoplasmic localization, derived from studies on higher organisms, have been described above. In asexual spheroids of Volvox the distribution of gonidia may be traced to the pattern of cleavage during their formation. Early cleavages produce cells which are morphologically identical, but unequal divisions differentiate the

larger gonidial initials from the prospective somatic cells at the division of the 16 celled stage in V. carteri f. weismannia (Kochert, 1968) or the 32 celled stage in V. carteri f. nagariensis (Starr, 1969, 1970). In both strains, the smaller cell continues to cleave and form somatic cells while the large cell usually ceases division and subsequently becomes a gonidium.

The symmetrical pattern of gonidial localization in Volvox is therefore established very early in spheroid formation, and Kochert and Yates (1970) have shown that it is probably established within the maturing gonidium itself. Unilateral irradiation of Volvox gonidia with ultraviolet (UV) light, just prior to the onset of cleavage, leads to the production of spheroids lacking one or more gonidia but whose existing reproductive cells subsequently produce spheroids with normal gonidial complements. The pattern of gonidial localization is therefore probably one which is predetermined and may exist in the form of cytoplasmic localization of "germ plasm" in the mature gonidium. Destruction of this germ plasm in Volvox by UV irradiation is comparable to results obtained for higher organisms where UV-labile germ plasm has been demonstrated in insect and amphibian eggs. UV treatment of these oocytes results in the formation of organisms lacking functional gametes (see above and Gurdon and Woodland, 1968 for review), thus demonstrating further similarities between gonidia and oocytes.

Kochert (1975) has also centrifuged mature gonidia to displace the cytoplasm and found that spheroids subsequently produced had altered gonidial distribution, results which again suggest the cytoplasmic localization of factors in the maturing reproductive cell

which determine the formation of future gonidia. Thus, the fascinating possibility of studying localized developmental determinants in a truly simple system is also provided by Volvox.

Purpose

At the time this study was undertaken most published reports on Volvox carteri had been related to morphological changes accompanying development and were reviewed by Starr (1970). (Also see Kochert and Yates, 1970; Sessoms and Huskey, 1973). However, a few studies of a biochemical nature had recently been conducted. Kochert and Sansing (1971) had isolated and separated total nucleic acids of Volvox carteri f. weismannia by polyacrylamide gel electrophoresis, and the processing of ribosomal RNA precursors in that Volvox strain had also been described (Kochert, 1971). Also, Tucker and Darden (1972) had recently studied the uptake or uridine-2-^{14}C into nucleic acids through the asexual life cycle in another Volvox species (V. aureus).

Thus, the patterns of macromolecular biosynthesis were as yet uncharacterized in Volvox. Studies related to DNA appeared to be a logical beginning for describing the biochemical events of development in Volvox carteri since these nucleic acids contain the information for the synthesis of RNAs and proteins and all these macromolecules have been shown to change both qualitatively and quantitatively at various developmental stages in other organisms.

The purpose of this work was to document the changes in nuclear and organelle DNA synthesis occuring during the development of Volvox carteri f. nagariensis in an attempt to discover how these changing patterns may be related to the processes of differentiation and development.

Volvox was, however, relatively new to the area of molecular biology, and before being used for more sophisticated research, its applicability for such studies had to first be rigorously demonstrated Thus much of my early research dealt with proving that the Volvox system was a feasible one for studies on DNA synthesis.

Numerous problems involved with growing Volvox and extracting its DNA had to be worked out even before the necessary basic DNA characterization studies could ensue; after nuclear and organelle DNA had been characterized, methods for studying their synthesis had to be devised. Only after proving that it is possible to extract DNA and study its synthesis in this as yet uncharacterized organism, could I proceed to study the temporal patterns of nuclear and organelle DNA synthesis that occur during Volvox development. These studies posed new methodology problems as well, since selection techniques for obtaining synchronous spheroid populations had to be devised. To exclude the possibility that the established imposed synchrony method itself was not causing changes in normal DNA metabolism, comparisons had to be made between results obtained using that method and those where more natural growth conditions were maintained. After elucidating the temporal patterns of DNA synthesis during the asexual life cycle of Volvox, I could then examine the consequence to development caused by their disruption.

The DNA species of several Volvox strains were to be characterized by analytical CsCl equilibrium density gradient centrifugation and thermal denaturation techniques, which would yield buoyant density and Tm determinations from which base composition could be calculated. The bulk of this project however, was to deal

with studies involving patterns of nuclear and organelle DNA synthesis and the main technique chosen for these analyses was preparative CsCl density gradient centrifugation of radioactively labeled material which could provide the required information in a relatively short time. Studies by Tucker and Darden (1972) on V. aureus had demonstrated the feasibility of achieving radioactive nucleic acid precursor uptake and incorporation in other Volvox species, and CsCl centrifugation had effected the separation of nuclear and organelle DNAs in numerous related organisms (see above).

Alterations of DNA synthesis patterns were studied using a developmental mutant, and two drugs, one of which, EB, had been well characterized and was known to specifically affect cytoplasmic DNA metabolism (see above). The other drug used in these studies, delta-9-tetrahydrocannabinol, had been shown to inhibit total DNA synthesis in numerous organisms (see Discussion); but its possible specific effects on DNA metabolism had not yet been studied.

Since 1973 Volvox has become increasingly popular for biochemical studies, and recently, there have been numerous publications involving Volvox research in that area. Three papers on the characterization of the sexual inducer in various strains of the organism appeared concurrently (Kochert and Yates, 1974; Starr and Jaenicke, 1974; Pall, 1974), and Volvox histones have been extracted and characterized as well (Bradley, Goldin, and Claybrook, 1974). Numerous studies involving macromolecular synthesis in V. carteri f. weismannia are reviewed by Kochert (1975) and many are described, where relevant, in this text. Biochemical studies using synchronized cultures and separated cell types of that strain have been conducted by Yates et

al. (1975) who measured DNA by spectrofluorometric assays. Kirk and Kirk (1976) have also employed light synchronized cultures to study amino acid uptake and protein synthesis throughout the asexual life cycle of V. carteri f. nagariensis, and portions of this dissertation have been published as well (Margolis-Kazan and Blamire, 1976; 1977). Further studies on the molecular events that accompany development in Volvox are currently being conducted in several laboratories.

MATERIALS AND METHODS

Source of Chemicals and Materials

Radioisotopes and Liquifluor were obtained from New England Nuclear; ethidium bromide (EB), protease (type VI), glycylglycine, polyoxyethylene sorbitan monopalmitate (Tween 40), and polyoxyethylene sorbitan mono-oleate (Tween 80), from Sigma; pancreatic RNase and T1 RNase, from Worthington; Miracloth, from Chicopee Mills (also Fisher); Nitex mesh (in sizes indicated in text) from Tobler, Ernst and Traber; Kieselguhr (Hyflo Super Cell), toluene (certified A.C.S.), and trichloroacetic acid (TCA), from Fisher; Sarkosyl NL-97, from Ciba-Geigy; CsCl (preparative grade), from Kawecki Berylco Industries; analytical CsCl (optical grade), from Harshaw Chemical; cellulose nitrate ultra-centrifuge tubes, from Beckman; paper filters (no. 3; 2.4 cm) and glass fiber (GF/A) filters were products of Whatman; delta-9-tetrahydrocannabinol (THC) was supplied by DHEW, Public Health Service, NIDA; marker DNAs used in analytical CsCl centrifugations were kindly supplied by Dr. R.A. Eckhardt; and the H-C Microhomogenizer was invented and produced by Howard Caplen, Brooklyn College.

Strains

The three strains of *Volvox* used in this study are *Volvox carteri* f. *nagariensis* HK-10 (supplied by R.C. Starr), *Volvox carteri* f. *weismannia* NB-7, and KA-1 (supplied by G. Kochert).

Solutions

Volvox Medium-SVM (Provasoli and Pinter, 1959)

For each liter of medium the following amounts of stock solutions are added to 981 ml of distilled water.

# of ml	Stock Solution	Per 100 ml
1	$Ca(NO_3)_2$ 4HOH	11.8 g
1	$MgSO_4$ 7HOH	4.0 g
1	Na_2glycerophosphate	5.0 g
1	KCl	5.0 g
10	Glycylglycine	5.0 g
1	Biotin	25.0 ug
1	B_{12}	15.0 ug
3	P_{IV} trace metal solution	see below

Adjust pH to 7 or 8 with 1 N NaOH.

P_{IV} Trace Metal Solution

Add the following amount of salts and chelating agent to 500 ml of distilled water.

Na_2EDTA (add and dissolve first)	0.750 g
$FeCl_3$ 6HOH	0.097 g
$MnCl_2$ 4HOH	0.041 g
$ZnCl_2$	0.005 g
$CoCl_2$ 6HOH	0.002 g
Na_2MoO_4	0.004 g

Stock Solutions, Used for Drug Experiments

Ethidium bromide was dissolved in sterile SVM, pH 7 (1-10 mg/ml) and stored in the dark at -20C until required.

THC was supplied as a solution of 20 mg/ml in 95% ethanol; stock solutions (stored in the dark at -20C), prepared by transferring 1 ml of the THC-ethanol solution to 1 ml tween 40/80, contained 10 mg/ml THC in 47.5% ethanol, 25% tween 40, 25% tween 80.

A stock solution containing 50% (95%) ethanol, 25% tween 40 and 25% tween 80, termed "carrier" hereafter, was stored at -20C until required.

Scintillation Fluid

Toluene-based liquid scintillation fluid was prepared by removing 100 ml liquid from a 1 gallon bottle of toluene and replacing it with 155 ml Liquifluor.

Asexual Life Cycle of Volvox carteri; Identification of Stages

There are several easily distinguishable periods of time in the life cycle of Volvox during which distinct morphological changes take place. For the purpose of this study, these stages, depicted in figure 1, (see also plate 1) are defined as follows:

a) Total Expansion: The continuous process of new spheroid enlargement which follows cleavage and inversion. Expansion is mainly due to secretion of matrix material by the somatic cells. Gonidial enlargement occurs during this period (from about 13u to 90u) but since gonidia are situated inside the spheroid they contribute little to its expansion. (Stages 7, 8 and 1)

b) Release: The time during expansion when newly formed, maturing spheroids are individually liberated from the parent. (Stage 8).

c) Gonidial Condensation: The time period just preceding cleavage during which the numerous large vacuoles normally present in maturing gonidia disappear and the previously spherical gonidia become slightly flattened on the side toward the surface of the parent (Starr, 1971). (Stage 2)

d) Cleavage: The precise pattern of successsive divisions of "condensed" gonidia described by Starr (1971) which yields a hollow sphere of 3-5000 somatic cells (with flagellar bases and nuclei facing inward) and 8-16 gonidial initials on the outer surface. (Stages 3 through 4)

e) Preinversion: The stage following cleavage and preceding inversion. (Stage 5)

f) Inversion: The time during which the preinversion form undergoes morphogenetic movement and turns itself "inside-out". At the completion of inversion, gonidia are internal to the surface of somatic cells and flagella are pointed outward in the newly formed spheroid. (Stage 6)

The life cycle of a Volvox carteri spheroid is usually completed in about 72 hr under conditions of growth in SVM, pH7 on a cycle of 16 hr light, 8 hr darkness. Imposed synchrony conditions (SVM, pH8; 30 hr light, 18 hr darkness) accelerate growth and the complete life cycle is only 48 hr in duration.

Growth and Maintenance

Axenic stock cultues of all strains were maintained at about 25C in 15ml screw cap tubes containing 5 or 10 ml of Synthetic Volvox Medium (SVM) adjusted to pH7 (Provasoli and Pintner, 1959) or pH8 (Starr and Jaenicke, 1974) at a light intensity of 300 ft candles (16

hr light, 8 hr dark). Cultures were routinely transferred at least twice a month to fresh media.

Radioisotope and drug treated cultures used in this study were incubated in 20x150mm borosilicate glass tubes, aerated by gentle shaking on a reciprocal shaker, unless otherwise specified. Cultures were prepared, either by transferring 1 ml of growing culture into 4 ml of SVM or by concentrating spheroids (by filtration or photoactic response described below) and resuspending them in a final volume of 5 ml SVM.

A. Asynchronous populations

Asynchronous growth conditions were obtained by transferring aliquots of stock cultures to vessels containing from 5ml to 10 litres sterile SVM, pH7. Cultures were aerated either by bubbling cotton filtered air (flasks) or by shaking (tubes), and illuminated with 400 ft candles of cool white fluorescent light, (25C-30C) on a light/dark cycle of 16 hr light/8 hr dark.

B. Synchronization of Populations of Spheroids

1. Imposed synchrony:

Synchronous cultures were started by innoculating a single spheroid into either 5 ml or 125 ml SVM, pH8, aerating, and growing on a cycle of 30 hr light, 18 hr darkness at a minimum of 600 ft candles of light intensity (28C-32C) (Starr, personal communication). Synchronous populations were maintained by transferring spheroids to new flasks (250 ml or 1 l) of sterile SVM, pH8 every 48 hours (either 25 ml of spheroid containing culture to 125 ml new media or 100 ml to 500 ml). Aliquots removed from stock synchrony flasks during particular stages of the life cycle provided cultures for synchrony

studies (referred to as SYNC in text).

2. Size sorting of asynchronously grown cultures:

The increase in spheroid size that occurs during the course of the asexual life cycle was employed to aid in the isolation of spheroid populations at the same developmental stage. Utilizing Nitex mesh (Yates, Darley and Kochert, 1975), asynchronously grown, heterogeneous spheroid cultures (125 ml) were filtered through particular pore sizes as described below, and the spheroids collected (all at a similar stage) were resuspended in 5 ml SVM, pH7. This method of synchronization is referred to as NIT in text.

a) Isolation of prerelease expansion spheroids used to study early expansion: Vacuum filtration through 60 u mesh Nitex caused disruption of larger spheroids and liberated free large gonidia (see b. below) and small prerelease spheroids which were isolated on the basis of their motility and phototactic response; light was directed on the upper portion of a tube containing the mixture and these spheroids removed from the top with a pasteur pipet.

b) Isolation of "in vitro" gonidia used in cleavage studies: "In vitro gonidia" were obtained by vacuum filtration disruption of spheroids as described above. After the removal of prerelease spheroids as described, the portion remaining contained mainly enlarged mature gonidia. These were removed from the bottom of the tube with a pasteur pipet.

c) Isolation of newly released spheroids used to study late expansion: Small newly released spheroids were obtained by filtration (no vacuum) through 100 u mesh Nitex. The spheroids which passed through the mesh were collected.

Mutagenesis

Spheroids of strain HK-10 were mutagenized by a modification of the procedure of Sessoms and Huskey (1973). N-Nitrosoguanidine (50 ug/ml) was added to asynchronous spheroid populations, newly innoculated into 100 ml SVM, pH7 (about 300 spheroids/ml). After 12 min the media was removed by filtration and the spheroids washed 3 times with SVM and resuspended in 300 ml SVM, pH7. Aliquots (10 ml) of this spheroid suspension were transferred to sterile culture tubes and incubated under asynchronous growth conditions for 6 days (2 life cycles). The enrichment of cultures for morphological mutants was enhanced by the removal of most positively phototactic swimming spheroids prior to examination. The lower 2/3 of each culture tube was covered and the tube illuminated from one side. After 10 min, spheroids were removed from the upper 1/3 of the tube near the light source and discarded. The remaining portion of the culture was transferred to a sterile petri dish and examined under a dissecting microscope. Apparent mutants (only one of a particular phenotype from the same tube) were then individually transferred to culture tubes containing 5 ml SVM (pH7). All procedures were carried out under sterile conditions.

Preparation of Lysates

All cultures used were checked for contamination by streaking aliquots of the culture medium onto nutrient agar plates. Non-radioactive spheroids were harvested by filtration through Miracloth, washed twice with SVM and resuspended in 1/4 to 1/3 the original volume of SVM at pH 7.5. Spheroids were dissociated into individual gonidial and somatic cells using modifications of the

method described by Kochert and Sansing (1971). The spheroid suspension was treated with Protease (Sigma, Type VI) at a final concentration of 80-100 ug/ml for 20-90 min at 25 or 37C with agitation or aeration. Dissociation was monitored by periodic microscopic examination, and usually terminated when all but the newly inverted juvenile spheroids were separated into free gonidia and somatic cells.

Small radioactive cultures were dissociated in a similar manner but the filtering and washing steps were omitted and the protease added directly to the growth medium which had been diluted with an equal volume of SVM. In many cases the treatment of resuspended or diluted cultures with 1% Tween 40/80 (0.5% Polyoxethylene sorbitan monopalmitate/0.5% Polyoxyethylene sorbitan mono-oleate) (referred to as "tween pretreatment" in text) for 1 hr (25C, agitation) prior to protease treatment was employed to aid in spheroid dissociation.

Dissociated cells were harvested by centrifugation at 4,000 x g for 10 min, washed at least twice in saline EDTA (0.15M NaCl, 0.1M ethylenediaminetraacetate, pH8) and resuspended in saline EDTA. Larger, non-radioactive samples were generally lysed in 2-10 ml saline EDTA containing 2% sodium lauryl sulfate (SLS) at 60C for 10-20 min. Radioactive samples which were to be analyzed by preparative CsCl density gradient centrifugation were lysed in 0.7 ml saline EDTA containing 2-4 drops (approximately 5-10%) 30% Sarkosyl NL-97. Lysates were heated to 60C for 20-60 min, usually frozen (-20C) and reheated (60C, 10 min) two times, and stored at -20C until required.

Extraction and Partial Purification of DNA

Total DNA from non-radioactive lysates was purified using modification of the method of Marmur (1961). Lysates were made 1M with respect to NaCl, centrifuged at 4000 x g for 10 min and the nucleic acids precipitated from the supernatant by adding two volumes of cold 95% ethanol (final ethanol concentration, 75%). The pellet was resuspended in 1.0-2.0 of either saline-EDTA or one-tenth the concentration of standard saline citrate (SSC) (0.15M NaCl, 0.015M Na citrate, pH 7.0), brought to a final concentration of 2% SLS and heated at 60C for 10 min. NaCl concentration was made 1M and the lysate was recentrifuged at 4000 x g for 10 min. Nucleic acids were precipitated from the supernatant using cold 95% ethanol as before, and the reextraction of the pellet repeated until no nucleic acid precipitate could be observed upon addition of the cold alcohol. Three sequential reextractions of the original pellet were usually performed. The ethanol precipitated material was collected in each case by either spooling it onto a glass rod or centrifuging at 7,700 x g for 10 min. The precipitates were pooled, washed with 95% ethanol and dissolved in 1.0-5.0 ml of 0.1 x SSC. The suspension was cleared by centrifugation and the clear supernatant adjusted to 1M NaCl. The solution was transferred to a stoppered round-bottomed flask and deproteinized by adding an equal volume of chloroform-isoamyl alcohol (24:1) solution and shaking on a wrist-action shaker for 20 min at 4C. The emulsion produced was separated by centrifugation at 27,000 x g for 20 min, and the upper aqueous layer containing the DNA removed and deproteinized as before. The proteinaceous interface layers from the first and second extractions were combined, resuspended in 1 ml of 0.1

x SSC, adjusted to SSC, reextracted and centrifuged as described above. The aqueous layers were pooled and deproteinized until no interface appeared upon centrifugation. The RNA was removed from the aqueous layer by incubation at 37C for 30 min with pancreatic RNase and T1 RNase (stock RNase solution, previously heated to 80C for 10 min and stored at -20C) at a final concentration of 40 ug/ml each. A final deproteinization was carried out and the DNA precipitated by adding 2 volumes of cold 95% ethanol. The DNA was redissolved in 0.1 x SSC, adjusted to SSC by adding 10 x SSC and stored at -20C.

When deproteinizing very small quantities of DNA, the final ethanol precipitation was replaced by repeated dialysis against SSC to remove nucleotides and other UV absorbing materials.

Determination of DNA concentration

Samples were placed in quartz cuvettes and UV absorbance at 260 nm measured in a Gilford Model 2400 spectrophotometer. DNA concentration (ug/ml) was determined from these values using the E_{260} of 20 (for native DNA). Protein contamination in DNA samples was determined from the ratio of absorbance at 280 nm to that at 260 nm. Pure DNA is expected to have a ratio of 0.5.

Preparative CsCl Density Gradients

Lysates or partially purified DNA stock solutions (0.8-1 ml) were transferred to cellulose nitrate ultra-centrifuge tubes and 4.1 ml of stock preparative grade CsCl solution (130 g of CsCl in 70 ml of 0.01 M Tris buffer, pH 6.8) was added to each. The density was adjusted to 1.710 g/cm^3 and mineral oil added to fill each tube. Samples were centrifuged at 33,000 rpm in a Spinco 50 Ti

rotor for 45-65 hr at 19-20C. Approximately 30 fractions (0.18-0.2 ml each) were collected through a 22 gauge, 1.5 inch needle from the bottom of each tube.

Non-radioactive fractions were diluted with 0.2 ml of distilled H_2O and monitored for UV absorbance at 260 nm in quartz semi-micro cuvettes using a Gilford model 2400 spectrophotometer.

Radioactive fractions were adjusted to 0.5M KOH, hydrolyzed at 65C for 2 hr or 25C for 18 hr, and then cooled to 4C. 100 ug of bovine serum albumin were added to each tube, and the KOH neutralized with 20% trichloroacetic acid (TCA). Excess TCA was added to a final concentration of 5%, and after 20 minutes at 4C, the precipitates were collected on Whatman GF/A glass fiber filters, washed once with cold 5% TCA, once with 95% ethanol, dried and placed in vials containing 4 ml of toluene based scintillant (liquifluor). Samples were counted in a Nuclear Chicago Liquid Scintillation Spectrometer. In many cases the identity of each DNA species was determined from its position relative to that of ^{14}C Escherichia coli DNA (1.710 g/cm^3) included in the same gradient as a reference density marker.

Radioactive Labeling

Spheroids under investigation were labeled with 2-^{3}H-adenine, methyl-^{3}H-thymidine or ^{14}C-adenine at concentrations ranging from 5-40 uCi/ml in SVM. Synchronous or heterogeneous cultures were grown in the presence of radioisotope for time periods varying from 4 hr to 6 days. Uptake of label was monitored by assaying the supernatant growth media at intervals of time after inoculation and comparing counts obtained with those at zero time; 20 ul aliquots were dried on Whatman No. 3 (2.4 cm) filter circles, transferred to vials

containing 4 ml of toluene based scintillant (liquorfluor) and counted in a Nuclear Chicago Liquid Scintillation Spectrometer.

Studies on uptake and incorporation of radioactive precursors under various conditions were usually carried out using newly innoculated 7.5 ml cultures containing 300-400 spheroids/ml in SVM. At time zero 15 uCi/ml of either 2-^{3}H-adenine or methyl-^{3}H-thymidine (and drug or carrier if indicated) were added and 20 ul samples (triplicate) of the supernatant growth media immediately withdrawn and assayed for radioactivity as described above. Samples (0.5 ml), removed from the growing culture every 1-2 hr for the first 8-9 hr and again after 24-25 hr, were filtered through 20 u Nitex mesh and the growth media (filtrate) assayed directly, as described above, (triplicate 20 ul aliquots). The collected spheroids were washed, resuspended in .2 ml saline EDTA and 1 drop 20% SLS, homogenized (H-C Microhomogenizer) and brought to a final volume of 1 ml with saline EDTA. The homogenate was divided into two 0.5 ml samples, one of which was combined with an equal volume of 10% TCA and incubated at 4C for 30 min. The other sample was KOH hydrolyzed as described (0.5 M KOH, 65C, 2 hr), cooled, neutralized (with 10% TCA) and incubated at 4C for 30 min. In both cases TCA precipitates were collected on Whatman GF/A glass fiber filters and processed (washed with 5% TCA and ethanol, dried, placed in 4 ml toluene based scintillant) and counted as described above.

Polylysine Keiselguhr Chromatography (PLK)

Partially purified DNA samples were loaded on and eluted from PLK columns using modifications of the methods described by Blamire,

Finkelstein and Marmur (1972). Standard PLK columns were prepared from 10 g kieselguhr (previously boiled with 14 ml 0.4 M buffered (0.02 M KH_2PO_4) saline), stirred with 0.4 ml of polylysine stock solution (10 mg/ml in 0.4 M buffer) and an aliquot of partially purified DNA (deproteinized, RNase treated, ethanol precipitated and resuspended as described above). (The proportion of polylysine to the amount of DNA estimated to be present should be at least 10:1.) The column was loaded with this material (above a layer of nylon mesh and glass wool or glass beads) under air pressure, and washed free of unbound material with 0.4 M buffered saline. (UV absorbing material was continuously monitored at 254 nm using an ISCO model UA-2 ultraviolet analyzer.) After washing, the column was eluted using a linear gradient of 0.4-4.0 M buffered saline and fractions were collected in 30 drop aliquots (approximately 1.8 ml). Individual fractions were monitored for absorbance at 260 nm and the salt concentration (in M NaCl) determined by refractive index measurements as compared to standard solutions.

Analytical CsCl Density Gradients

Analytical centrifugations were carried out in a Beckman model E ultracentrifuge equipped with ultraviolet optics. DNA samples (1-10 ug) were adjusted to a refractive index of 1.4000 by adding optical grade CsCl, and centrifuged at 44,000 rpm for 18-20 hr at 25C (Meselson, Stahl and Vinograd, 1957). The buoyant density of the DNA was determined by its position relative to that of *Micrococcus lysodeikticus* DNA (1.731 g/cm^3) and *Clostridium perfringens* DNA (1.691 g/cm^3). All densities are related to the buoyant density of *Escherichia coli* DNA in CsCl, taken to be 1.710 g/cm^3.

The base compositions of the DNAs were calculated using the formula Schildkraut, Marmur, and Doty (1962),

$$p=0.098\ (G+C) + 1.660\ g/cm^3$$

(where p = buoyant density and (G+C) = mole fraction guanine + cytosine), relating buoyant density in CsCl to base composition in % G+C.

Thermal Denaturation of DNA

Samples of DNA to be analyzed were dialyzed for 18 hr against 0.1 x SSC, (3 changes of 1-2 l at 4C), sheared (15 passages through a 22 gauge needle), adjusted to a concentration of 10-25 ug/ml, transferred to Teflon-stoppered cuvettes, and the initial absorbance at 260 nm (A_{260}) at 25C determined.

The increase in absorbance at 260 nm with increasing temperature was monitored using a Gilford 2400 recording spectrophotometer equipped with temperature sensor and reference compensator accessories. The thermal denaturation profiles and hyperchromicity values obtained (relating A_{260} values to initial, 0%, and final 100%, values) were related to those of calf thymus DNA prepared under identical conditions and denatured simultaneously. Calf thymus DNA was taken to have a Tm of 71.6C in 0.1 SSC and all values obtained were corrected accordingly. (Marmur and Doty, 1962; Mandel and Marmur, 1968).

Base composition was calculated from corrected thermal denaturation data using the formula of Mandel and Marmur, (1968).

$$G+C = (Tm - 53.9)\ 2.44$$

relating thermal denaturation (Tm = temperature at which 50% hyperchromicity occurs) in 0.1 x SSC to % G+C (Marmur and Doty, 1962).

RESULTS

Chapter 1. Identification and Characterization of DNA Species

Three strains of Volvox carteri were used in this study: Volvox carteri f. weismannia, strains NB-7 (female) and KA-1 (male) and Volvox carteri f. nagariensis, strain HK-10 (female). DNA was isolated and purified from all three strains and the various components present characterized with regard to their buoyant densities, thermal denaturation properties and base compositions. The results of these determinations can be seen in Table 1 and are discussed in this chapter.

Strain HK-10 was studied in greatest detail. Work concerning DNA synthesis during development was, for the most part, confined to HK-10 spheroids, and the results of those studies are presented in later chapters.

When purified total DNA from asynchronously grown Volvox carteri f. nagariensis (strain HK-10) spheroids is banded by analytical CsCl density gradient centrifugation, (see Materials and Methods) profiles such as that shown in figure 2a are obtained. Two species of Volvox DNA can be discerned from such tracings -- a major component with a buoyant density of 1.715 g/cm^3 and a minor component with a density of 1.705 g/cm^3 (as calculated from banding positions of marker DNAs from M. lysodeikticus, ρ=1.731 g/cm^3, and C. perfringens, ρ=1.691 g/cm^3; see figure legend).

Figure 3B shows that banding larger amounts of total DNA from HK-10 in preparative CsCl density gradients (see Materials and

Methods) yields essentially similar results. When aliquots of both species of DNA from these preparative profiles (see figure legend) are rebanded in analytical CsCl gradients, results confirming two distinct DNA species (with bouyant densities of 1.715 g/cm^3 and 1.705 g/cm^3 as compared to density markers) can be seen in figure 3B. The major and minor DNA species have been termed nuclear and cytoplasmic respectively on the basis of their similar CsCl banding properties to these species in other eukaryotic alga such as Chlamydomonas and Chlorella (Chun, Vaughan, and Rich, 1963), and the minor DNA species is presumed to be mainly of chloroplast origin (see Discussion).

Analytical CsCl density gradient banding profiles of total DNA from Volvox carteri f. weismannia, strains NB-7 and KA-1 have also been elucidated and the results are shown in figures 2b and 2c. These profiles are similar to those obtained with strain HK-10, and demonstrate a major DNA component (1.714 g/cm^3) and at least one minor peak (1.704 g/cm^3). In strain KA-1, a second minor species is observed banding at a density of 1.693 g/cm^3 (see discussion).

Total DNA has also been purified from strain NB-7, banded on preparative CsCl gradients as before, and then rebanded in analytical CsCl density gradients. Results, similar to those found for HK-10, can be seen in figure 4. Buoyant densities calculated from such tracings (figure 4B) are 1.714 g/cm^3 (b) and 1.704 g/cm^3 (c) for nuclear and cytoplasmic DNA, respectively (see figure legend).

Purified total DNA from each strain was subjected to thermal denaturation in 0.1X SSC as described in Materials and Methods. Profiles of % hyperchromicity at 260 nm vs. increase in temperature

showed that the DNA denatures over a range of values, starting at 59-62C and reaching a maximum at 83-87C, and gives values for Tms (50% hyperchromicity) of 76-77C (see Table 1). In all three strains it was noted that the melting curves were biphasic, with an inflection occurring at lower temperatures. This indicates heterogeneity in the DNA preparations being tested.

When enriched nuclear and cytoplasmic DNA fractions from strain HK-10 (previously purified on preparative CsCl gradients and prepared as described) are subjected to thermal denaturation, results such as those seen in figure 5 are obtained. While the purified nuclear DNA melts almost as a single species (5a), the "cytoplasmic" DNA still gives a mixed profile (5b) however, with considerable enrichment for the lower temperature denaturing material. Tm values for nuclear and enriched cytoplasmic DNA differ by about 5C; nuclear DNA is 50% denatured at 77.8C and cytoplasmic DNA at 72.5C. The Tm of total DNA from HK-10 is 77C (see Table 1).

Similar results, seen in figure 6, are obtained by the thermal denaturation of purified nuclear and enriched cytoplasmic DNA from strain NB-7. The Tm values are 77C and 72.5C for enriched nuclear (6a) and enriched cytoplasmic DNA (6b) respectively, whereas total DNA from that strain is 50% denatured at 76C (see Table 1).

The base compositions of the nuclear and cytoplasmic DNAs were calculated from buoyant density and thermal denaturation data as described in Materials and Methods. The results of these calculations, shown in Table 1, indicate that the nuclear and cytoplasmic DNAs from all three strains give similar buoyant density and Tm values, but small consistent differences are detectable

between determinations for HK-10 and those for NB-7 and KA-1 (HK-10 DNA species are higher in p, Tm and % G+C). These values translate into base compositions in the range of 55-58% G+C for nuclear DNA of all 3 strains. Similarly, the base composition of the cytoplasmic DNA is about 45-46% G+C, and that of the KA-1 secondary satellite, 34% G+C. The close correspondence between base composition estimated from both buoyant density and Tm values indicates the probable absence of unusual bases (Mandel et al., 1970).

Asynchronously grown, cultures of strain HK-10 which were to be used for the DNA characterizations described above were routinely monitored for the total number of cells present (haemocytometer counts), and the % recovery of DNA/cell determined. Cells were counted and weighed following spheroid dissociation procedures described and 10 l cultures generally yielded $1\text{-}2 \times 10^9$ cells weighing 0.8-1.2g. When cell counts (made on combined separated cells of both types subsequent to spheroid dissociation) were related to the ug DNA (as calculated from A_{260}, see Materials and Methods) recovered following purification procedures described in Materials and Methods, values averaging 2.5×10^{-7} ugDNA/cell were obtained. [The % recovery of this DNA following preparative CsCl density gradient centrifugation was generally high (95-98%).]

Individual Volvox carteri spheroids contain 3,000-5,000 somatic cells and 10-16 gonidia. These cell number variations are a function of the size of the cleaving gonidium, which is determined, to a large extent, by such growth conditions as the intensity of the light and culture density. The proportion of the two cell types present within

a particular spheroid is more constant, however, and the ratio of somatic cells to gonidia is approximately 300:1.

The quantity of total DNA present in isolated somatic or gonidial cells of V. carteri has been assayed spectrophotometrically by others. Reported values for strain NB-7 are 1.14×10^{-7} ugDNA/somatic cell and 27.5×10^{-7} ugDNA/gonidium (Kochert, 1975), and those for HK-10, 1.84×10^{-7}ug DNA/somatic cell and 69.4×10^{-7} ugDNA/gonidium (Kirk, personal communication). Based on the ratio of 300 somatic cells/gonidium, these values translate to 1.23×10^{-7} (NB-7) and 20.6×10^{-7} (HK-10) for ugDNA/cell that would be present in combined populations of gonidial and somatic cells from dissociated spheroids. Such quantities suggest that losses of total DNA encountered during the purification procedures used in this study were low, since estimated values even greater than these were found for the ugDNA recovered/cell described above (2.5×10^{-7}).

Methods other than preparative CsCl density gradient centrifugation have been attempted for effecting the separation of Volvox nuclear and cytoplasmic DNAs. Polylysine kieselguhr (PLK) chromatography of lysates of Volvox spheroids did not yield these desired results. When partially purified preparations of DNA from strain HK-10 were loaded on and eluted from standard PLK columns as described in Materials and Methods, only one peak of DNA (eluting at about 2.0 M NaCl) was observed (data not presented). When this material was later analyzed by thermal denaturation procedures described, the Tm and % G+C calculated from these results (84C, 73% G+C) were significantly higher than values obtained using DNA purified for such melts as described above (77C, 56% G+C for HK-10 total DNA;

see Table 1). Also, when duplicate aliquots of DNA preparations were run on preparative CsCl gradients and PLK columns, calculations of the amount of DNA recovered showed that only a small fraction (7-10%) of the total DNA banded by CsCl centrifugation was being eluted from the PLK column. After repetitions and variations of the procedures described failed to improve the yields of DNA obtained by this method, PLK chromatography of Volvox DNA was discontinued.

The DNAs used in these studies were isolated from a number of different strains grown under a wide variety of culture conditions. The protocols described in Materials and Methods represent those finally adopted after characterizing several different alternatives.

The major problems encountered during the manipulation of Volvox spheroids for the subsequent analysis of their DNA have been those associated with the removal of the extracellular matrix material which encapsulates the spheroids. This material is composed mainly of glycoproteins (Miller et al., 1972), and its presence during cell lysis appears to interfere with subsequent DNA purification; yields of DNA recovered from samples which contained substantial quantities of this matrix (sheets of somatic cells and smaller intact spheroids present prior to lysis) were sometimes as much as 60-80% lower than those of equivalent samples where cell separation was more complete. The elimination of this material subsequent to cell lysis has been attempted by incubating lysates with self-digested proteases and amylase. However, similarly low recoveries were observed following this treatment.

Various alternatives to the protease (type VI, Sigma) mediated dissociation of spheroids (Kochert and Sansing, 1971) described in

Materials and Methods, have been attempted in this laboratory and in others (Starr, Kirk, Kochert, Huskey, personal communications). Mechanical disruption of spheroids (Dounce homogenizer, Kirk and Kirk, 1976; French pressure cell press, Yates, 1975) followed by filtration or centrifugation have further decreased yields of DNA/cell purified by methods used in this study. The use of various concentrations of other proteolytic enzymes (trypsin, "pronases" and other proteases) and amylases, alone and in various combinations have been tried as well, but the protease (type VI) treatment described, achieves the greatest amount of dissociation in the shortest time. However, for optimum yields and in order to avoid selective losses of DNA, the following factors should be taken into consideration when attempting to purify Volvox DNA.

Spheroids from different strains and spheroids of the same strain at different points in their life cycle show considerable variations in their sensitivity to protease mediated dissociation. It was found, for example, that strains NB-7 and KA-1 dissociate more rapidly and more completely than HK-10. Under conditions described, times of dissociation for KA-1 and NB-7 are about 20-30 min whereas those for HK-10 are commonly in the range of 60-120 min. Also, unreleased spheroids which have not yet begun to expand, require treatments significantly longer than those normally necessary for their strain and may never completely dissociate. For the reasons indicated above, it is necessary to removed as much of the matrix as possible before proceeding to cell lysis. "Tween treatment" prior to spheroid dissociation accelerates cell separation and aids in the breakdown and subsequent elimination of contaminating matrix material

(see Chapter 2 and Discussion). Washing dissociated cells with either media or buffer several times before proceeding to the lysis of cells appears to aid in the removal of this matrix material as well.

The lysis of large quantities of cells requires the use of 2% ionic detergent (SLS) in saline-EDTA (pH 8) at 55-60C. Such treatment serves to promote the lysis of cells and organelles while inhibiting the action of nucleases. Repeated reextraction of material precipitated from the lysate at 1M NaCl concentration was also found to increase the total yield and aid in avoiding the selective loss of organelle DNA.

Chapter 2. Normal Patterns of DNA Synthesis

The asexual life cycle of Volvox carteri spheroids is described in Materials and Methods and diagrammed in figure 1 (also see plate 1 for certain life cycle stages. DNA synthesis was studied in strain HK-10 for various periods of time at different stages in this life cycle. These studies required a) that it be possible to use radioactive precursors as a probe to measure the amount of DNA synthesis at any particular interval, b) that a sufficiently large number of spheroids could be isolated which were all at the same stage in the life cycle and c) that results obtained using different methods of stage separation be comparable at the macromolecular level.

The incorporation of various nucleic acid precursors into Volvox DNA was examined by incubating known quantities of spheroids in the presence of each radioisotope for 18 hr (1/4 life cycle), lysing the spheroids, and banding the nucleic acids on preparative CsCl equilibrium density gradients (see Materials and Methods). Banding profiles of radioactively labeled DNA from nonsynchronous populations of HK-10 spheroids using 2-^{3}H-adenine were similar to those of purified, unlabeled DNA and were therefore investigated further. Figure 7 shows results typical of those obtained when cultures of strain HK-10 are labeled with concentrations of 2-^{3}H-adenine ranging from 10 to 40 uCi/ml for periods of time from 16 to 72 hr. Profiles of the DNA from such lysates, banded in preparative CsCl gradients and plotted as cpm ^{3}H vs. fraction number, closely resemble those of purified HK-10 DNA banded in either preparative or analytical

gradients (see Chapter 1, figures 2a and 3). As noted previously, 2 peaks are evident which, when related to the density of ^{14}C E. coli DNA included in the same gradient as a marker, band with buoyant densities previously assigned to the nuclear and cytoplasmic DNA species (see Table 1).

Figure 7 also demonstrates the beneficial effects of "tween pretreatment" of spheroids mentioned in Chapter 1. Figure 7B shows a banding profile of radioactive DNA from spheroids which were incubated in the presence of 1% tween 40/80 for one hour prior to protease mediated dissociation, as described (see figure legend). A better resolution of the nuclear and cytoplasmic DNA species is visible following such treatment than can be seen in profiles from similar cultures which had not been treated with tween (figure 7A) (see Discussion).

Other radioactive DNA precursors do not necessarily give comparable incorporations. For example, results have shown that methyl-^{3}H-thymidine is incorporated almost exclusively into the nuclear DNA of HK-10 spheroids. Figure 8 shows the differing profiles obtained when equivalent heterogeneous 5 ml cultures are labeled with either 2-^{3}H-adenine or methyl-^{3}H-thymidine for 42 hr (see figure legend). When lysates of such cultures are centrifuged to equilibrium in preparative CsCl density gradients, very different DNA banding profiles emerge. While both the nuclear and cytoplasmic DNA peaks, such as those described above, are visible after labeling with ^{3}H-adenine (figure 8A), ^{3}H-thymidine labeling of spheroids results in the appearance of only one radioactive Volvox DNA species which, when compared with marker ^{14}C E. coli DNA, bands

in the position of nuclear DNA (figure 8B). Thymidine labeling studies have been repeated numerous times under various conditions of synchrony and asynchrony, and at no time has a detectable cytoplasmic DNA banding species been visible.

Growth of Volvox spheroids in the presence of ^{14}C-adenine subsequently produces CsCl density gradient DNA profiles with both nuclear and cytoplasmic banding species present in proportions similar to those obtained using ^{3}H-adenine. When asynchronous cultures of HK-10 spheroids are grown in the presence of both ^{3}H-thymidine and ^{14}C-adenine, results such as those shown in figure 9 are obtained (see figure legend). Both nuclear and cytoplasmic DNA banding species have incorporated ^{14}C radioactivity, but radioactivity from ^{3}H-thymidine is again present only in the nuclear DNA. (The minor shoulder often visible on the lighter portion of the ^{3}H-thymidine labeled nuclear DNA peak only in such double label experiments is as yet unexplained). Such results substantiate the findings that methyl-^{3}H-thymidine is incorporated almost exclusively into nuclear DNA whereas radioactive adenine will label both the nuclear and the cytoplasmic DNA species. For this reason 2-^{3}H-adenine, and not methyl-^{3}H-thymidine, was used as the radioactive DNA precursor for studies on DNA synthesis during the life cycle of Volvox.

Other strains of V. carteri have been labeled with 2-^{3}H-adenine (NB-7 and KA-1) and preparative CsCl profiles, exhibiting both nuclear and cytoplasmic DNA banding species, have been obtained. (The second minor DNA species, consistently seen in analytical CsCl profiles of purified DNA from strain KA-1 (see Chapter 1; figure 2c) was not

resolvable in preparative CsCl profiles obtained for either purified or radioactively labeled DNA (data not presented).) As was demonstrated for strain HK-10, banding profiles of DNA from asynchronous cultures of strain NB-7 grown in the presence of 2-^{3}H-adenine (figure 10; see figure legend) resemble those seen when purified DNA of that strain is banded in preparative (figure 4) and analytical (figure 2b) CsCl density gradients. Two peaks of radioactively labeled material are evident, which, when compared to the density of marker ^{14}C E. coli DNA, band with the buoyant densities assigned to the nuclear and cytoplasmic DNAs of that strain (see Table 1).

Numerous other labeling studies have been conducted using this radioactive DNA precursor. Strain HK-10 has been labeled with 2-^{3}H-adenine under a variety of conditions (for synchrony studies, see below). Heterogeneous cultures, containing as few as 100 spheroids have been grown in the presence of 2-^{3}H-adenine (20-40 uCi/ml) for time periods as short as 4 hr and subsequent profiles exhibiting two DNA banding species have still been obtained (data not presented). It therefore appears that ^{3}H-adenine will be taken up by Volvox spheroids and incorporated into their DNA in a manner which reflects the overall DNA content and ratios of nuclear to cytoplasmic species visible when other techniques are used.

In this study, two different approaches, described in Materials and Methods, were employed to obtain synchronous populations of spheroids. Method one, (SYNC), involves the imposition of synchrony on cultures of spheroids by growth on a cycle (48 hr) of 30 hr light (minimum 600 ft candles) and 18 hr darkness using media (SVM) at pH 8

(Starr, personal communication; see Materials and Methods). This method is similar to the 72 hr light, 36 hr dark method used by Yates et al., (1975) to synchronize NB-7, and produces a population of spheroids which are all temporally at the same stage of development. Using this method, spheroids required at any particular point in the life cycle can be reproducibly and predictably obtained every two days.

Method two (NIT), takes advantage of the facts that the diameter of a spheroid increases during the course of the asexual life cycle and that this size change reflects the developmental stage to which a particular spheroid has advanced. Populations of individuals at the required stage may therefore be separated from an asynchronously growing, heterogeneous spheroid culture by filtration through Nitex mesh of particular pore sizes (as described in Materials and Methods).

Although the SYNC method is a simple means of obtaining spheroid populations which are more homogeneous (in tighter synchrony) than those obtained by filtration (NIT), it is an imposed technique and forces spheroids into an accelerated life cycle (48 hr vs. natural 3-5 days). There are, however, many more technical problems associated with the more natural NIT procedure. Maintenance of axenic conditions is more difficult than in the SYNC method since NIT requires more equipment and manipulatory steps (see Materials and Methods). The number of spheroids present within the homogeneous populations obtained following NIT filtration is more varied and less predictable than within those obtained by SYNC, and unknown quantities of spheroids and gonidia may be damaged during the NIT filtration process

itself. However, the NIT method yields relatively homogenous populations of spheroids, which had been and continue to be, grown under more "natural" conditions. It was necessary, therefore, to make comparisons between results obtained using these two differing synchronization techniques.

Patterns of DNA synthesis during various periods of time at different stages of the asexual life cycle of Volvox carteri f. nagariensis were studied by following the incorporation of 2-^{3}H-adenine into the DNA of populations of spheroids synchronized by both methods described above. The results of such experiments (see figure legend) are shown in figure 11 (see figure 1 for life cycle stages quoted below).

Preparative CsCl density gradient profiles of lysates from spheroids labeled during the prerelease expansion stage (figure 1, stage 7; plate 1) can be seen in figures 11a and 11b. In both the SYNC and NIT profiles two major peaks of radioactively labeled material, banding in the positions of nuclear and cytoplasmic DNA, can be seen. The proportion of these two DNA species is, however, very different from the proportions present in profiles from asynchronous cultures (see figure 7). The ratio of cpm ^{3}H incorporated into cytoplasmic DNA (cDNA) to those incorporated into total (t) DNA (sum of cpm ^{3}H in cDNA and nuclear DNA) will be termed c/t ratio. During the course of this study such ratios have been found to vary between 5 and 20% when banding profiles from heterogeneous, asynchronously grown cultures were used for these calculations. In profiles obtained from synchronous populations of spheroids labeled during the stage following

inversion and preceding release (figures 11a and 11b) however, these c/t ratios have always been much higher (c/t's usually greater than 60%). Such results imply that this prerelease expansion stage is one of preferential cDNA synthesis.

Profiles obtained from synchronous cultures labeled during the portion of the expansion phase following release (stage 1) are depicted in figures 11c (SYNC) and 11d (NIT). Only one peak of radioactively labeled material banding in the position of nuclear DNA is observed whether spheroids were isolated by the SYNC or the NIT procedure. Using these methods it has thus far not been possible to demonstrate the synthesis of any other DNA species during this stage in the Volvox life cycle.

Figure 11e shows results obtained when enlarged, fully expanded spheroids, isolated using the SYNC method, are labeled during the period encompassing cleavage and inversion (stages 3-6); nuclear DNA is the predominant species synthesized here. However, for this stage, results obtained using the NIT method (figure 11f) are somewhat different. NIT studies were done using "in vitro" gonidia (free gonidia, developing outside the parental spheroid) isolated by the disruption of enlarged parental spheroids as described (see figure legend and Materials and Methods). The DNA labeled using this method bands as indicated in figure 11f, showing that both nuclear and cytoplasmic DNA are being made. The proportion of cytoplasmic DNA in this profile (c/t = 30%) is greater than that seen during cleavage of gonidia within the parental spheroid using the SYNC method (c/t = 15%). However, the increased cytoplasmic DNA synthesis observed here

(NIT), may well be a reflection of the limitations of this synchronization method (see Discussion), and therefore artifactual for this stage of the life cycle.

The period of total expansion includes the stages before and after juvenile spheroid release (stages 7, 8 and 1). The DNA banding profiles from spheroids isolated by both the SYNC and the NIT methods and labeled during this portion of their life cycle (figures 11g and 11h), show that large proportions of both DNA species are being synthesized (c/t SYNC = 37%; c/t NIT = 34%). Such proportions would be expected from results described above since this period of the life cycle combines both the prerelease stage, when cDNA is the predominant species synthesized (see figures 11a and 11b), and the postrelease expansion stage when only nuclear DNA is made (see figures 11c and 11d). The preparative CsCl density gradient profiles of radioisotope labeled Volvox DNA presented above demonstrate that the two synchronization procedures employed in this study yield spheroid populations which exhibit similar patterns of ^{3}H-adenine incorporation into nuclear and cytoplasmic DNA during the various stages of the life cycle examined. The porportions of the two DNA banding species (c/t ratios) seen in such profiles are similar during the same life cycle stage, whether populations are isolated by the imposed (SYNC) or the more natural, selection synchrony (NIT) method. The more standardized SYNC method was therefore employed for all further experiments necessitating the use of synchronous cultures.

Chapter 3. Alterations in DNA Metabolism

After the "normal" temporal patterns of DNA synthesis in strain HK-10 spheroids had been determined, studies were then conducted to learn the morphological and biochemical consequences of interfering with DNA metabolism, in the hope of correlating these two effects. Such studies employed normal HK-10 spheroids treated with drugs (ethidium bromide: EB; and delta-9-tetrahydrocannabinol: Δ^9 THC or THC), and a "somatic cell regenerator" mutant of strain HK-10. The aberrant developmental patterns, as well as the changes in DNA metabolism which result from such drug treatments and mutation are described in this chapter.

The Effect of THC and EB on Morphological Development During The Asexual Life Cycle of *Volvox carteri* Spheroids.

The asexual life cycle of *Volvox* has, for these studies, been divided into four parts, three of which are diagrammed in figure 12. Stage 1 illustrates the period before and after release during which the newly inverted juvenile spheroid with a peripheral sheath of somatic cells, and internal immature gonidia begins the process of expansion and maturation. During this period, the spheroid enlarges as shown due to somatic cell synthesis and excretion of matrix material, while the gonidia also expand and become highly vacuolated. At the end of Stage 1 the spheroid is fully "mature" and ready to begin asexual reproduction. Stage 2 depicts the period just prior to the onset of cell division and embryogenesis during which a marked morphological change takes place within the gonidia. The vacuoles

disappear and the contents of the cells become highly granular and darker in color. This has been termed the phase of gonidial 'condensation' and it is readily observed under the light microscope. Stage 3 shows the period of gonidial cleavage. The mature, condensed gonidia flatten slightly and begin the process of semisynchronous cell division which ultimately leads to a preinversion form (not shown); the preinversion form, with all cell division completed, carries out a morphogenetic movement known as inversion during which the embryo turns inside out (not shown.)

The effects of various concentrations of THC and ethidium bromide on the morphology, growth, embryogenesis and development at the different stages of the asexual life cycle described above have been investigated, and these observations are presented below (see plates 2 and 3).

Studies performed on asynchronous populations were conducted by incubating 2 ml and 5 ml cultures (SVM, pH 7) in the presence or absence of carrier (see below), THC or EB under conditions of constant light (THC) or a light/dark cycle of 16/8 (EB). Synchronous cultures were incubated under SYNC conditions described above (pH 8, 30 hr light/18 hr dark).

[9] THC is not soluble in water. Therefore, in order to test the various effects of this drug on Volvox spheroids, a suitable "carrier" (see Materials and Methods) had to be devised as a vehicle to suspend the THC in the SVM media. Since all drug treated cultures contained this carrier, the morphological and biochemical effects of equal concentrations of carrier alone had to be elucidated. All experiments and studies involving THC were therefore performed using

carrier as well as non-carrier controls.

THC Treatment

Studies performed on asynchronous spheroid populations revealed that at concentrations of THC between 5-25 ug/ml all stages of the life cycle appear to take place normally over the first 24 hours of treatment. At concentrations of 50 ug/ml THC some cells already committed to embryogenesis show signs of aberrant cleavage; the 2-8 cell stages show evidence of unequal division, and some separation of the cells is visible.

At 125 ug/ml THC (12.5 ul/ml carrier) embryogenesis appears normal in control samples. In the drug treated cultures those embryos which have fully inverted look normal, but those inverting during this period appear distorted (see below). At this (125 ug/ml) THC concentration all enlarged gonidia appear condensed and very dark in color and the culture becomes arrested in this condition. When the drug concentration is raised above 250 ug/ml (up to 500 ug/ml) all gonidia (large and small) become condensed and arrest at this stage within the first 16-18 hr of treatment (plate 2A). After 24 hr in the presence of the drug some enlarged gonidia appear to degenerate (become highly granular and distort and lose some of their color).

When experiments were performed on synchronous and semi-synchronous cultures, similar points of morphological block could be detected (execution points), with one additional observation. When low concentrations of THC are added to spheroids which are in later stages of embryogenesis (time zone E) the new embryos do not invert properly. If, THC is added after the completion of cell division, inversion proceeds normally but the release of the new juvenile spheroids is inhibited.

The results of morphological studies using spheroids treated with THC can be summarized as follows (see plate 2). In the continuous presence of the drug (up to 400 ug/ml) no point of morphological block can be detected in time zone A (figure 12). At concentrations of 125 ug/ml and higher, time zone B contains the first execution point with a block such that all gonidia arrest at the condensation stage (2), and at higher concentrations, reach this block prematurely.

Treatment of spheroids with even lower concentrations of the drug (about 50 ug/ml) shows the existence of a second execution point in time zone D or E, with the consequence that embryogenesis is affected (aberrant cleavage and inversion), and in many cases the release of juvenile spheroids is significantly delayed.

EB Treatment

A distinct morphological effect is produced in HK-10 spheroids incubated for 18-24 hr in the presence of EB over a narrow concentration range (25-30 ug/ml). When Volvox cultures are thus treated for up to 24 hr, spheroids accumulate at stage 3 (figure 12). This block appears to be one which allows mature gonidia to begin cleavage, but causes cessation of cell division between the 4 and 32 cell stage (see plate 3). The point at which the drug affects development (execution point), therefore, appears to be somewhere in time zone E. EB concentrations of 20 ug/ml and below, produce no visible effects during the first 24 hr of spheroid treatment, whereas concentrations of the drug above 30 ug/ml appear to cause degeneration of all gonidia (shape distortion, loss of green color and appearance of orange dye color).

The Effects of THC and EB on DNA Metabolism

THC and DNA Synthesis

DNA synthesis can be followed in Volvox spheroids by employing the incorporation of 2-^{3}H-adenine into DNA molecules which are then resolvable into nuclear and cytoplasmic origin by CsCl density gradient centrifugation (Chapter 2 and Margolis-Kazan and Blamire, 1976). These techniques were used to follow the effects of THC on DNA synthesis in both heterogeneous and synchronous cultures of V. carteri f. nagariensis strain HK-10.

Asynchronous cultures have been incubated with 2-^{3}H-adenine in the presence and absence of THC (400 ug/ml) or carrier (40 ul/ml) for 18 hr. Results of such experiments, (seen in figure 13) show that carrier alone has no appreciable effect on DNA synthesis during this time period. Two peaks of radioactively labeled DNA can be seen banding in the positions of the nuclear and cytoplasmic DNA species in both the control (figure 13A) and the carrier treated (figure 13B) profiles. In the profile from THC treated spheroids, however, only one peak, banding in the position of nuclear DNA, is visible (figure 13C). Under the various conditions tested (synchrony and asynchrony, long and short term treatment, see below), synthesis of cytoplasmic DNA has consistently been differentially inhibited in the presence of 400 ug/ml THC.

Studies were conducted to learn the effects of carrier and THC on the uptake and total incorporation of 2-^{3}H-adenine by HK-10 spheroids. Asynchronous cultures, containing about 400 spheroids/ml, were incubated with ^{3}H-adenine in the presence and absence of 30-40 ul/ml carrier and 300-400 ug/ml THC, as described in Materials and

Methods. Samples were withdrawn at 0 hr and every 1-2 hr for the first 8-10 hr and again after 25 hr, and the growth media, TCA precipitable material from total lysates and hot KOH hydrolyzed lysates of spheroids, monitored for cpm ^{3}H as described. The results on the uptake of ^{3}H adenine (as analyzed by depletion of radioactivity in the growth media) in such an experiment are presented in figure 14. For the first 4 hr the uptake of adenine is fairly linear in the THC treated, carrier treated and control cultures. Relatively linear uptake continues in both carrier-treated and control samples for 8 hr from time 0. (The "uptake lag" seen in figure 14 in the carrier control, between 4 and 6 hr, is commonly seen somewhere between 4 and 7 hr and is as yet unexplained.)

Results on the incorporation of 2-^{3}H-adenine from this same study (see legend to figure 14 and Materials and Methods) are presented in Table 2. They show, first of all, that at least 95% of the ^{3}H-adenine present in TCA precipitable material from lysates of HK-10 spheroids is not incorporated into DNA. At the various time points tested, control samples show that the counts incorporated into KOH hydrolyzed material are only about 1-5% of those incorporated into TCA precipitable unhydrolyzed material. A major portion of the tritium counts present in unhydrolyzed samples, therefore, probably eminate from adenine incorporated into RNA.

As was found for uptake, described above, the first 4 hr of drug treatment has very little effect upon the total incorporation of 2-^{3}H-adenine as well. After 4 hr, tritium counts in TCA precipitates of unhydrolyzed or KOH hydrolyzed lysates are similar to those of the control whether spheroids have been treated with carrier (30 ug/ml) or

THC (300 ug/ml). After 6 hr of THC treatment, however, unhydrolyzed material shows less than half of the incorporation seen in the control, and after 8.5 hr tritium counts present in KOH hydrolyzed samples are only one-third of those found in the control. Carrier treatment begins to significantly decrease the incorporation of adenine into unhydrolyzed samples relative to the control, at a time (8.5 hr) when KOH hydrolyzed material is still virtually unaffected. Results presented in Table 2 would, therefore, suggest that THC causes a decrease in the incorporation of 2-^{3}H-adenine into RNA sooner and more dramatically than it does incorporation into DNA. It should also be noted that with time (8.5 hr) and especially after 25 hr of growth in the presence of carrier, incorporation of label into RNA is significantly reduced.

Similar uptake and incorporation studies were conducted using ^{3}H-thymidine. In all cases media depletion analysis showed no appreciable reduction in cpm ^{3}H present in the growth media during the 25 hr time period tested (data not presented). Incorporation studies employing ^{3}H-thymidine (see Table 3), however, yielded results which are significantly different from those described for adenine. The incorporation of 3H-thymidine does not appear to be inhibited by treatment of spheroids with carrier or THC for at least 10 hr. In fact, after the first 8.5 hr of exposure, in this experiment, cpm ^{3}H in both the total and the KOH hydrolyzed TCA insoluble material are highest in THC treated spheroids and very similar in both carrier treated and control samples. These results are in accord with findings described previously. Since methyl-^{3}H-thymidine is incorporated only into Volvox nuclear DNA (Chapter 2) and 18 hr treatment of

spheroids with 300-400 ug/ml THC interferes mainly with cytoplasmic DNA synthesis (above and Margolis-Kazan and Blamire, 1977) one would not expect the drug to interfere with ^{3}H-thymidine incorporation (which is only into nuclear DNA), during this same time period.

In this particular experiment, however, 25 hr treatment of spheroids with THC in the presence of either ^{3}H-adenine or ^{3}H-thymidine (and with carrier as well in the ^{3}H-adenine labeled spheroids) substantially reduced (80-95%) the cpm present in the TCA insoluble, KOH hydrolyzed material relative to those of untreated controls.

The effects of increasing THC concentration upon DNA synthesis were followed in HK-10 spheroids using protocols described above. Figure 15 shows the CsCl profiles obtained after spheroids were treated for 18 hr with 40 ul/ml carrier (15A), 100 ug/ml THC (15B), 200 ug/ml THC (15C) and 400 ug/ml (15D), in the presence of 20 uCi/ml 2-^{3}H-adenine. Two peaks of radioactively labeled DNA, banding with buoyant densities previously assigned to the nuclear and cytoplasmic species, are present in similar proportions (see Table 4 and below) in profiles from spheroids treated with either 40 ul/ml carrier, or 100 or 200 ug/ml THC (figures 15A, B, and C). When THC concentration is raised to 400 ug/ml, however (15D), there is almost total inhibition of adenine incorporation into cytoplasmic DNA while incorporation into nuclear DNA continues.

Experiments of this type were generally conducted using "heterogenous" Volvox cultures with a semi-synchronous bias to the prerelease expansion stage in order to enrich for cytoplasmic DNA synthesis and thereby make that species easily discernable above

"background." The ratios of cpm ^{3}H incorporated into cytoplasmic DNA to those incorporated into total DNA (c/t ratio) may, therefore, vary from about 5-15% for asynchronous cultures to over 30% for enriched semi-synchronous populations.

The c/t ratios calculated from the results described above (figure 15) are presented in Table 4. 18 hr treatments of equivalent cultures with 40 ul/ml carrier or 100, 200 and 400 ug/ml THC yielded c/t ratios of 21%, 20.5%, 21% and 0 respectively. Repetition of experiments of this type showed that during the first 18-24 hrs of exposure to THC, cytoplasmic DNA synthesis is differentially interfered with only when the drug is present in the growth media in concentrations between 300 and 400 ug/ml. Concentrations of the drug greater than 500 ug/ml prevent nuclear DNA synthesis as well (data not presented).

Experiments were then performed to learn the effects of THC upon DNA synthesis in synchronous Volvox cultures during various stages of the asexual life cycle. Equivalent 5 ml synchronous cultures (SYNC) at particular points of development were incubated in the presence of 2-^{3}H-adenine alone, or label and carrier (40 ul/ml) or THC (400 ug/ml). Preparative CsCl density gradient profiles of cultures thus treated are presented in figure 16. (Profiles of the untreated controls are not presented but they are similar to those of carrier treated controls.) The near perfect synchrony obtained in the untreated samples was not always present in carrier and drug treated spheroid populations. The presence of carrier appears to cause a slight decrease in the rate of development so that these cultures were therefore only semi-synchronous at the time of spheroid dissociation

and lysis.

Spheroids incubated with 2-^{3}H-adenine and carrier or THC during the prerelease expansion stage yield CsCl density gradient profiles such as those seen in figure 16. This stage (figure 12, first half of time zone A) was shown to be the one of maximum cDNA synthesis in untreated spheroids (see figures 11a and 11b) and treatment with 40 ul/ml carrier for 17 hr yields similar profiles (figure 16a). When THC (400 ug/ml) is present during this same labeling period, however, no cDNA synthesis is evident. Figure 16b shows that only one peak, banding in the position of nuclear DNA, is present after THC treatment, even during this period of normally maximum cDNA synthesis.

Profiles obtained from juvenile spheroids, treated during the period just following release are similar in carrier (16c) and THC treated (16d) samples. This period of late or postrelease expansion (figure 12, second half of time zone A) is one in which only nuclear DNA is synthesized (see also figures 11c and 11d). The single peak of radioactively labeled DNA visible in both 16c and 16d, bands in the position of Volvox nuclear DNA, and shows once again that nDNA synthesis may continue in the presence of THC. Profiles of synchronous Volvox cultures, treated with carrier or THC during the period of gonidial cleavage (figures 16e and 16f), again show nuclear and cDNA banding species present in the carrier control and only nDNA in the THC treated sample.

Results obtained from THC treatment of synchronous cultures are therefore, consistent with those described above for heterogeneous populations, and show that only cDNA synthesis is inhibited when the

drug is present for up to 18 hr.

The Effect of EB on DNA Synthesis

These experiments were performed in a manner similar to those described for THC. Cultures of heterogeneously growing Volvox (5 ml SVM, pH 7) were simultaneously treated with 2-^{3}H-adenine (20 uCi/ml) and increasing concentrations of EB. These cultures were allowed to grow for 18 hr, harvested, lysed, and the amount of radioisotope found in the nuclear and cytoplasmic DNA analyzed after preparative CsCl density gradient centrifugation of lysates as described above. Results are presented as c/t ratios in Table 5.

These experiments were repeated several times using either heterogeneous or semi-synchronous cultures and the results can be summarized as follows. At 0 ug/ml and 1 ug/ml EB there appears to be little or no effect on the extent of radioisotope incorporation or on the c/t ratio. At 2.5 ug/ml EB, however, the total incorporation of isotope falls to only 15% of the control. This drop in incorporation is more marked at higher EB concentrations and at 5, 15, and 25 ug/ml the percent incorporations relative to those of the control are 2-7%, 1-2% and 0.5% respectively. Thus, in Volvox, eithidium bromide appears to inhibit incorporation of adenine into both nuclear and cytoplasmic DNA.

When the c/t ratio is calculated for this residual incorporation it is found that at 0 ug/ml and 1 ug/ml the ratio is the same. However, at concentrations of EB of 2.5 ug/ml and higher this ratio doubles and in some cases goes up to three times that of the control. It is not known whether or not this change in c/t ratio is significant, as the total incorporation is very low at these high

values of EB concentration, but the same phenomenon has been observed in many experiments, for example when the c/t ratio of the control varied from 4.8% to 13% (see Table 5).

Similar experiments (5 ml asynchronous cultures, 20 uCi/ml 2-^{3}H-adenine) were then performed to determine how rapidly EB acts to produce an effect on DNA synthesis. The amount of label being incorporated into nuclear and cytoplasmic DNA during the first 6 hr of EB treatment was examined and the results are shown in Table 6. In control samples a c/t ratio of 6% was obtained, however at 2.5 ug/ml EB and 10 ug/ml EB this ratio increased to 15% and 56% respectively. Similarly, if the Volvox spheroids were pre-treated with EB for 6 hr before the addition of 2-^{3}H-adenine (present for 18 hr) then the c/t ratio obtained at 2.5 ug/ml EB was 56% while the 10 ug/ml pre-treatment inhibited all incorporation. Ethidium bromide therefore appears to inhibit the incorporation of adenine into nuclear DNA during the first 6 hr of treatment while having a more delayed effect on incorporation into cytoplasmic DNA. Such observations may explain the increased c/t ratios of the residual DNA synthesis found present in the 18 hr EB concentration experiments described above.

The Effect of THC and EB on Pre-existing DNA in Volvox

The results obtained above indicate that both THC and EB have inhibitory effects on DNA synthesis in Volvox, but that, whereas the THC is somewhat selective for the inhibition of cytoplasmic DNA synthesis, EB is a much more general inhibitor, and in fact seems to inhibit nuclear DNA synthesis more rapidly than it does that of cytoplasmic DNA.

Other experiments conducted on THC treated cultures yielded somewhat unexpected results. Spheroids exposed to concentrations of THC between 300-400 ug/ml (without radioactive label) for 15 or more hours, were then washed, resuspended in fresh SVM, and allowed to recover for varying periods of time. Morphological observations showed that such spheroids were incapable of forming new offspring, and in addition, remained arrested at the stage they reached when the drug was removed. When incubated in the presence of 2-^{3}H-adenine no detectable incorporation of label into cytoplasmic DNA could be observed in these "recovering" cultures although its incorporation into nuclear DNA continued for some time (data not presented). Similarly treated carrier controls showed none of these morphological or biochemical abnormalities.

Other puzzling results have been briefly mentioned above. During the later stages of spheroid expansion (second half of time zone A, figure 12) nuclear DNA is the predominant species synthesized. Thus profiles of ^{3}H-adenine incorporation into DNA during this stage of the life cycle were shown to be similar in semi-synchronous THC treated and carrier control cultures (figures 16c and 16d). However, spheroids treated with THC during this period still show morphological distortions similar to those exhibited by prerelease spheroids exposed to the drug. This morphological arrest at condensation caused by THC treatment during a period when DNA synthesis is hardly altered, coupled with the inability of treated spheroids to recover when the drug is removed, suggested the possibility that other aspects of DNA metabolism may be affected by treatment with this drug.

It was therefore important to see if THC, as well as EB, was

having any effect on pre-existing nuclear and cytoplasmic DNA species. Volvox cultures (5 ml, SVM, pH 7) were labeled with 2-^{3}H-adenine (10 uCi/ml at 0 hr and again at 24 hr) for a minimum of one complete generation (3-4 days). These cultures were then transferred to fresh medium without isotope and treated with increasing concentrations of THC or EB as before. At lower concentrations of either THC (0-100 ug/ml) or EB (0-5 ug/ml) no apparent alteration in the c/t ratios could be detected (see Table 7). However, at higher concentrations (THC>200 ug/ml, EB>15 ug/ml) it was found that there were decreasing amounts of radioactive tritium counts in the area of cytoplasmic DNA. It appeared as if the drugs were aiding in the selective degradation of the cDNA.

Results of preparative CsCl gradients showing radioactively (2-^{3}H-adenine) prelabeled Volvox DNA after in vivo treatment with THC (300 ug/ml) show the presence of nuclear DNA alone (figure 17B), while similar treatment with carrier (30 ul/ml) yields profiles in which both nuclear and cytoplasmic DNA peaks are visible (figure 17A). These results indicate that prelabeled cytoplasmic DNA has been selectively and completely degraded in the presence of THC.

Similar results can be seen in preparative CsCl density gradient profiles from spheroids prelabeled and subsequently treated with 25 ug/ml EB. While the untreated control (figure 18A) shows the presence of both nuclear and cytoplasmic banding DNA species, treatment with EB yields profiles in which only nDNA is present (figure 18B).

Analytical CsCl profiles of DNA from spheroids grown in the presence and absence of EB can be seen in figure 19. EB (25 ug/ml) was added to a 10 liter culture of Volvox in SVM. After 20 hr of drug

treatment the culture was harvested and the DNA extracted and purified as described. This sample of DNA was then banded in analytical CsCl density gradients and the presence or absence of DNA species monitored by ultraviolet absorbance. Once again, cytoplasmic DNA is the drug's target. While the DNA from the control cultures produces bands of absorbance at 260 nm that can be identified as nuclear and cytoplasmic by their buoyant densities in neutral CsCl (figure 19b), no cytoplasmic DNA is detectable in the EB treated sample (figure 19c). The specific degradation of pre-existing cytoplasmic DNA in the presence of ethidium bromide was thus confirmed.

Treatment of HK-10 spheroids with either THC or EB has similar consequences on pre-existing Volvox DNA. Both drugs appear to eliminate the cytoplasmic DNA species within 24 hr of treatment. Preliminary results of time course exposures of spheroids to the two drugs (data not presented) suggest the possibility that THC may be causing the specific degradation of cDNA more rapidly (within 14-19 hr) than EB (20-24 hr).

Studies on a Somatic Cell Regenerator Mutant of Strain HK-10

The somatic cells in a normal spheroid perform a variety of functions but because they have lost the capacity to divide they eventually die not long after the parental spheroid has released the next generation of juvenile spheroids. Mutant strains of Volvox termed "somatic cell regenerator" (SR) have been isolated (Starr, 1970; Sessoms and Huskey, 1973; Griffin and Huskey, 1974) in which these events are altered. An SR mutant (HMK-01 reg), induced and isolated for this study as described under Materials and Methods, (see plate 4) carries out what appears to be a normal developmental

sequence of events up to and including embryogenesis and inversion of original developing gonidia (as shown in figure 20, stage 1; plate 4). At this point, however, instead of releasing the new individuals and subsequently dying, the somatic cells of the parental spheroid begin a sequence of "dedifferentiation" and "regeneration" (plate 4). The course of events follows that illustrated in figure 20, stages 2-7: Stage 2 shows differentiated somatic cells with extended flagella; in Stage 3 these cells begin dedifferentiation, which includes loss of flagella and therefore spheroid mobility; in Stage 4 the cells enlarge and become vacuolated; in Stage 5 they "condense;" Stage 6 depicts the onset of cell division; Stage 7 shows groups of small newly inverted spheroids (formed from "regenerated" somatic cells) being released from the matrix.

DNA synthesis has been studied in the SR mutant HMK-01 reg, using protocols described above. Since SR mutants develop more slowly than wild-type strains, a complete life cycle and hence the labeling period could be as long as five or more days. When DNA from HMK-01 reg is banded in preparative CsCl density gradients, results such as those presented in figure 21 are obtained.

Two major peaks can be detected banding with buoyant densities characteristic of the nuclear and cytoplasmic DNA from strain HK-10 (the shoulder on the second, lighter, cytoplasmic peak is unexplained as yet). The unusual finding from this profile is the higher ratio of counts found in the cytoplasmic DNA to those of the total DNA counts; in this mutant the c/t ratio is always greater than 30% in long-term (> 2 days) labeling profiles.

Results presented above indicate that Δ^9 THC preferentially interferes with cytoplasmic DNA metabolism in wild-type Volvox spheroids. Experiments were therefore performed using the SR mutant and following DNA synthesis for 18 hr in the presence and absence of THC or carrier. Figure 22A shows a profile obtained from an 18 hr pulse-label (2-^{3}H-adenine) of a heterogeneous SR culture treated with 40 ul/ml carrier. Both nuclear and cytoplasmic DNA banding species are present in proportions similar to those in the untreated control (not presented). Figure 22B, however, shows the effect of THC (400 ug/ml) treatment on this process. The nuclear DNA incorporation appears unaffected although the incorporation of label into the cytoplasmic DNA is dramatically halted. The THC is therefore acting in a manner similar to that observed in wild-type cells.

Asynchronous cultures of this SR strain have been treated with THC and examined microscopically for two days. Both gonidia formed from somatic cells which had "regenerated" as well as "original" internal gonidia, react to the drug in a manner similar to that exhibited by gonidial cells in wild-type (HK-10) cultures. At concentrations of THC of 400 ug/ml, all enlarged gonidial cells become arrested at condensation and many smaller ones are seen to reach this stage prematurely.

Conditions supporting synchronous growth of SR cultures have not yet been attained. The longer life cycle of these mutants, as well as the great variations in the times of regeneration of different somatic cells within a particular individual may indeed make complete synchrony impossible in cultures of this strain. However, newly inverted spheroids of HMK-01 reg which still possess flagella have

been isolated on the basis of their motility. Such individuals have been incubated in the presence of carrier (30-40 ul/ml) or THC (300-400 ug/ml) and observed microscopically for two days. During this time control and carrier treated cultures showed the "dedifferentiation" (loss of flagella, rounding and enlarging) of somatic cells typical of this mutant, while THC treated cultures did not appear to begin this process.

Preliminary "THC recovery" studies were also performed on SR cultures. When heterogeneous populations of these mutants were exposed to the drug (400 ug/ml THC) for 18-24 hr and washed and resuspended in fresh SVM, many individuals survived the treatment, and after a lag of 2-3 days developed at a rate characteristic of that strain. Similarly treated, newly inverted spheroids described above, however, did not recover.

These results appear consistent with preliminary findings on cDNA degradation by THC in SR mutants (data not presented). The same 24 hr treatment with 300-400 ug/ml THC which totally eliminates pre-existing cDNA prelabeled with 2-^{3}H-adenine as described for HK-10 spheroids, appears to only lower the c/t ratio relative to the control in SR cultures. Recovery would, therefore, seem more plausible for these mutants since a template for the synthesis of cDNA may still exist. Since the c/t ratio is much higher in these mutants (over 30%) than in HK-10 spheroids (5-15%), cDNA degradation is likely to occur by the same mechanism but longer treatment is probably required for total elimination of this DNA species.

DISCUSSION

This project was undertaken to demonstrate the feasibility of using Volvox as a model system for research involving the molecular control of differentiation and development. The specific purpose of this study was to elucidate the temporal patterns of nuclear and organelle DNA synthesis which occur during the asexual life cycle of Volvox carteri f. nagariensis and to examine the developmental consequences caused by disrupting or modifying these natural patterns.

In this section the experimental results presented previously will be summarized; these will then be discussed in greater detail and compared with relevant studies by other researchers. A theoretical model concerned with the regulation of development will be presented, and the limitations of the experiments and the model will be considered. Insights gained through this study on the relevance of research employing Volvox to studies concerning animal development will be discussed and the directions for future research explored.

An individual Volvox spheroid has a well defined life cycle which includes embryogenesis, "birth", growth, reproduction and death. In this respect it resembles higher multicellular eukaryotes more than it does single celled microorganisms which have only a simple cell cycle of growth and mitotic division. It is known that during the cell cycle of these organisms there are periods of nuclear and cytoplasmic DNA synthesis, which need not occur simultaneously but which must be coordinated in some manner so as to insure that upon cell division each offspring receives its appropriate genetic complement (see Introduction). Similarly, therefore, a multicellular organism must

coordinate the patterns of nuclear and cytoplasmic DNA synthesis that occur during its life cycle .

This study has demonstrated that it is possible to follow the synthesis of nuclear and cytoplasmic DNA in Volvox by monitoring the incorporation of radioisotope precursors into these DNA species, and that the temporal patterns of their synthesis do indeed vary during the course of the asexual cycle. Various methods of disrupting or modifying these patterns of DNA synthesis were studied. They included treatment with two drugs (EB and Δ^9THC) and the use of a genetic lesion (SR mutant) known to alter the morphology of the spheroid. It was demonstrated that both EB and THC mediate in the selective degradation of existing cytoplasmic DNA; also, THC was shown to selectively inhibit the synthesis of this DNA species. Studies on DNA synthesis in a developmental mutant (SR) demonstrated that its cells contain higher proportions of cytoplasmic DNA than those of wild-type Volvox spheroids, and that THC also inhibits cytoplasmic DNA synthesis in this mutant.

Before proceding to studies involving DNA synthesis it was necessary to first determine the nature of the various DNA species present in Volvox cells. Chapter 1 demonstrated that it is possible to isolate, purify and characterize at least two distinct species of DNA from the two types of cells found in Volvox carteri spheroids, and that minor differences in base composition exist between the DNA from the three different strains examined. The two DNA components which are present in all three strains have been assigned to nuclear and cytoplasmic (presumably mainly chloroplast) origin (see below). A third DNA component was found in strain KA-1. EM

studies by Kochert and Olson (1970) have demonstrated the presence of an endosymbiotic bacterium in this strain and it is possible that the second minor DNA species found in KA-1 represents the DNA of this bacterium.

In simple eukaryotes, DNA of cytoplasmic (chloroplast and mitochondrial) origin is generally present in lower proportions per cell, is of lower G+C content, and bands at lower buoyant density values in neutral CsCl, than corresponding nuclear DNA (see Introduction). Equilibrium density gradient centrifugation profiles of DNA obtained from such organisms will therefore exhibit a heavier major peak of nuclear origin and at least one minor peak containing organelle DNA.

Numerous structural similarities exist between the somatic cells of Volvox carteri spheroids and the unicellular alga Chlamydomonas (Kochert and Olson, 1970) and it has also been demonstrated that they both contain similar quantities of total DNA/cell (Yates, 1975). The nuclear and chloroplast DNA species of Chlamydomonas have been positively identified (Chun et al., 1963) and the assignments of nuclear and cytoplasmic origin to the major and minor banding species in CsCl profiles of Volvox DNA have therefore been made on the basis of their qualitative and quantitative similarities to the known DNA banding species present in Chlamydomonas and other related organisms. Positive identification of these DNA species, however, can not be made until DNA extracted from isolated organelles yields similar results when characterized (see Introduction).

Cottrell (personal communication) has recently developed a methodology to isolate and purify various organelles from the cells of

Volvox carteri spheroids. These techniques will make the unequivocal identification of the mitochondrial, chloroplast and nuclear DNA of this organism more readily attainable.

After the various classes of DNA present in three strains of Volvox carteri had been characterized, studies were then conducted to monitor the incorporation of radioactive precursors into the DNA species of strain HK-10 (Chapter 2). Results obtained using methyl-^{3}H-thymidine to label Volvox carteri f. nagariensis spheroids were somewhat surprising. In organisms as closely related to Volvox as Chlamydomonas reinhardi, methyl-^{3}H-thymidine is specifically incorporated only into chloroplast DNA (Swinton and Hanawalt, 1972). This phenomenon of selective thymidine incorporation (typical of most algae and believed due to a lack of cytoplasmic thymidine kinase), appears to be reversed in Volvox, and in HK-10 spheroids, ^{3}H-thymidine is incorporated almost exclusively into nuclear DNA. This discrepancy is as yet unexplained and warrants further investigation.

2-^{3}H-adenine was found to be incorporated into both the nuclear and cytoplasmic DNA species, and it was therefore chosen as the DNA precursor used to study the temporal patterns of incorporation that occur during the asexual life cycle of Volvox. Homogeneous populations of spheroids (all at the same stage of their life cycle) obtained by different techniques were employed in these studies. Both synchronization methods yielded comparable results, demonstrating that variations in the patterns of ^{3}H-adenine incorporation into nuclear and cytoplasmic DNA occur at different life cycle stages (see below). The accord between results obtained using either the imposed or the more "natural" selection

synchronization procedures, also justified the use of the more standardized imposed synchrony method for later studies (see Chapter 2).

The major problems encountered during the isolation of DNA from the cells of Volvox carteri have been those associated with the removal of the sheath or matrix material which encapsulates the spheroids. It was found that Protease (Sigma, type VI) mediated dissociation of spheroids into individual somatic and gonidial cells, as well as the elimination of contaminating glycoprotein matrix material prior to lysis, are enhanced by pretreating spheroids with 1% tween 40/tween 80 for 1 hr as described in Materials and Methods. Much of the variability otherwise encountered in the times and extents of protease mediated spheroid dissociation (described in Chapter 1 results) is eliminated by preincubation with this tween mixture, and such treatment also appears to reduce possible differential losses of cytoplasmic DNA, and glycoprotein contamination following nucleic acid extraction, as indicated in figure 7, Chapter 2.

Templeman and Blamire (personal communication) have found that a time course treatment of Volvox (HK-10) spheroids with tween 40/tween 80 at varying concentrations differentially extracts protein moieties from the glycoprotein sheath matrix. The subsequent electrophoresis of these proteins on polyacrylamide gels yields distinct reproducible bands. The increased ease of sheath dissolution that is observed when spheroids are treated with tween prior to protease digestion, must therefore be due to the tween-mediated elimination of many of the sheath proteins even before the spheroids are exposed to protease. A more complete separation of cells may thus occur during shorter

periods of exposure to the enzyme. This technique is most valuable in cases where dissociation times are longest (newly inverted spheroids of all strains and most spheroids of strain HK-10, see Chapter 1), since many free gonidial cells exhibit morphological distortions when exposed to protease (80-100 ug/ml) for periods of time longer than 1.5 hr. Since such damage may result in the preferential loss of gonidial DNA, dissociation in such cases often has to be terminated before cell separation is complete. The accelerated dissociation observed after tween treatment thus provides enhanced matrix removal without causing gonidial damage.

The technique of "tween pretreatment" was devised after employing a mixture of tween and ethanol as a "carrier" to solubilize Δ^9 THC (see Chapter 3). Subsequent to finding that "carrier" treated control cultures dissociated more rapidly than untreated controls upon exposure to protease, and that short term incubation of cultures with 1% tween could mimic these beneficial carrier effects, the technique of tween pretreatment was used routinely to facilitate cell separation in Volvox spheroids.

Other aspects of the methodology involved in the extraction of DNA from the cells of Volvox spheroids are considered in Chapter 1 and in Materials and Methods. The possibility that particular DNA species have been lost in whole or in part during the purification processes cannot be entirely ruled out. However, considerable effort was made to ensure that no systematic or selective losses occurred during isolation (see Chapter 1). The fact that purified DNA gives profiles similar to those obtained using radioactive unpurified DNA, also strengthens the conclusion that no selective losses occurred.

No measurements have thus far been made on pool sizes of DNA precursors in the nucleus and/or cytoplasmic organelles. Although differences in pool sizes could account for differences seen in the amount of incorporation into nuclear and cytoplasmic DNAs, labeling of asynchronous cultures has been carried out for a wide variety of time periods (4 hr to 5 days), and remarkably similar profiles have been obtained. This appears to indicate that the radioactive precursors equilibrate rapidly with the pools of unlabeled precursors and that the amount of incorporation into DNA closely reflects the amount of DNA seen using other criteria. The differences in adenine incorporation into DNA during the life cycle, though, could still be a reflection of preferential sequestering of precursors in organelle pools, and this possibility should be investigated.

The studies of the incorporation of 2-^{3}H-adenine into the DNA of synchronous Volvox cultures demonstrated that nuclear DNA synthesis occurs to some extent throughout the entire course of the asexual life cycle, while cytoplasmic DNA appears to be made cyclically, with its major synthetic period during the prerelease expansion stage immediately following inversion (see figures 11a and 11b and figure 1, stage 7). Some cytoplasmic DNA may also be made during cleavage, but it is difficult to divorce this synthesis from the onset of the later synthetic period since synchronization techniques are not yet precise enough to terminate labeling before every newly formed spheroid has undergone inversion. Also, the amount of cytoplasmic DNA synthesis seen during this period in "in vitro" gonidia, isolated by the NIT method (figure 11f) is greater than that seen during cleavage of gonidia within parental spheroids isolated by the SYNC method (figure

11e). The enlarged free gonidia used for the NIT studies are more heterogeneous than those within SYNC spheroids, since enlarged gonidia of the same size may still exhibit various states of maturation (precondensation or condensation). Thus, while it was possible to label SYNC cultures during cleavage and terminate labeling before appreciable prerelease expansion occurred, it was not found possible to reproducibly prevent the further advanced *in vitro* gonidia (NIT) from attaining the postinversion stage during which the synthesis of significant quantities of cytoplasmic DNA (see figures 11a and 11b) takes place.

These findings on the temporal patterns of DNA synthesis substantiate suggestions made by Kochert (1975) to account for the higher ratio of chloroplast to nuclear DNA in gonidia and the higher amount of total DNA per cell that he finds present in mature gonidia as compared to somatic cells of *V. carteri* f. *weismania* (strain NB-7). This study has shown that a period of differential synthesis of cytoplasmic (chloroplast) DNA, like the one that Kochert hypothesizes for NB-7, does indeed exist in strain HK-10, Yates' (1974) measurements of total $^{32}PO_4$ DNA/spheroid in synchronized cultures of NB-7 indicate that there is a period of DNA synthesis immediately after inversion; it is likely that this is the period of preferential synthesis of chloroplast DNA in NB-7, and that it is similar to the period of cytoplasmic DNA synthesis shown here to occur in HK-10 spheroids during the same stage of their life cycle. It is therefore probable that patterns of DNA synthesis are similar in the different strains of *Volvox carteri*.

These studies were carried out using either intact spheroids, or,

in some cases "in vitro" gonidia, however no attempt was made to distinguish between gonidial and somatic cell DNA when spheroids were employed. The bulk of DNA synthesis observed during expansion and embryogenesis is assumed to be taking place in the gonidia. However this should be tested further by labeling spheroids during these time periods and separating the two cell types before analyzing the DNA.

Numerous similarities between embryogenesis in Volvox and early development in higher organisms were presented in the Introduction and comparisons were drawn between the maturation of gonidia and oocytes. It was shown that both the Volvox gonidium and the amphibian oocyte synthesize quantities of rRNA sufficient to supply all cells formed during cleavage. It has also been demonstrated that the amount of organelle DNA present in mature amphibian oocytes could supply mitochondrial genomes for the entire embryo (Chase, 1970). The relatively high amount of Volvox cytoplasmic DNA synthesis detected during early gonidial enlargement may well be related to the amplification of organelle DNA that has been demonstrated in maturing oocytes of other organisms whose early embryos also show division without growth. The shorter cell cycles of such dividing embryos (Mitchison, 1971) may not provide sufficient time to allow coordinated organelle DNA replication (see Introduction) and the synthesis of large quantities of cytoplasmic DNA prior to cleavage may have therefore evolved simultaneously with this type of rapid cell division.

Results presented here for Volvox show that relatively little synthesis of organelle DNA occurs during cleavage and that even the amount demonstrated may be artifactual (see above). Similarly, during

the cleavage of amphibian oocytes, no mitochondrial DNA synthesis has been detected (Chase and Dawid, 1972). However, Kochert (1975) has found that in V. carteri f. weismannia, the amount of ctDNA present in a mature gonidium represents an amplification of only 100-150 times that found in somatic cells. These results suggest that, in that strain at least, ctDNA synthesis must occur at some time during gonidial cleavage in order to provide every cell of the embryo with a complete genome.

Kochert (1975) has also reported that mature gonidia of V. carteri f. weismannia contain about 25 times as much DNA as do somatic cells and that only about 30% of this DNA is of organelle origin. Results presented here indicate that significant amounts of DNA synthesis occur during gonidial enlargement. Studies performed on cultures containing similar numbers of spheroids have demonstrated that ^{3}H cpm from adenine incorporated into nuclear DNA during spheroid expansion are generally between 15-25% of those incorporated into nuclear DNA during cleavage (data not presented). These results suggest that more nuclear DNA than the 2C complement is present in the mature gonidia of the haploid (C) Volvox spheroid prior to the first cleavage.

As described in the Introduction, oocyte development usually involves transcription from at least twice the normal DNA complement present in diploid organisms. In oocytes requiring long maturation periods (weeks to months), lampbrush chromosomes, which contain a 4 C complement of DNA, are the centers of great transcriptional activity. However, in meroistic insects, where oocytes are connected to nurse cells, only days are required for oocyte development; nurse cells are

highly polytene and therefore usually several thousandfold more genomic DNA is active in the preparation of their oocyte RNA (see Davidson, 1976). Volvox gonidia undergo full maturation in a relatively short time (1-2 days) and since the organism is haploid, the presence of high levels of nuclear DNA in the mature gonidia may indicate that in this organism, amplification of the entire nuclear genome is necessary for accelerated transcription in preparation for cleavage and embryogenesis. Studies on V. carteri f. weismannia have demonstrated that stages of gonidial enlargement prior to the first cleavage are highly sensitive to actinomycin-D treatment (Weinheimer, 1973; Kochert, 1975). Small gonidia do not enlarge and mature gonidia fail to cleave, whereas a 16-celled embryo placed in the drug can undergo about 5 more cleavage divisions. These differential effects of a drug known to inhibit nuclear RNA synthesis may also be in accord with the idea that there is accelerated transcriptional activity in the rapidly maturing Volvox gonidium and that it therefore may be provided for by an amplified genome.

Further studies should be conducted on possible qualitative and quantitative changes in the Volvox genome occurring during the process of gonidial maturation. The nuclear DNA complement present in gonidia at various stages of maturation should be determined. Also, the recent demonstrations of the presence of lampbrush chromosome forms (morphologically similar to those of oocytes) in the primary nuclei of various species of the alga Acetabularia, (Spring et al., 1974; Scheer et al., 1976), suggest the possibility that the active transcription necessary during gonidial maturation in Volvox may also involve this altered chromosome form. EM studies of maturing gonidia

should therefore be conducted as well.

THC and EB Studies

Interference with DNA metabolism in Volvox was studied by treating spheroids with THC and EB. The widespread use of marijuana has stimulated research on the interactions between its major psychoactive ingredient, Δ^9 THC, and DNA. Changes in DNA synthesis in cultured human lung cells after exposure to marijuana smoke (Leuchtenberger et al., 1973), as well as aberrent chromosomal changes in chronic marijuana users (reviewed by Matsyama et al., 1977), have been reported. Δ^9 THC induced inhibition of DNA synthesis has been demonstrated in Tetrahymena (Zimmerman and McClean, 1973), and mitogen stimulated lymphocytes from marijuana users have been reported to incorporate ^{3}H-thymidine at a lower rate than those from control subjects (Nahas et al., 1974). More recently, in studies related to its antineoplastic activity in vivo (Munson et al., 1975), Δ^9 THC was shown to inhibit DNA synthesis in Lewis lung (adenocarcinoma) cells in tissue culture, as measured by ^{3}H thymidine incorporation into acid insoluble material (Carchman et al., 1976), without decreasing precursor uptake (White et al., 1976). The possibility of differential inhibition of nuclear or organelle DNA synthesis by THC has not been investigated in these systems.

This study (Chapter 3 and Margolis-Kazan and Blamire, 1977) has shown that in Volvox, Δ^9 THC selectively affects cytoplasmic DNA metabolism; results have been obtained which demonstrate both the degredation of existing cytoplasmic DNA and the selective inhibition of its synthesis.

The effects of EB on DNA metabolism have been studied extensively

in other systems and EB is known to selectively inhibit organelle DNA synthesis in numerous organisms. The drug has also been shown to mediate in the breakdown of preexisting mtDNA in yeast and ctDNA in Chlamydomonas. (See Introduction.)

These studies (Chapter 3) have indicated that while EB induced degradation of existing cytoplasmic DNA is similar to that mediated by THC, the actions of the two drugs on DNA synthesis in Volvox are very different. Treatment with Δ^9 THC inhibits cytoplasmic DNA synthesis but has little effect upon the synthesis of the nuclear species during this same time period. The biosynthetic effect of EB, however, is one which drastically depresses all DNA synthesis and its most immediate action is to inhibit the synthesis of the nuclear species while allowing some cytoplasmic DNA synthesis to continue.

The biosynthetic effect of EB on Volvox DNA is therefore different from its effect on DNA synthesis in Chlamydomonas where ctDNA synthesis is selectively inhibited by the drug. While ctDNA is degraded by EB in both organisms, Chlamydomonas can recover from such treatment but Volvox does not (Flechtner and Sager, 1973; see Introduction). Although these two organisms are believed to be related evolutionarily and exhibit numerous structural similarities described above, the life cycle of Volvox studied here includes the developmental processes of embryogenesis and somatic cell senescence and death, whereas that studied in Chlamydomonas is basically a cell cycle, followed from one division to the next. Many of the differences, encountered here, between the two organisms, (including the inability of Volvox and the ability of Chlamydomonas to recover and continue their respective cycles after ctDNA synthesis has been

inhibited or that DNA species degraded, as well as their differing EB induced DNA synthesis inhibitions) may indeed be a function of these differing developmental patterns and reflect the increased importance of organelle DNA in the regulation of development in multicellular organisms.

The temporal patterns of DNA synthesis described above reveal that most cytoplasmic DNA is synthesized at an early period (prerelease) in the asexual life cycle of a Volvox carteri spheroid (figures 11a and 11b; Life cycle stage 7, figure 1). The gonidia (reproductive cells) are plausible sites for such synthesis and as discussed above, these cells probably require a sufficient amount of DNA and correct proportions of the nuclear and cytoplasmic DNA species before commiting themselves to cleavage and embryogenesis. In an attempt to understand the regulation of these processes, the consequences of interference with the natural DNA metabolism of Volvox (SR mutation and THC and EB treatment) were investigated. The various treatments of Volvox spheroids with EB and THC yielded morphological and biochemical results, which, when correlated, suggest possible models in which the amounts and proportions of nuclear and cytoplasmic DNA play a role in the regulation of embryogenesis in this organism. One such model is presented below.

After release of Volvox carteri f. nagariensis spheroids, the ratio of cytoplasmic DNA to total DNA (c/t ratio) decreases steadily from a high value at release (caused by high levels of cDNA synthesis before this time) to a much lower value at condensation (figure 12, stage 2, time zone C), due to the fact that while the amount of cDNA remains constant after release, the amount of

nuclear DNA (nDNA) increases (because of its continuous synthesis). At some point during this period of spheroid expansion (time zone B, figure 12) this decreasing c/t ratio may reach a critical level which in some way triggers the gonidial cell to begin the processes of condensation and embryogenesis. If this c/t ratio is one which allows cleavage to begin, nDNA synthesis must naturally continue for normal cell divisions and embryogenesis to take place.

Ethidium bromide promotes changes in the c/t ratio of Volvox DNA by two mechanisms. At low EB concentrations nDNA synthesis is inhibited and residual cDNA synthesis continues. This results in a gradual rise in the c/t ratio. After some time, however, (6-18 hrs.) cDNA synthesis is also inhibited and this DNA species begins to degrade (higher concentrations of EB promote cDNA breakdown and synthesis inhibition earlier), and the c/t ratio begins to decrease. According to this model, gonidial cells could therefore pass through condensation and begin cleavage, but because of the absence of nDNA synthesis, arrest after only a few divisions. (This morphological arrest in early cleavage most marked at 25 ug/ml EB.)

Delta-9-tetrahydrocannabinol, which is more specific for Volvox cDNA metabolism than EB, allows nDNA synthesis to continue over a wide range of concentrations. At THC concentrations between 300 and 400 ug/ml, cytoplasmic DNA synthesis is arrested earlier, and the degradation of cDNA probably begins sooner and takes place faster than that induced by EB. The net effect of these phenomena is to rapidly decrease the c/t ratio in all gonidia, which according to this model, causes then to condense, often prematurely. Upon

treatment of Volvox spheroids with THC (300-400 ug/ml) small immature gonidia as well as enlarged ones, reach and remain arrested at condensation. This absence of cell division after condensation of gonidia, may be due to the very low amount of cDNA present in these cells and/or to other phenomena induced by THC treatment (see below).

The effects of THC treatment on the DNA of somatic cell regenerator mutants (HMK-01 reg) are similar to those exhibited by wild-type HK-10 cultures and many of the morphological changes exhibited by this mutant in the presence of the drug may also be explained in accordance with the model of changing c/t ratios presented above. Since cDNA synthesis is inhibited and cDNA degredation also promoted by THC in this mutant, the sharply falling c/t ratio may again be acting to trigger the premature condensation and gonidial arrest that is observed. Since these same effects were noted in both types of gonidial cells present in this mutant strain, the "original" internal gonidia, as well as peripheral ones formed from "regenerated" somatic cells, one may therefore infer either the universality of the morphological consequences of THC treatment to different types of Volvox gonidia or the true "gonidia like" nature of regenerated somatic cells.

The possibility that targets other than DNA metabolism are causing the morphological changes observed upon treatment of Volvox with EB or THC should not be overlooked. The drugs may be acting directly on cell division or producing other effects which lead to the reproducible morphological arrests observable at the particular stages of development reported here.

Also, at lower concentrations of THC, different morphological

effects were noted, namely, embryonic arrest during cleavage or inversion and delayed release of juvenile spheroids. Assuming DNA metabolism is not the immediate target at these drug concentrations, then some other sites must be the areas of sensitivity. The effects of THC on RNA metabolism in Volvox are currently under investigation and preliminary studies demonstrate an inhibition of RNA synthesis by Δ^9 THC at concentrations well below 300 ug/ml (Howard Caplen, personal communication). Also results presented here indicate that at THC concentrations of 400 ug/ml the incorporation of 2-^{3}H adenine into Volvox RNA is severely inhibited.

It is well established that EB inhibits transcription from mtDNA as well as interfering with its synthesis (reviewed by Corcoran and Hahn, 1975). One must therefore consider the possibility that it may again be the lack of gene products, in this case specifically those of organelles, which lead to the morphological changes observed in EB treated spheroids. It is not yet certain from these studies whether the naturally occurring changes in c/t ratios, as well as those induced by drugs, are cause, effect, or coincidental to the asexual development cycle of Volvox carteri spheroids. However, these fluctuations during the normal course of development do indeed follow consistent temporal patterns suggesting important temporal regulation, and interference with these natural changes in proportions of nuclear and cytoplasmic DNA species does yield reproducible and consistent morphological abnormalities. It is clear that further studies in this area are necessary to gain insight into the integration of these events and their action in the regulation of development.

All Δ^9 THC studies presented were performed using carrier

treated as well as untreated controls, since "carrier" was used as the vehicle to solubilize THC. Although spheroids can survive growth in carrier, and patterns and extents of DNA systhesis are similar to those of controls (using protocols described for the time periods studied), certain carrier effects, noted in the text, should not be ignored. Carrier treatment of Volvox spheroids does act to reduce the uptake and incorporation of 2-^{3}H-adenine relative to that found in untreated controls, and this reduction in incorporation appears to be primarily into RNA. The asexual developmental cycle is somewhat delayed and the induction and maintenance of tight synchrony inhibited in the presence of this tween 40/80-ethanol mixture. These molecular and morphological observations on carrier effects could very well be related. Although the consequences of spheroid treatment with THC are different from those exhibited by carrier-treated cultures, the possibility does exist that it is the carrier, present as part of THC treatment, that is acting with the drug in a synergistic manner to produce some of the morphological and biochemical effects noted, and not the drug alone. This possibility would be eliminated if THC made soluble with other agents, or water soluble derivatives of Δ^{9} THC, were tested and produced the same effects.

SR Mutant Studies

The SR mutants used in this study exhibit higher proportions and rates of synthesis of cDNA than do wild-type HK-10 spheroids. This is not surprising, since gonidia of Volvox spheroids contain higher proportions of cDNA than somatic cells and SR strains possess many more "gonidia" during portions of their life cycle.

The synthesis of cDNA in "regenerated" gonidia may occur during a

developmental period similar to the prerelease expansion phase of high cDNA synthesis observed in wild-type gonidia. Thus the "regenerated" gonidia may also exhibit a discrete period of high cDNA synthesis during the process of maturation, in preparation for cleavage.

The timing of periods of cDNA synthesis in regenerating cells should therefore be investigated further. Although synchronization using intact spheroids may not be possible in this strain, short term studies employing newly inverted SR spheroids (isolated as described in text) and relatively homogeneous populations of dissociated "in vitro" regenerated gonidia, at various stages of maturation (feasibly isolated by filtration through differing sizes of Nitex mesh), should be conducted. DNA synthesis patterns during the period of dedifferentiation and the various stages of gonidial maturation could then be elucidated. Knowledge of the timing of organelle DNA synthesis in the SR mutant would also aid in the determination of which effect on DNA metabolism (degradation of organelle DNA or the inhibition of its synthesis) is operative in producing the absence of dedifferentiation observed in the presence of Δ^9 THC (flagellated somatic cells do not become regenerated gonidia upon treatment with 300-400 ug/ml THC).

The existence and availability of "somatic regenerator" mutants adds further dimension to the range of biochemical and genetic studies of differentiation and development possible within the "Volvox system." In wild-type individuals, flagellated somatic cells of post inversion spheroids are terminally differentiated and do not divide again. In regenerator mutants, however, these cells undergo a

series of phenotypic changes, including loss of flagella and change of shape and proceed to form reproductive cells. Such events provide a unique illustration of the total reversibility of the genomic changes which occur during the process of differentiation.

Other examples of redifferentiation, where obviously differentiated somatic cells change their specialized roles and assume new states of differentiation, do indeed exist. These phenomena, termed transdifferentiation, occur under numerous natural and experimentally induced conditions including eye and limb regeneration, silk worm metamorphosis and the culture of blood lymphocytes (reviewed by Davidson, 1976); they do not, however, demonstrate that the total potential is retained by the genome after differentiation has occurred. Experiments, such as those in which nuclei from differentiated cells, injected into mature eggs, were shown to direct the development of a complete new organism have been necessary to illustrate this principal (see Introduction).

Volvox SR mutants provide a system in which the retention of the full genomic capacity following differentiation may be studied under more "natural" circumstances. Not only does transdifferentiation occur in the somatic cells of these mutants, but their new state is one which allows them to direct the complete course of development.

Genetic studies have been performed on other Volvox regenerator mutants. Griffin and Huskey (1974) have reported two non-linked mutations carrying this phenotype and have demonstrated that in individual crosses the regenerator trait segregates as a single allele. The possible existence, in Volvox, of a single gene product with major epigenetic regulatory potential is thus

inferred. Further genetic and molecular studies should certainly be conducted on this mutant in the hope of isolating this gene product and determining its mechanism of action. An understanding of the regulation involved in the dedifferentiation of this mutant, and possible comparisons with the allelic wild-type gene product, would surely provide insight on the mechanisms of differentiation.

Future Studies

In addition to the areas warranting further investigation which have been described throughout this discussion, this study of Volvox DNA has not included certain genomic characterizations which may be indicated for further developmental studies.

Popular models of gene regulation, and hence differentiation, in higher eukaryotes are based on the organization of DNA sequences in the genome (see Introduction). An ordered pattern of interspersion of single copy and repetitive DNA sequences has been demonstrated in all forms of metazoa studied (reviewed by Davidson et al., 1975).

The organization of the Volvox genome should therefore be elucidated. This study has demonstrated that the DNA of Volvox may be radioactively labeled and purified. Studies involving the reassociation of DNA fragments and subsequent hydroxyapatite chromatography (see Davidson et al., 1973 for methodology) should now be conducted to verify the existence of repetitive and unique sequences in Volvox DNA and to elucidate their patterns of interspersion.

The feasibility of using Volvox for research involving macromolecular synthesis during development has been demonstrated (see

Introduction for other recent Volvox studies). The "Volvox system" now awaits further molecular developmental studies of the type (see Introduction) currently being conducted on the oocytes and early embryos of other "more popular" organisms.

SUMMARY

This study has shown that it is possible to isolate, purify and characterize at least two distinct species of DNA from the two cell types present in Volvox carteri spheroids. The DNA from three strains (HK-10, NB-7 and KA-1) was compared, and all strains were shown to contain at least two distinct DNA species which band at densities of 1.714-1.715 and 1.704-1.705 g/cm^3 in neutral CsCl and correspond to nuclear and "cytoplasmic" DNA, respectively. Base compositions calculated from these densities, 55-56% G+C for nuclear DNA and 45-46% G+C for cytoplasmic DNA, are in close agreement with % G+C values estimated from thermal denaturation data. DNA from KA-1 has a third component with a buoyant density of 1.693 g/cm^3 which corresponds to 34% G+C and probably represents the DNA of the endosymbiotic bacteria which are present in this strain.

It was demonstrated that it is possible to follow the synthesis of nuclear and cytoplasmic DNA in Volvox by monitoring the incorporation of radioisotope precursors into these DNA species. Preparative CsCl density gradient profiles obtained from spheroids grown in the presence of 2-^{3}H-adenine demonstrated that this precursor is incorporated into both nuclear and cytoplasmic DNA in proportions similar to those obtained using other preparative and analytical techniques. Methyl-^{3}H-thymidine, however, was found to be incorporated almost exclusively into Volvox nuclear DNA.

Studies to elucidate the temporal patterns of nuclear and cytoplasmic DNA synthesis during the asexual life cycle of strain

HK-10 were performed using homogeneous spheroid populations obtained by either imposed or size-sorting synchronization procedures. Both methods yielded comparable results which demonstrated that variations in the patterns of ^{3}H-adenine incorporation into nuclear and cytoplasmic DNA occur at different life cycle stages. While nuclear DNA synthesis takes place to some extent throughout the life cycle, cytoplasmic DNA appears to be made cyclically, with its major synthetic period occurring during the prerelease expansion stage immediately following inversion.

Interference with DNA metabolism was studied by treating spheroids with THC and EB. THC was shown to selectively affect cytoplasmic DNA metabolism. Treatment of spheroids with 400 ug/ml THC was demonstrated to mediate in both the degradation of existing cytoplasmic DNA and the selective inhibition of its synthesis. While EB (25 ug/ml) induced degradation of existing cytoplasmic DNA is similar to that mediated by THC, its biosynthetic effect is one which substantially depresses all DNA synthesis, and its most immediate action is to inhibit the synthesis of the nuclear species while allowing some cytoplasmic DNA synthesis to continue.

Treatment of Volvox spheroids with THC and EB produced morphological effects characteristic to each drug. The reproductive cells (gonidia) of THC treated spheroids became arrested at the condensation stage of the life cycle just prior to cleavage and at drug concentrations between 300 and 400 ug/ml, often reached this stage prematurely. The gonidia of EB (25 ug/ml) treated spheroids passed through condensation and began cleavage, but arrested after a few divisions.

Studies performed on a developmental mutant of strain HK-10, "somatic cell regenerator" mutant HMK-01, have shown that its cells contain a higher proportion of cytoplasmic DNA than those of wild-type spheroids, and preliminary studies indicate that the effects of THC on the DNA metabolism and gonidial morphology of this mutant are similar to those observed in strain HK-10. In addition, the "dedifferentiation" and "regeneration" of somatic cells characteristic of mutant HMK-01, appeared to be inhibited by treatment with 400 ug/ml THC.

Changes in proportions of Volvox nuclear and cytoplasmic DNA occurring during normal life cycle stages as well as those found after drug treatment or mutation were correlated with observed developmental changes, and a theoretical model was proposed in which the ratios of cytoplasmic DNA to total DNA (c/t ratio) play a role in the regulation of Volvox development.

TABLE 1

BASE COMPOSITIONAL ANALYSIS OF NUCLEAR AND CYTOPLASMIC DNAs FROM THREE STRAINS OF *Volvox carteri*

Analytical CsCl

	DNA	Density (g/cm^3) in CsCl	% G + C
KA-1	nuclear	1.714	55%
KA-1	cytoplasmic	1.704	45%
KA-1	"endosymbiont"	1.693	34%
NB-7	nuclear[a]	1.714	55%
NB-7	cytoplasmic[a]	1.704	45%
HK-10	nuclear[a]	1.715	56%
HK-10	cytoplasmic[a]	1.705	46%

Thermal Denaturation

	DNA	Tm(C) in 0.1 x SSC	% G + C
KA-1	total	76.5	55%
NB-7	total	76.0	54%
NB-7	enriched nuclear	77.0	56%
NB-7	enriched cytoplasmic	72.5	45%
HK-10	total	77.0	56%
HK-10	enriched nuclear	77.8	58%
HK-10	enriched cytoplasmic	72.5	45%

Values presented above were obtained using DNA purified as described in Materials and Methods. "Enriched" DNAs used for denaturations were taken from preparative CsCl gradients (see Chapter 1). Values in table are averages, with differences no greater than $\pm$ .001 g/cm^3 or $\pm$ 1 C ($\pm$ 2% G + C) found upon repetition of procedures indicated.

[a]These values for buoyant density are calculated from enriched nuclear and enriched cytoplasmic DNAs taken from preparative CsCl gradients (see legends to figures 3 and 4). Kochert and Jaworski (1972) reported buoyant densities of 1.715 g/cm^3 and 1.707 g/cm^3 for nuclear and chloroplast DNAs of NB-7, using total DNA in analytical CsCl density gradient centrifugations.

TABLE 2

INCORPORATION OF 2-^{3}H-ADENINE

	Control			Carrier Treated					THC Treated				
Time	^{3}H cpm			^{3}H cpm					^{3}H cpm				
Hr	Total	Alkali Stable	%Alkali Stable	Total	%Control (total)	Alkali Stable	% Control (Alk.Stab.)	%Alkali Stable	Total	%Control (total)	Alkali Stable	% Control (Alk.Stab.)	%Alkali Stable
2	55,000	2,280	4%	49,500	90%	1,170	51%	2%	49,000	89%	1,310	58%	3%
4	66,500	2,100	3%	58,800	88%	2,450	117%	4%	61,000	92%	1,800	86%	3%
6	77,500	2,260	3%	62,600	81%	2,250	100%	4%	30,600	40%	1,180	52%	4%
8.5	220,000	2,550	1%	142,000	65%	2,400	94%	2%	29,000	13%	840	33%	3%
25	550,000	20,200	4%	47,600	9%	2,900	14%	6%	8,000	2%	1,900	9%	24%

See Materials and Methods (Radioactive Labeling) and Chapter 3 for explanations and experimental details.

TABLE 3

INCORPORATION OF METHYL-^{3}H-THYMIDINE

	Control			Carrier Treated					THC Treated				
Time	^{3}H cpm			^{3}H cpm					^{3}H cpm				
Hr	Total	Alkali Stable	%Alkali Stable	Total	%Control (total)	Alkali Stable	% Control (Alk.Stab.)	%Alkali Stable	Total	%Control (total)	Alkali Stable	% Control (Alk.Stab.)	%Alkali Stable
2	1,900	410	22%	460	24%	380	93%	83%	470	25%	108	26%	23%
4	2,400	775	33%	2,450	102%	355	46%	15%	423	18%	211	27%	50%
6	1,190	250	21%	655	55%	477	190%	73%	3,160	266%	1,380	552%	44%
8.5	3,950	890	26%	4,600	117%	805	90%	18%	7,200	182%	1,070	120%	15%
25	2,350	940	40%	3,230	137%	1,600	170%	50%	5,350	228%	217	23%	4%

See Materials and Methods (Radioactive Labeling) and Chapter 3 for explanations and experimental details.

TABLE 4

DNA SYNTHESIS IN THE PRESENCE OF DELTA-9-TETRAHYDROCANNABINOL FOR 18 HOURS

	Control	100 ug/ml	200 ug/ml	400 ug/ml
c/t ratio	21%	20.5%	21%	0%

See legend to figure 15 and Chapter 3 for explanations and experimental details.

TABLE 5

DNA SYNTHESIS IN THE PRESENCE OF
ETHIDIUM BROMIDE FOR 18 HOURS

	Control	1 ug/ml	2.5 ug/ml	5 ug/ml	15 ug/ml
% Synthesis	100			2.4	0.8
c/t ratio	4.8%			8.3%	13%
% Synthesis	100	104	15	7.2	
c/t ratio	13%	15%	38%	35%	

See chapter 3 for explanations and experimental details.

TABLE 6

DNA SYNTHESIS IN THE PRESENCE
OF ETHIDIUM BROMIDE (EB)

6 hr in the combined presence of EB and 2-^{3}H-adenine			
	Control	2.5 ug/ml	10 ug/ml
c/t ratio	6%	15%	56%
6 hr pretreatment with EB and subsequent 18 hr incubation in the combined presence of EB and 2-^{3}H-adenine			
c/t ratio	12.3%	56%	-

See Chapter 3 for explanations and experimental details.

TABLE 7

DEGRADATION OF PRELABELED DNA BY ETHIDIUM BROMIDE (EB) AND DELTA-9-TETRAHYDROCANNABINOL (THC)

4 days in the presence of 2-^{3}H-adenine and subsequent 20 hr incubation with EB				
	Control	5 ug	15 ug	25 ug
c/t ratio	27%	28%	17%	0

4 days in the presence of 2-^{3}H-adenine and subsequent 20 hr incubation with THC					
	Control	100 ug	200 ug	300 ug	400 ug
c/t ratio	22%	25%	15%	2%	0

See legends to figures 17 and 18 and Chapter 3 for explanations and experimental details.

Figure 1. Diagrammatic representation of the asexual life cycle of Volvox carteri.

For details and description, see text.

Stage 1: Postrelease expansion

Stage 2: Gonidial "condensation"

Stage 3: First cleavage

Stage 4: Cleavage

Stage 5: Postcleavage, preinversion

Stage 6: Inversion

Stage 7: Prerelease expansion

Stage 8: Release

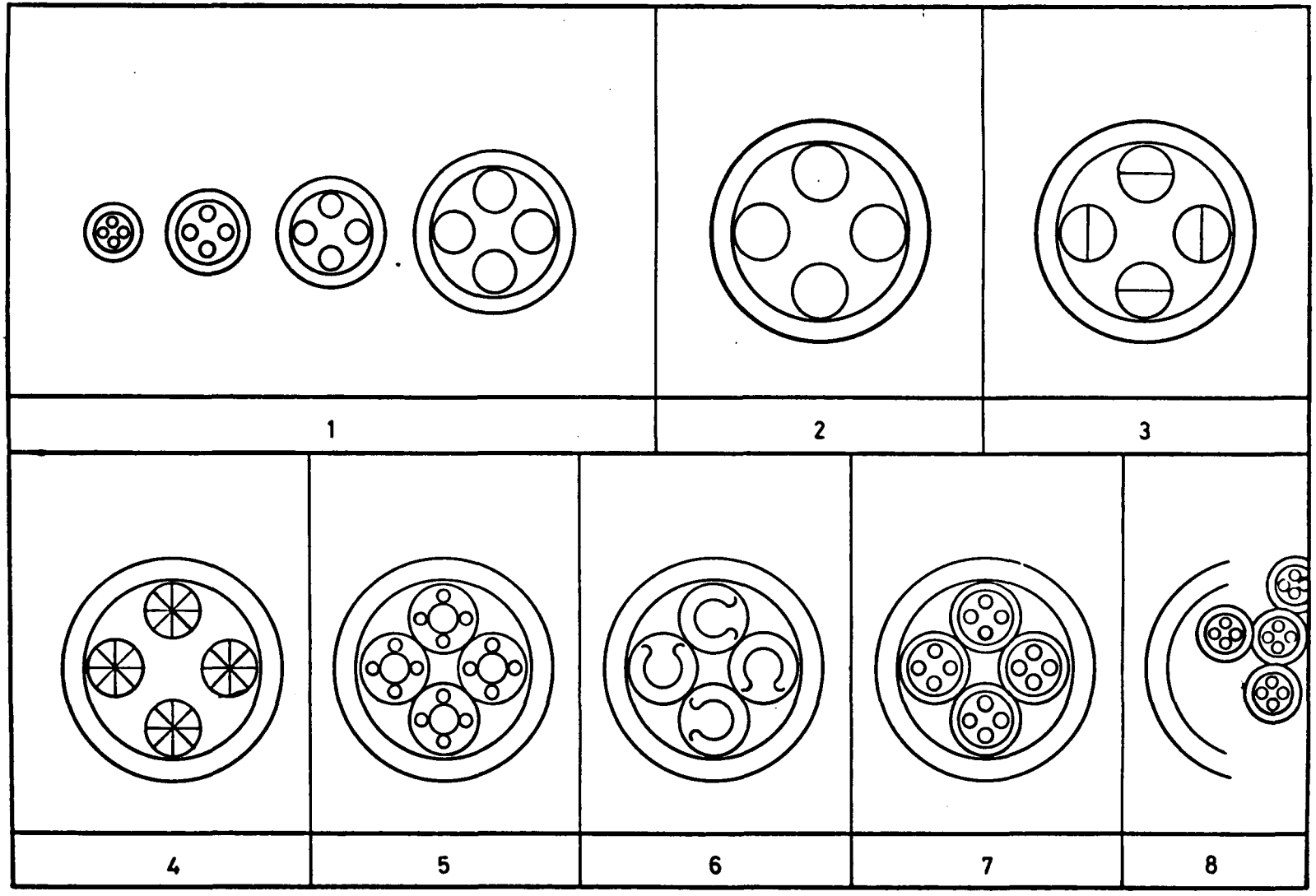

1
2
3
4
5
6
7
8

Figure 2. Microdensitometer tracings of purified DNA from three strains of Volvox carteri centrifuged to equilibrium in analytical CsCl density gradients.

One 10 l culture of each strain was grown and harvested as described for figure 3. Protease (100 ug/ml) mediated spheroid dissociation (30 min for NB-7 and KA-1; 90 min for HK-10) and lysis, and subsequent purification of DNA from the lysate were preformed as described for figure 3A. Aliquots from purified DNA stock solutions (containing 2-3 ug of DNA) were centrifuged to equilibrium in analytical CsCl density gradients containing Micrococcus lysodeikticus DNA (1-2 ug) as described in Materials and Methods (44,000 rpm, 20 hr at 25C in a Beckman An-F rotor). Densities of the DNA species (given in Table 1) were determined by their banding positions relative to those of marker DNAs.

a. Strain HK-10 DNA and M. lysodeikticus marker DNA.

b. Strain NB-7 DNA and marker.

c. Strain KA-1 DNA and marker.

d. Markers, M. lysodeikticus DNA (ρ=1.731 g/cm^3) and C. perfringens DNA (ρ=1.691 g/cm^3).

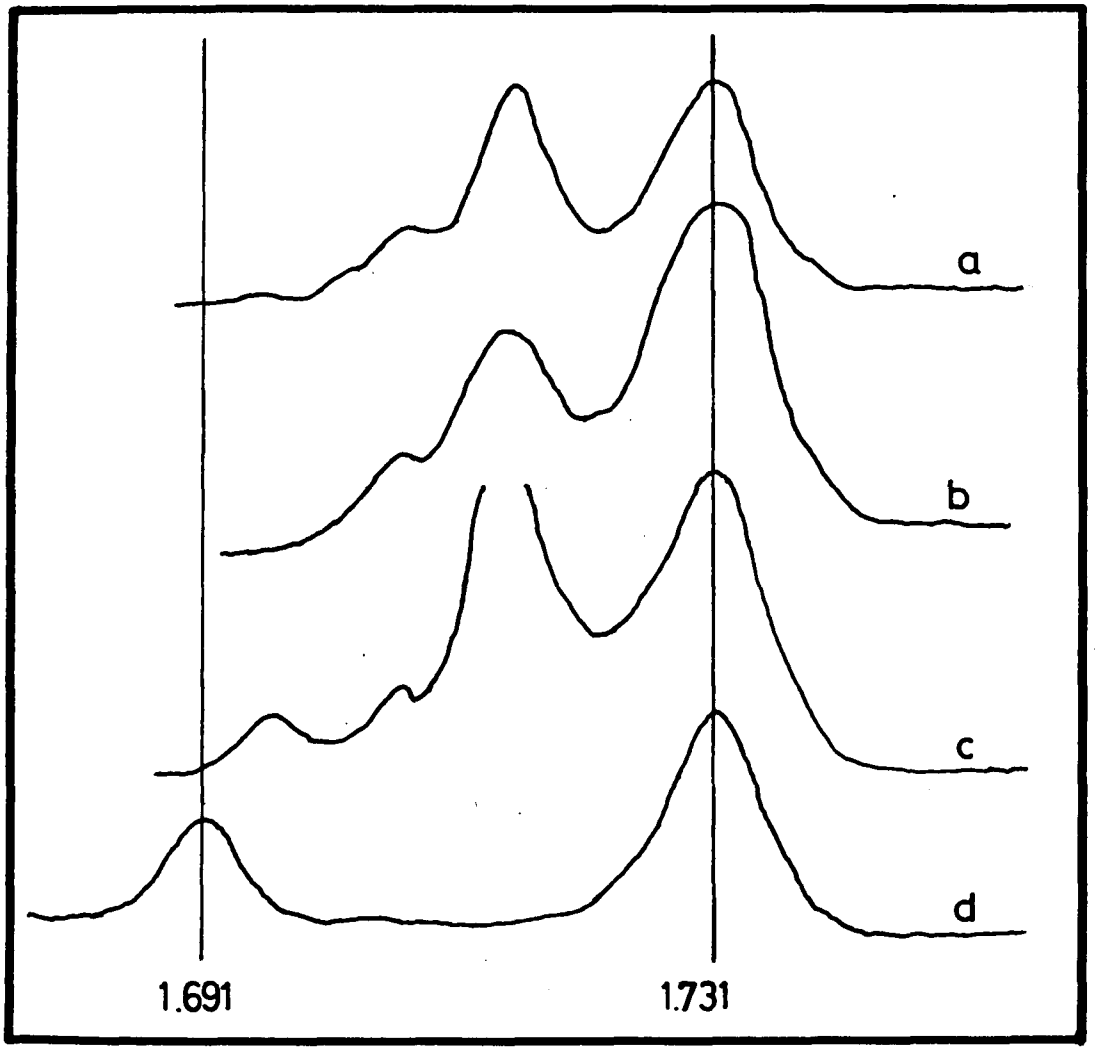

a
b
c
d
1.691
1.731

Figure 3A. Preparative CsCl density gradient centrifugation profile of DNA from Volvox carteri f. nagariensis, strain HK-10.

Two 10 l cultures of V. carteri strain HK-10 were asynchronously grown on a cycle of 16 hr light, 8 hr darkness. Each culture was harvested individually and its DNA extracted as indicated. In each case spheroids were collected by filtration, washed twice with SVM and resuspended in 3 l SVM, pH 7.5 as described in Materials and Methods. The suspensions were incubated with protease VI (100 ug/ml) at 37C for 90 min under constant aeration. Dissociation was monitored by microscopic examination every 10 min after the first 20 min. The cells were harvested by centrifugation, washed twice with saline EDTA and resuspended in 5 ml saline EDTA as described. The suspensions were made 2% with respect to SLS concentration and incubated at 60C for 20 min. DNA was purified from the lysates as described in Materials and Methods. The ethanol precipitates, sequentially extracted as described from both lysates, were combined and resuspended in 5 ml 0.1 x SSC, and salt concentration was adjusted as described. The final purified ethanol precipitate (following deproteinization and RNase treatments described) was dissolved in 2 ml 0.1 x SSC, adjusted to SSC, and stored at -20C. An aliquot (1 ml) of this stock solution of DNA (300 ug/ml) was centrifuged in CsCl as described (cellulose nitrate tube, 48 hr, 19C, 33,000 rpm, 50 Ti rotor). About 30 fractions (approximately 0.18 ml each) were collected from the bottom of the tube and each fraction diluted with water and monitored for UV absorbance at 260 nm as described in Materials and Methods.

Figure 3B. Microdensitometer tracings of HK-10, enriched nuclear and cytoplasmic DNA fractions centrifuged to equilibrium in analytical CsCl.

Aliquots of DNA (1-3 ug), recovered from preparative CsCl fractions indicated in Part A of this figure were centrifuged to equilibrium in analytical CsCl as described in Materials and Methods (44,000 rpm, 20 hr at 25C in a Beckman An-F rotor). Densities of the DNA species were determined by their banding positions relative to those of marker DNAs (see Table 1).

a. Fraction 11 (enriched nuclear DNA) plus marker M. lysodeikticus DNA.

b. Fraction 16 (mixed DNAs) plus marker.

c. Fraction 19 (enriched cytoplasmic DNA) plus marker.

d. Markers, E. coli DNA (ρ=1.710 g/cm^3) and M. lysodeikticus DNA (ρ=1.731 g/cm^3).

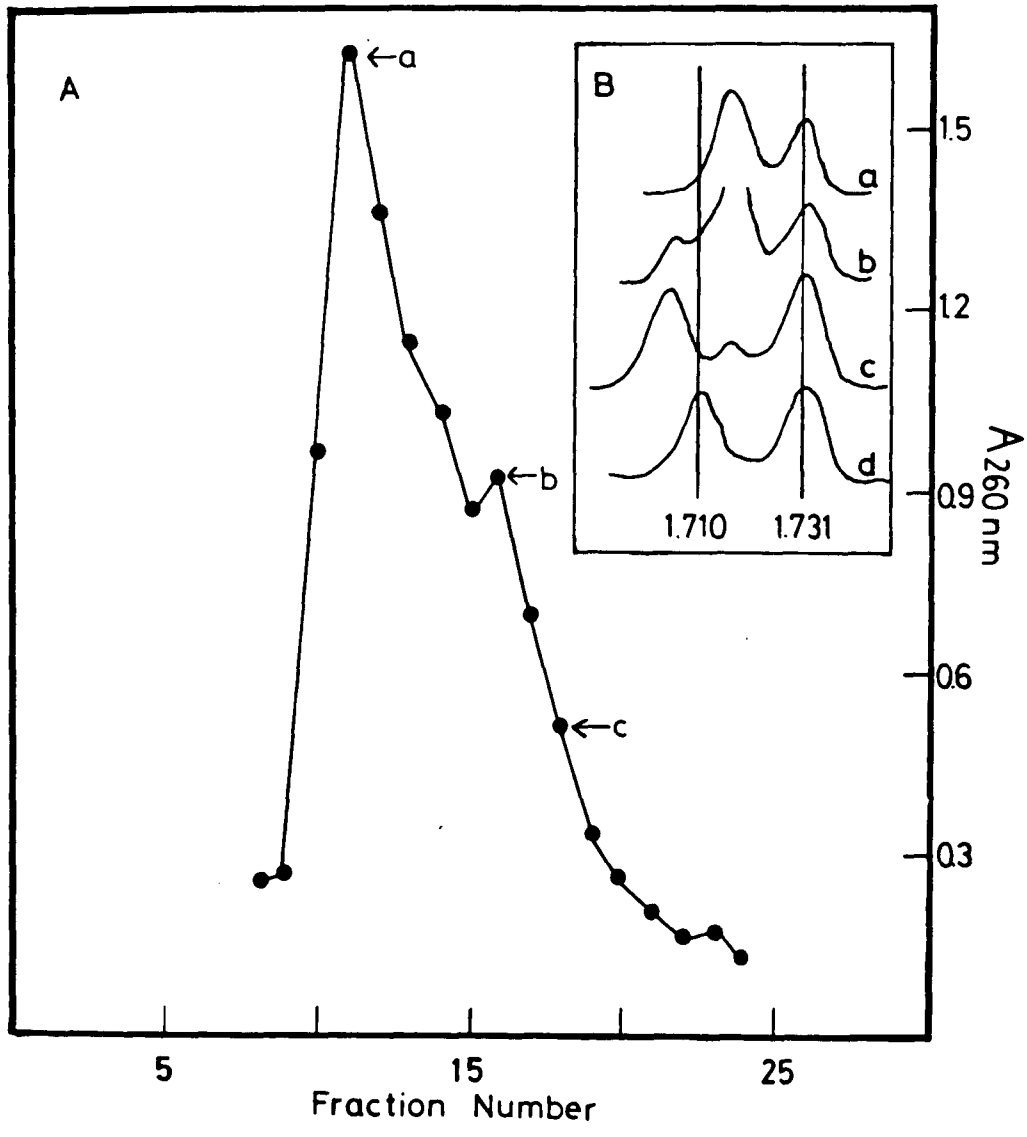

A
←a
←b
←c
B
a
b
c
d
1.710
1.731
1.5
1.2
0.9
0.6
0.3
A260 nm
5
15
25
Fraction Number

Figure 4A. Preparative CsCl density gradient centrifugation profile of DNA from Volvox carteri f. weismannia, strain NB-7.

DNA from Volvox carteri, strain NB-7 was purified and analyzed by preparative and analytical CsCl density gradient centrifugations as described in the legends to figure 3 with the following modifications:

One 10 l culture was grown and harvested as in figure 3. Spheroid dissociation by protease (80 ug/ml for 20 min) was monitored every 5 min and a lysate prepared from washed, resuspended cells as described. The ethanol precipitates, representing sequential extractions of the lysate, were combined, resuspended in 2 ml of 0.1 x SSC, adjusted to SSC, then to 1 M NaCl and deproteinized and further purified as described. After a final ethanol precipitation, the purified DNA was dissolved in 1 ml of 0.1 x SSC, adjusted to SSC and stored at a concentration of 350 ug/ml. An aliquot (0.5 ml) of this DNA stock solution was centrifuged in a preparative CsCl density gradient for 63 hr and analyzed as described.

Figure 4B. Microdensitometer tracings of NB-7 enriched nuclear and cytoplasmic DNA fractions from preparative gradients, centrifuged to equilibrium in analytical CsCl. (See figure legend 3B.)

a. Markers, C. perfringens DNA (ρ=1.691 g/cm^3) and M. lysodeikticus DNA (ρ=1.731 g/cm^3).

b. Fraction 13 (enriched nuclear DNA) plus markers.

c. Fraction 20 (enriched cytoplasmic DNA).

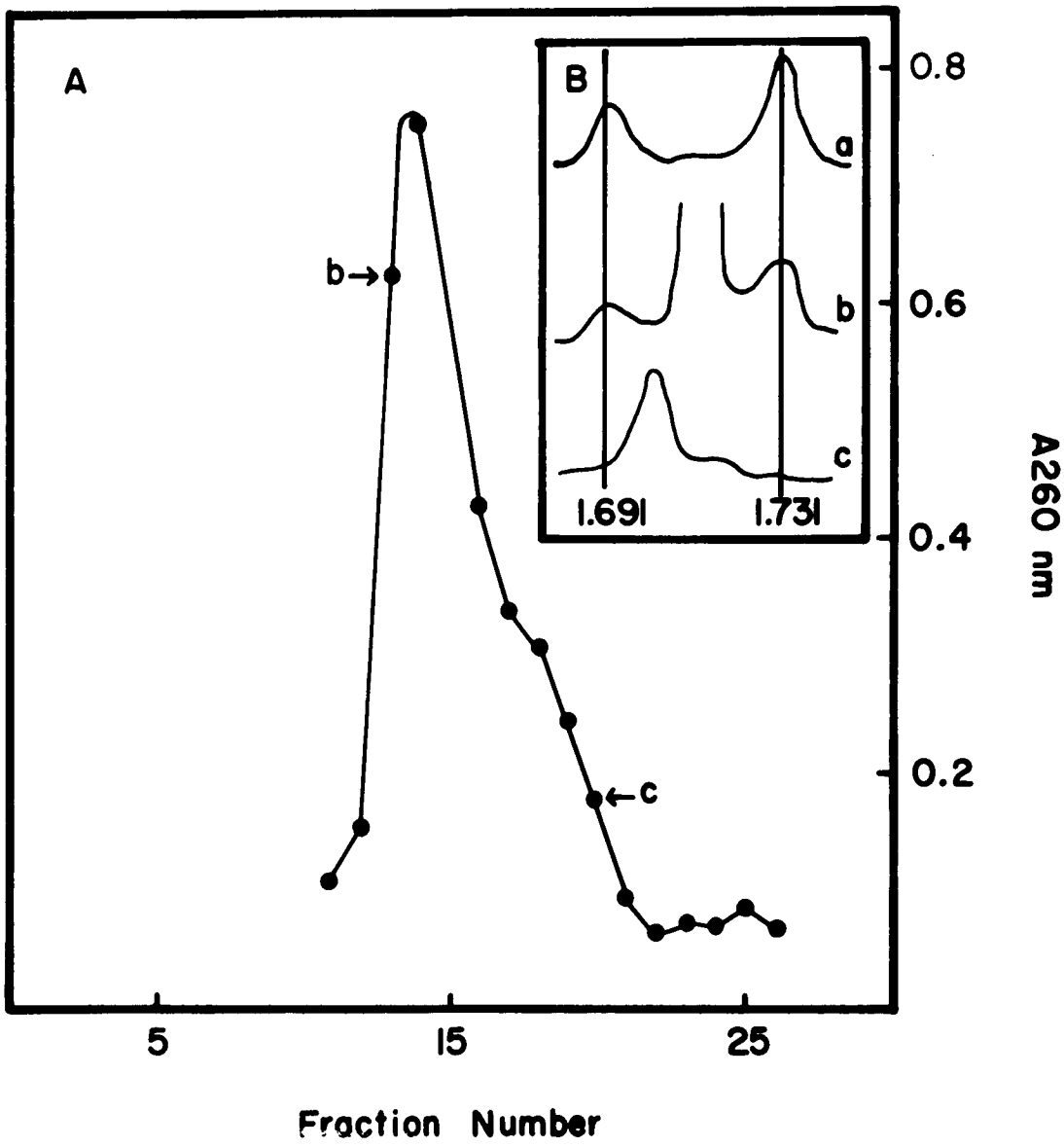

A
B
a
b
c
1.691
1.731
b→
←c
0.8
0.6
0.4
0.2
A260 nm
5
15
25
Fraction Number

Figure 5. Thermal denaturation profiles of enriched nuclear and cytoplasmic DNA samples from strain HK-10 spheroids.

Purified DNA from HK-10 was prepared as described for figure 3A. Enriched cytoplasmic DNA was purified by two rounds of centrifugation in preparative CsCl density gradients. (Fractions such as (c) in figure 3 were combined and recentrifuged. Only the fractions containing the lighter DNA species were used for "enriched cytoplasmic" thermal denaturations.) Enriched nuclear DNA was prepared directly from one preparative CsCl density gradient centrifugation. (Fractions such as (a) in figure 3 were used.) DNA samples were freed of CsCl by repeated dialysis against 0.1 x SSC (3 changes of 1-2 l 0.1 x SSC at 4C for 18 hr total), adjusted to concentrations of between 10 and 25 ug DNA/ml in 0.1 x SSC and heated in a Gilford Model 2400 spectrophotometer as described under Materials and Methods. Solid circles represent values for % hyperchromic increase in absorbance at 260 nm for cytoplasmic DNA and open circles indicate these values for nuclear DNA. Calf thymus DNA, taken to have a Tm of 71.6C in 0.1 x SSC, was denatured simultaneously and all values obtained were corrected accordingly.

a. Enriched cytoplasmic DNA: original A260 = .293 nm; final A260 = .390 nm.

b. Enriched nuclear DNA: original A260 = .472 nm; final A260 = .587 nm.

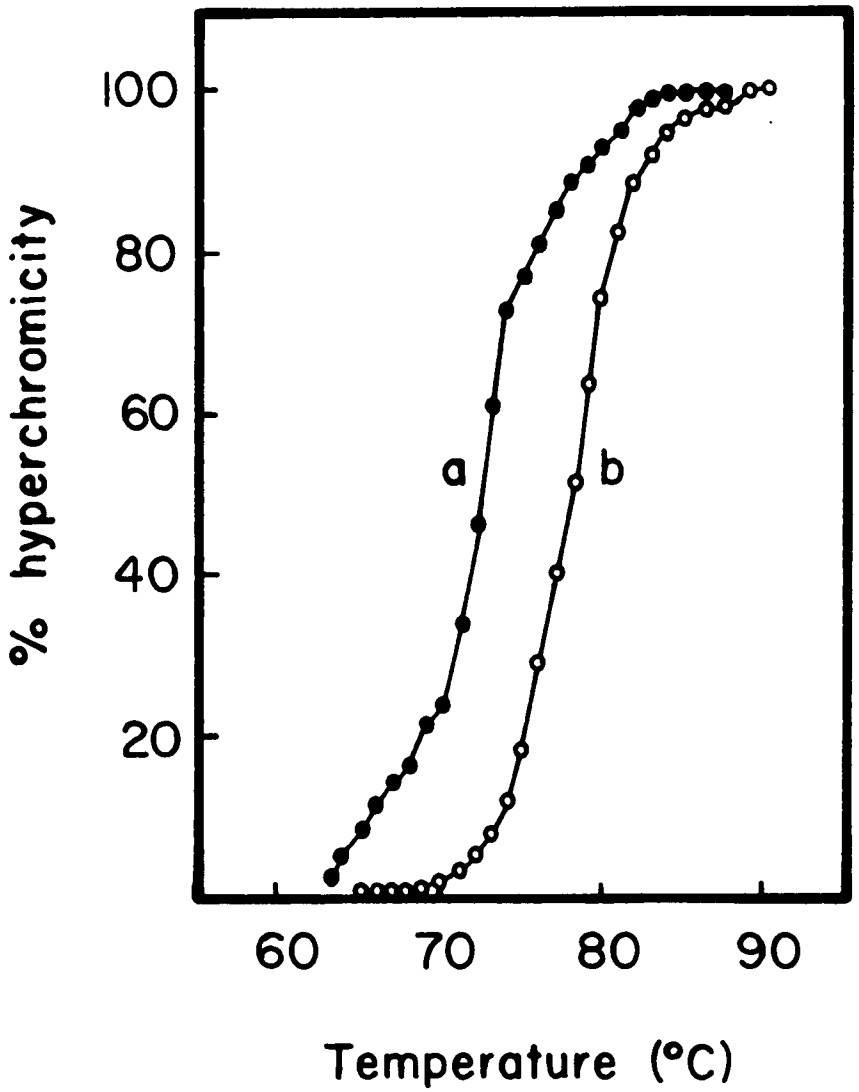

100
80
60
40
20
% hyperchromicity
a
b
60
70
80
90
Temperature (°C)

Figure 6. Thermal denaturation profiles of enriched nuclear and cytoplasmic DNA samples from strain NB-7 spheroids.

Samples of enriched cytoplasmic and enriched nuclear DNA from strain NB-7 were prepared and thermally denatured as described for figure 5. (See figure 4 for NB-7 preparative CsCl density gradient profile showing typical enriched nuclear and cytoplasmic fractions.) Solid circles represent values of % hyperchromic increase in absorbance at 260 nm for enriched cytoplasmic DNA and open circles indicate these values for nuclear DNA.

a. Enriched cytoplasmic DNA: original A260 = 0.228 nm; final A260 = 0.290 nm.

b. Enriched nuclear DNA: original A260 = 0.432 nm; final A260 = 0.583 nm.

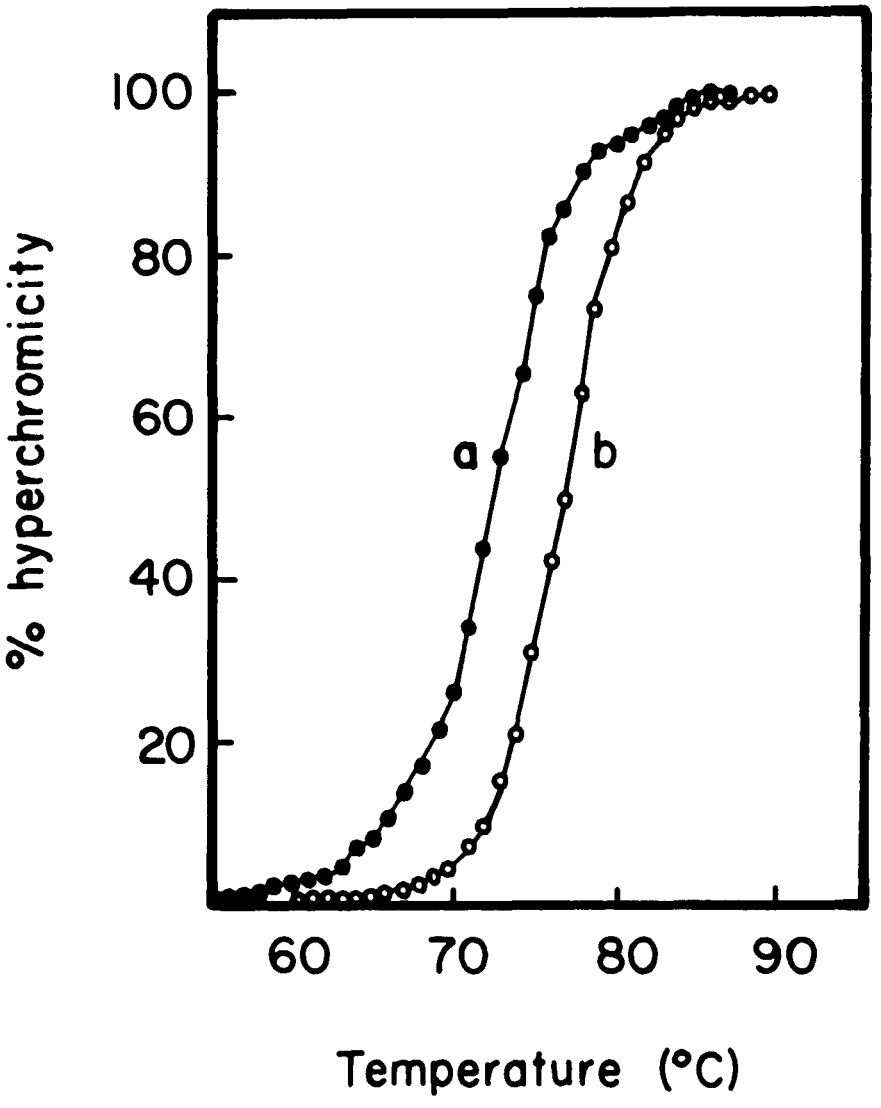

100
80
60
40
20
% hyperchromicity
a
b
60
70
80
90
Temperature (°C)

Figure 7. DNA synthesis in V. carteri f. nagariensis, strain HK-10: Preparative CsCl density gradient profile of DNA from radioactively labeled, asynchronously grown spheroids, dissociated in the presence or absence of tween.

A. Profile of DNA isolated from a culture dissociated in the absence of tween: 2-^{3}H-adenine (20 uCi/ml) was added to a newly innoculated culture (5 ml SVM, pH 7) at time 0 and again after 24 hr. Asynchronous growth was maintained on a 16 hr light/8 hr dark cycle under constant agitation. After 48 hr spheroid dissociation was initiated by the dilution of the culture to 10 ml with SVM (pH 8) and the addition of 1.0 mg protease VI. The culture was then incubated at 37C under constant agitation and dissociation monitored every 10 min as described. After 80 min (when only the largest spheroids had been completely dissociated), cells and spheroids were collected by centrifugation as described and resuspended in 0.5 ml saline EDTA. Further dissociation was achieved by repetitive (10 times) passages of the suspension into and out of a pasteur pipet. The suspension was then brought to 5.5 ml by the addition of saline EDTA, centrifuged, washed (saline EDTA), recentrifuged and the pellet resuspended in 0.7 ml saline EDTA. This suspension was incubated with Sarkosyl (3 drops of a 30% solution) at 60C for 45 min, and the lysate then frozen (-20C) and reheated (60C for 10 min) two times. The entire lysate (plus 20 ul ^{14}C E. coli lysate) was centrifuged as described in a preparative CsCl density gradient (19 C, 63 hr), collected and each fraction monitored for hot alkali stable, cold TCA-precipitable cpm ^{3}H as described in Materials and Methods.

Solid circles represent radioactivity from ^{3}H (Volvox DNA); open circles represent radioactivity from ^{14}C E. coli DNA (ρ=1.710 g/cm^{3}) included in the same gradient as a marker.

B. Profile of DNA isolated from a culture dissociated in the presence of tween: A 5 ml culture of strain HK-10 was innoculated labeled, and grown as described in legend A above. After 48 hr, 0.1 ml tween 40/80 was added and the suspension agitated in the light for 1 hr. The suspension was then diluted, incubated with protease and monitored for dissociation as described in A. After 40 min, when all but newly inverted spheroids were dissociated, the cells were collected by centrifugation and washed twice with saline EDTA as described. Further treatment and analyses were performed as in A, above.

Solid circles represent cpm ^{3}H (Volvox DNA); the vertical bar indicates the banding position of ^{14}C E. coli DNA included in the same gradient as a marker.

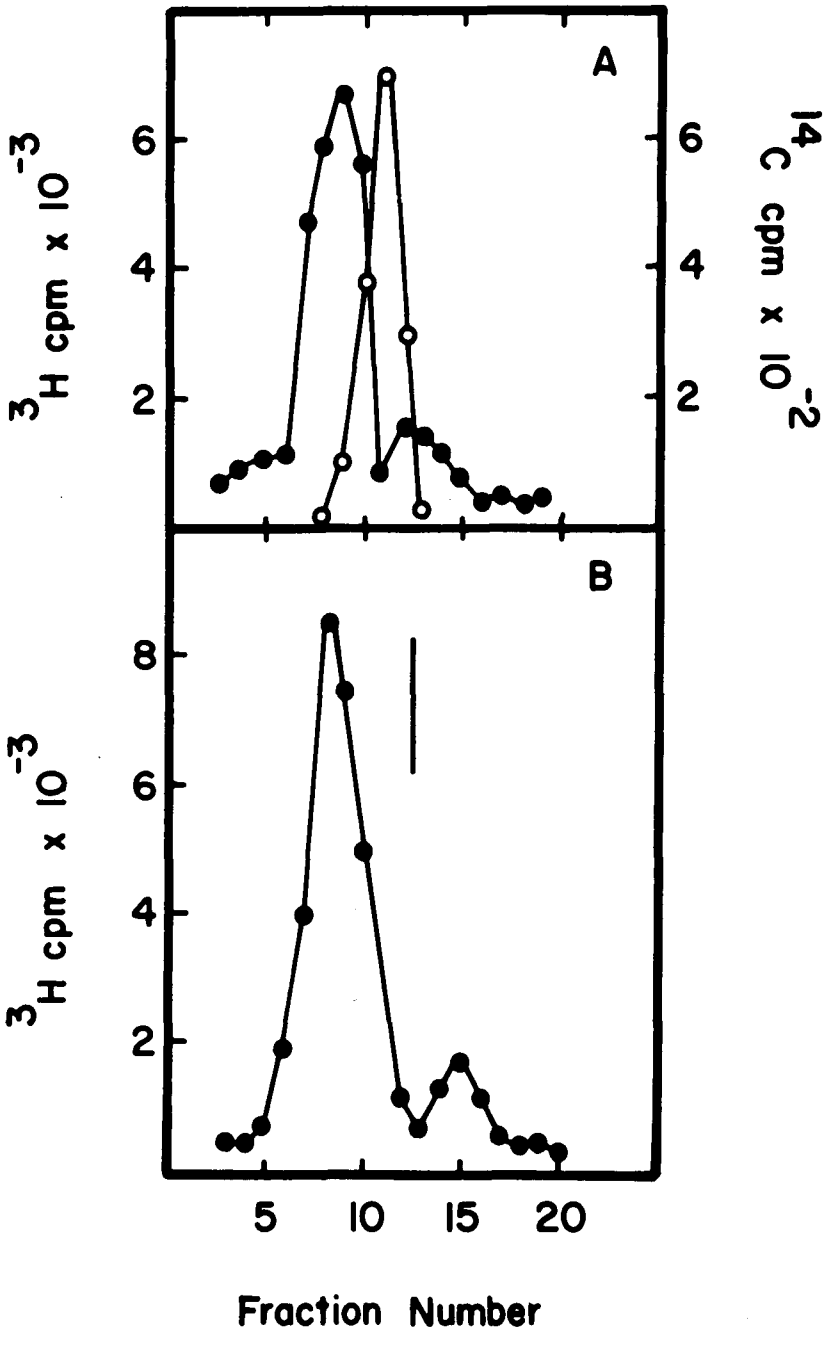

A
B
^{3}H cpm x 10^{-3}
^{14}C cpm x 10^{-2}
2
4
6
8
5
10
15
20
Fraction Number

Figure 8. Comparison between labeling profiles obtained using radioactive adenine and radioactive thymidine precursors. Preparative CsCl density gradient profiles of radioactively labeled DNA from strain HK-10.

Equivalent, newly innoculated 5 ml (SVM, pH 7) asynchronous cultures of HK-10 spheroids were incubated in the presence of 2-^{3}H-adenine or methyl-^{3}H-thymidine under asynchronous growth conditions for 42 hr. In each case radioactive precursor (20 uCi/ml) was added at time 0 and again after 24 hr. Lysates were prepared as described for figure 7B (1 hr tween, 40 min protease) and centrifuged as described (19C, 48 hr). Solid circles represent cpm ^{3}H (Volvox DNA) and vertical bars indicate the banding position of marker ^{14}C *E. coli* DNA included in the gradients.

A. 2-^{3}H-adenine profile

B. Methyl-^{3}H-thymidine profile

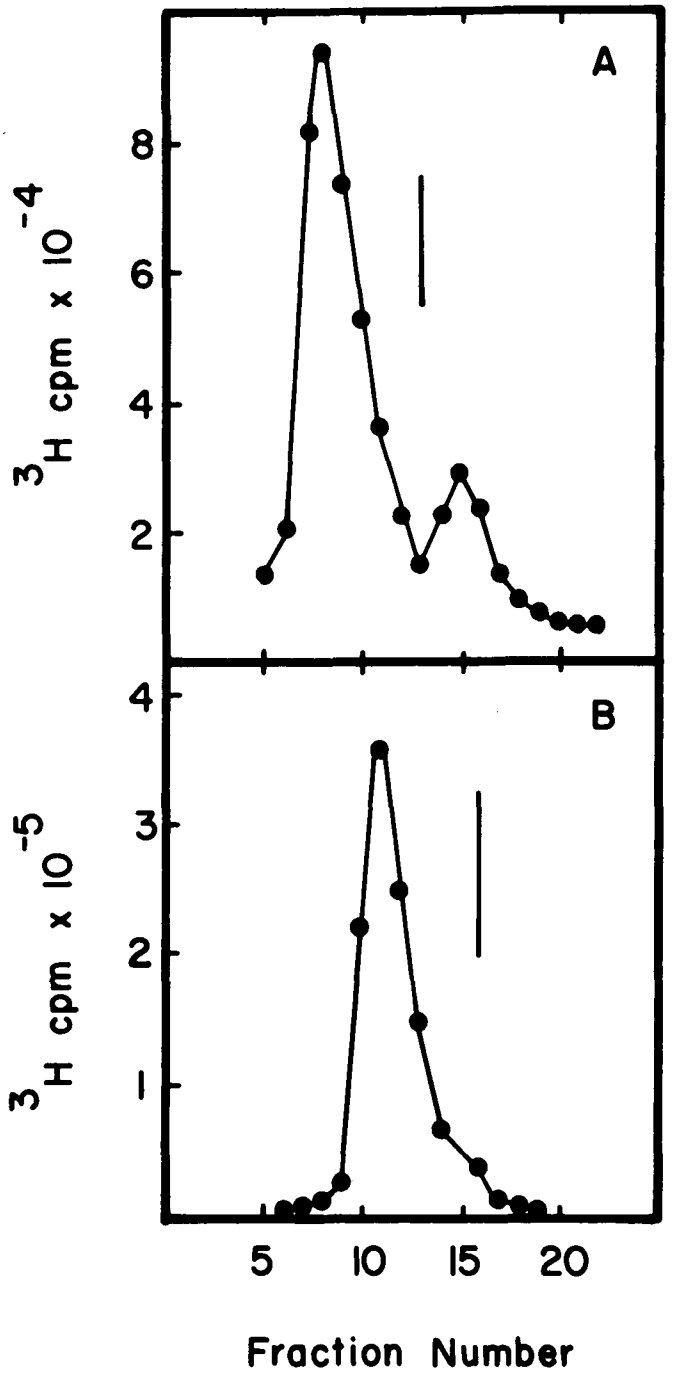

A
B
8
6
4
2
4
3
2
1
^{3}H cpm x 10^{-4}
^{3}H cpm x 10^{-5}
5
10
15
20
Fraction Number

Figure 9. Preparative CsCl density gradient profile of DNA from HK-10 spheroids simultaneously labeled with ^{3}H-thymidine and ^{14}C-adenine.

A newly innoculated 5 ml (SVM, pH 7) asynchronous culture of strain HK-10 was incubated under asynchronous growth conditions described, for 42 hr. Methyl-^{3}H-thymidine (20 uCi/ml) and ^{14}C-adenine (4 uCi/ml) were added to the culture simultaneously at time 0 and again at 24 hr. After 42 hr, a lysate was prepared as described for figure 7B (1 hr tween, 40 min protease dissociation). The sample was centrifuged in a preparative CsCl density gradient as described. (19C, 48 hr). Solid circles represent cpm from ^{3}H and open circles indicate cpm from ^{14}C. (^{3}H cpm indicated were corrected for "overlap.")

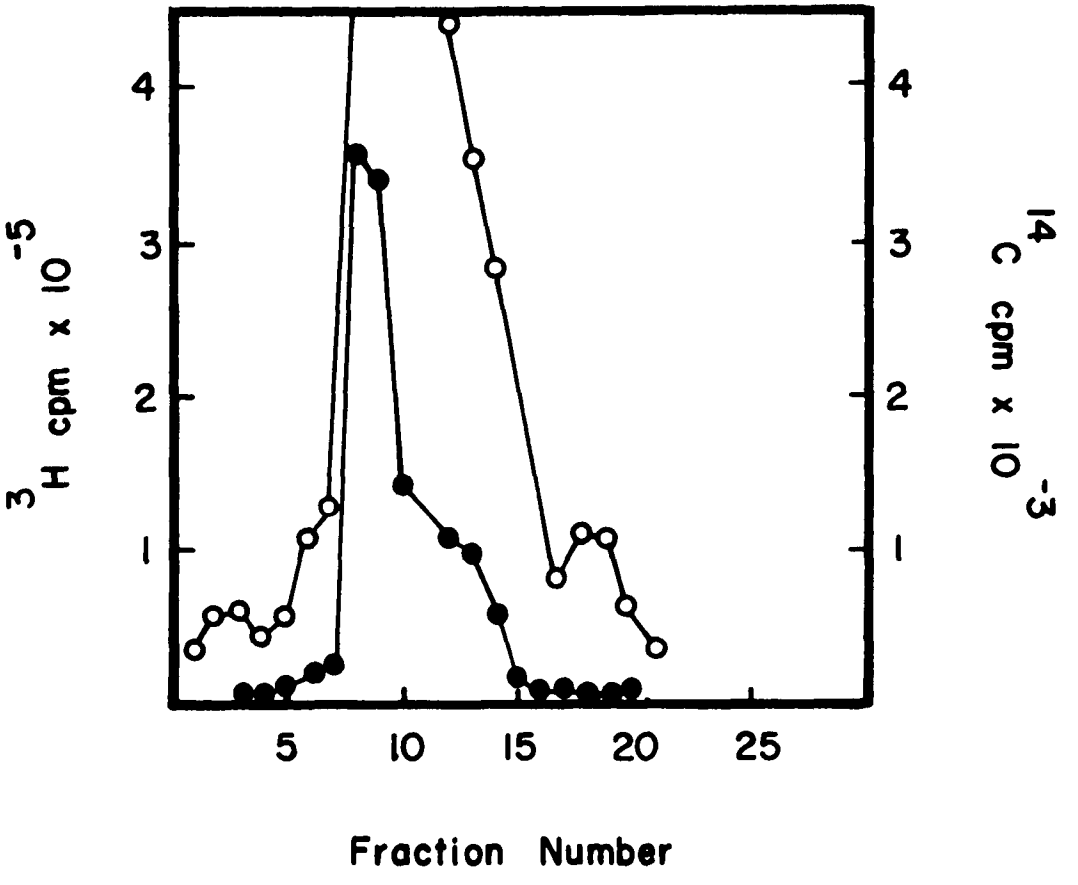

4
3
2
1
^{3}H cpm x 10^{-5}
^{14}C cpm x 10^{-3}
5
10
15
20
25
Fraction Number

Figure 10. DNA synthesis in V. carteri f. weismannia, strain NB-7: Preparative CsCl density gradient profile of DNA from radioactively labeled, asynchronously growth NB-7 spheroids.

A newly innoculated 5 ml (SVM, pH 7) culture of strain NB-7 spheroids was incubated under asynchronous growth conditions described. 2-^{3}H-adenine (20 uCi/ml) was added to the culture at time 0 and again after 48 hr. After 72 hr the culture was dissociated and lysed as described in the legend to figure 7B (30 min tween, 20 min protease). The sample was centrifuged in a preparative CsCl density gradient as described (19C, 48 hr). Solid circles represent cpm ^{3}H (Volvox DNA) and vertical bar indicates the banding position of ^{14}C E. coli DNA included in the same gradient as a marker.

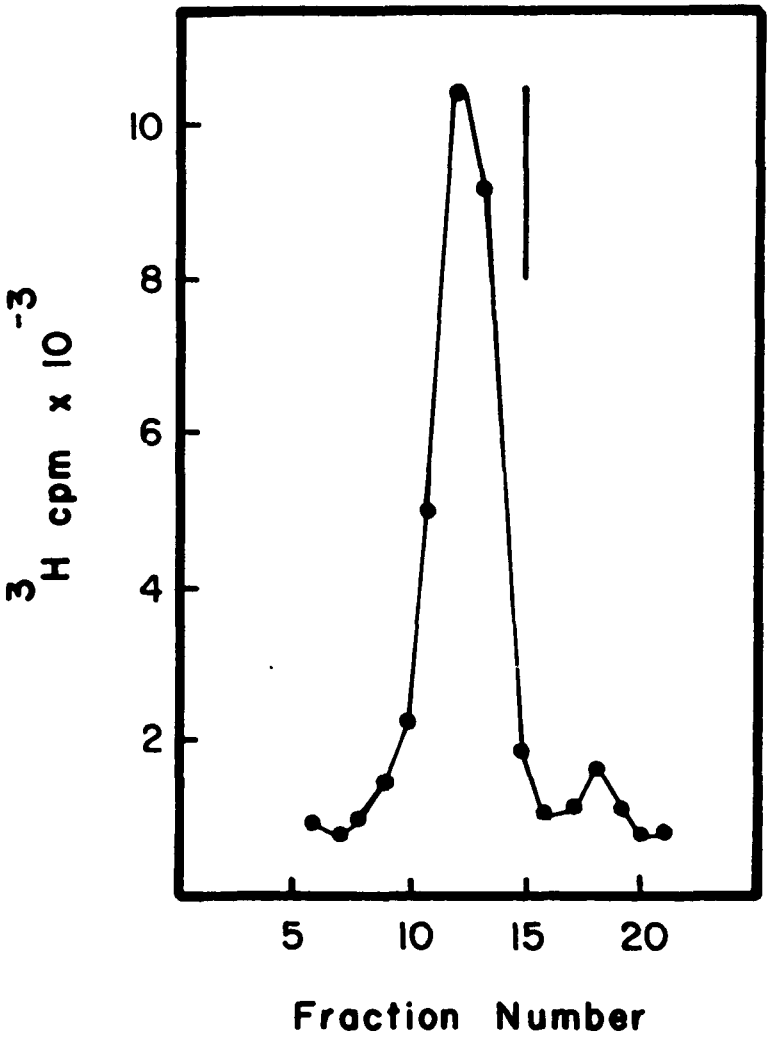

10
8
6
4
2
^{3}H cpm x 10^{-3}
5
10
15
20
Fraction Number

Figure 11. Patterns of nuclear and cytoplasmic DNA synthesis during the asexual life cycle of strain HK-10: Preparative CsCl density gradient profiles of DNA radioactively labeled during various stages of the life cycle in synchronous cultures.

(a), (c), (e) and (g) are preparative CsCl density gradient profiles obtained from lysates of 5 ml cultures removed at time 0 from aerated flasks of spheroids (125 ml SVM, pH 8) induced into (and maintained in) synchronous growth by a 30 hr light, 18 hr dark cycle (aerated) in SVM, pH 8 (SYNC). Populations of spheroids, removed at various stages of the life cycle, were incubated in the presence of 2-^{3}H-adenine (amounts and times specified below) at time 0 under synchronous growth conditions (30 hr light, 18 hr dark) with constant agitation. Lysates were prepared as described in the legend to figure 7A (no tween) and were centrifuged in preparative CsCl density gradients (containing ^{14}C *E. coli* DNA) as described (63 hr, 20C). Profiles (b), (d), (f) and (h) were obtained from lysates of synchronous cultures prepared by the filtration of asynchronously grown spheroid populations through various sizes of Nitex mesh as described and the resuspension of spheroids of similar size in 5 ml SVM, pH 7 at time 0 (NIT). Synchronous cultures thus obtained were incubated in the presence of 2-^{3}H-adenine on a 16 hr light, 8 hr dark cycle under constant aeration. Lysates were prepared as described above and centrifuged in preparative CsCl density gradients containing no marker DNA (63 hr, 20C).

All procedures for culture preparation were carried out under sterile conditions. Solid circles represent cpm ^{3}H (*Volvox* DNA) and vertical bars indicate the banding position of marker ^{14}C *E. coli* DNA.

a. Early expansion (SYNC): Postinversion prerelease spheroids incubated in the presence of 20 uCi/ml 2-^{3}H-adenine for 18 hr.

b. Early expansion (NIT): Prerelease spheroids isolated by vacuum filtration through 60 u mesh as described, incubated in the presence of 20 uCi/ml 2-^{3}H-adenine for 20 hr.

c. Late expansion (SYNC): Newly releases spheroids incubated in the presence of 40 uCi/ml ^{3}H-adenine for 10 hr.

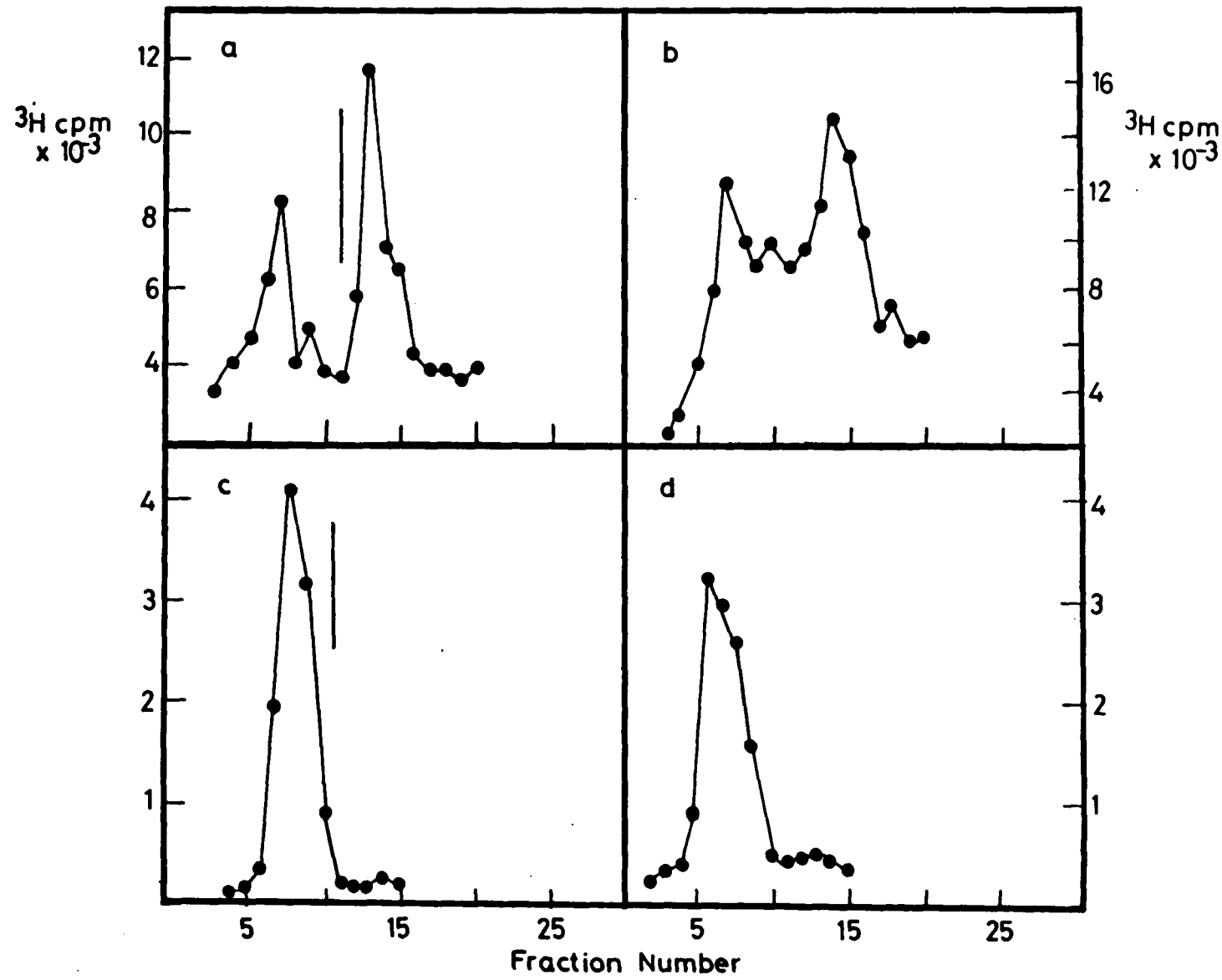
d. Late expansion (NIT): Small newly released spheroids, obtained by filtration (no vacuum) through 100 u mesh Nitex as described, incubated in the presence of 40 uCi/ml 2-^{3}H-adenine for 16 hr.

e. Cleavage (SYNC): Spheroids with gonidia about to cleave or beginning states of cleavage incubated in the presence of 40 uCi/ml 2-^{3}H-adenine for 16 hr.

f. Cleavage (NIT): "*In vitro* gonidia" formed by vacuum filtration disruption of spheroids as described incubated in the presence of 20 uCi/ml 2-^{3}H-adenine for 18 hr. (At the time of lysis most spheroids were in the preinversion or inversion stages.)

g. Total expansion (SYNC): Postinversion, prerelease spheroids incubated in the presence of 20 uCi/ml 2-^{3}H-adenine for 30 hr. (During this time the spheroids had undergone release and expansion.)

h. Total expansion (NIT): Isolated postinversion, prerelease spheroids, obtained as described in b, incubated in the presence of 20 uCi/ml 2-^{3}H-adenine for 50 hr.


a
b
c
d
^{3}H cpm x 10^{-3}
^{3}H cpm x 10^{-3}
12
10
8
6
4
16
12
8
4
4
3
2
1
4
3
2
1
5
15
25
5
15
25
Fraction Number

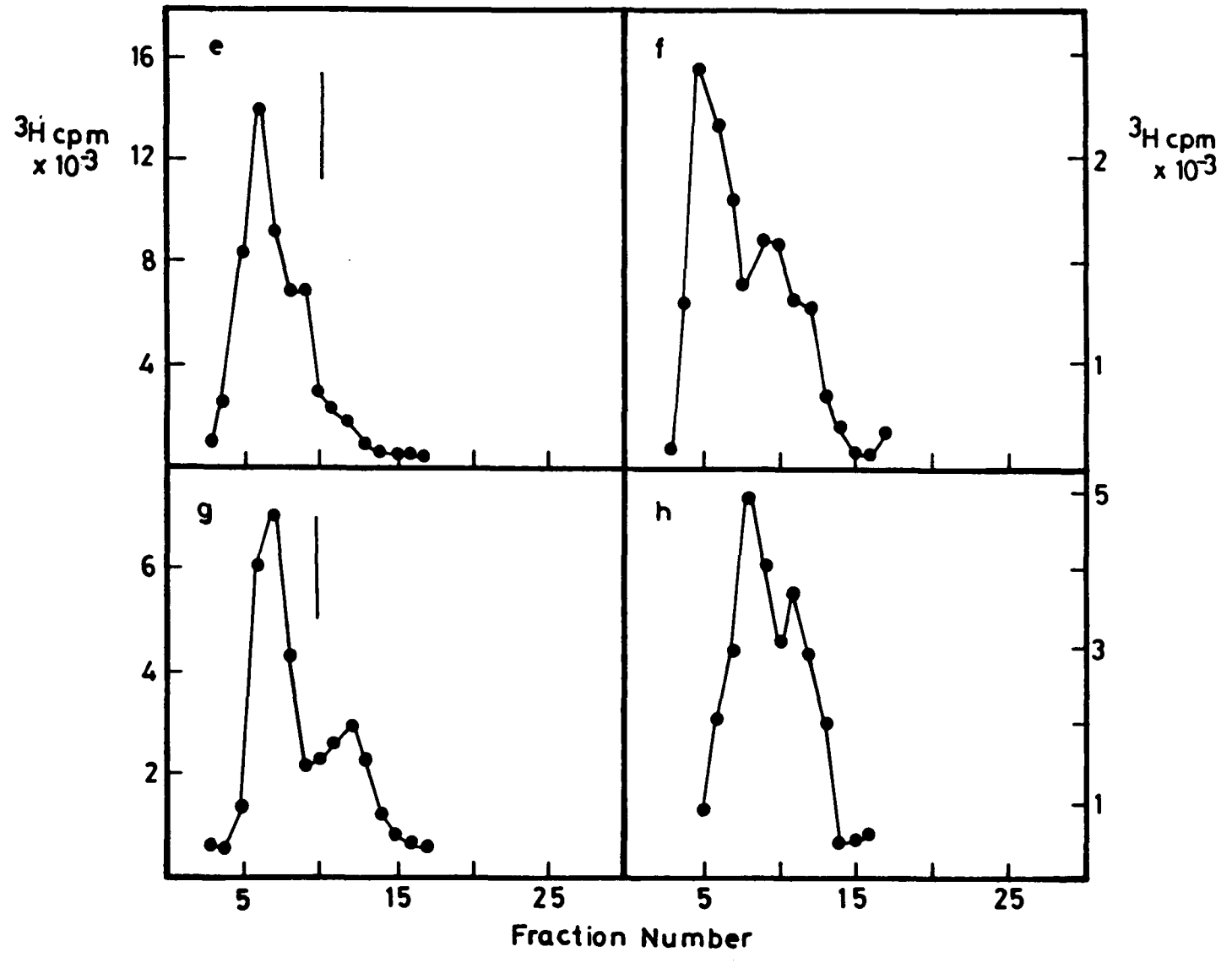

e
f
g
h
^{3}H cpm x 10^{-3}
^{3}H cpm x 10^{-3}
16
12
8
4
2
6
5
3
1
5
15
25
Fraction Number

Figure 12. Diagrammatic representation of three stages in the asexual life cycle of Volvox carteri.

For detailed explanation, see text.

Stage 1: Total expansion. (Includes stages 7, 8, and 1 in figure 1.)

Stage 2: Gonidial "condensation." (Stage 2 in figure 1.)

Stage 3: Cleavage (includes stages 3 and 4 in figure 1.)

The bars (A, B, C, D, E) represent various time zones during which experiments were conducted.

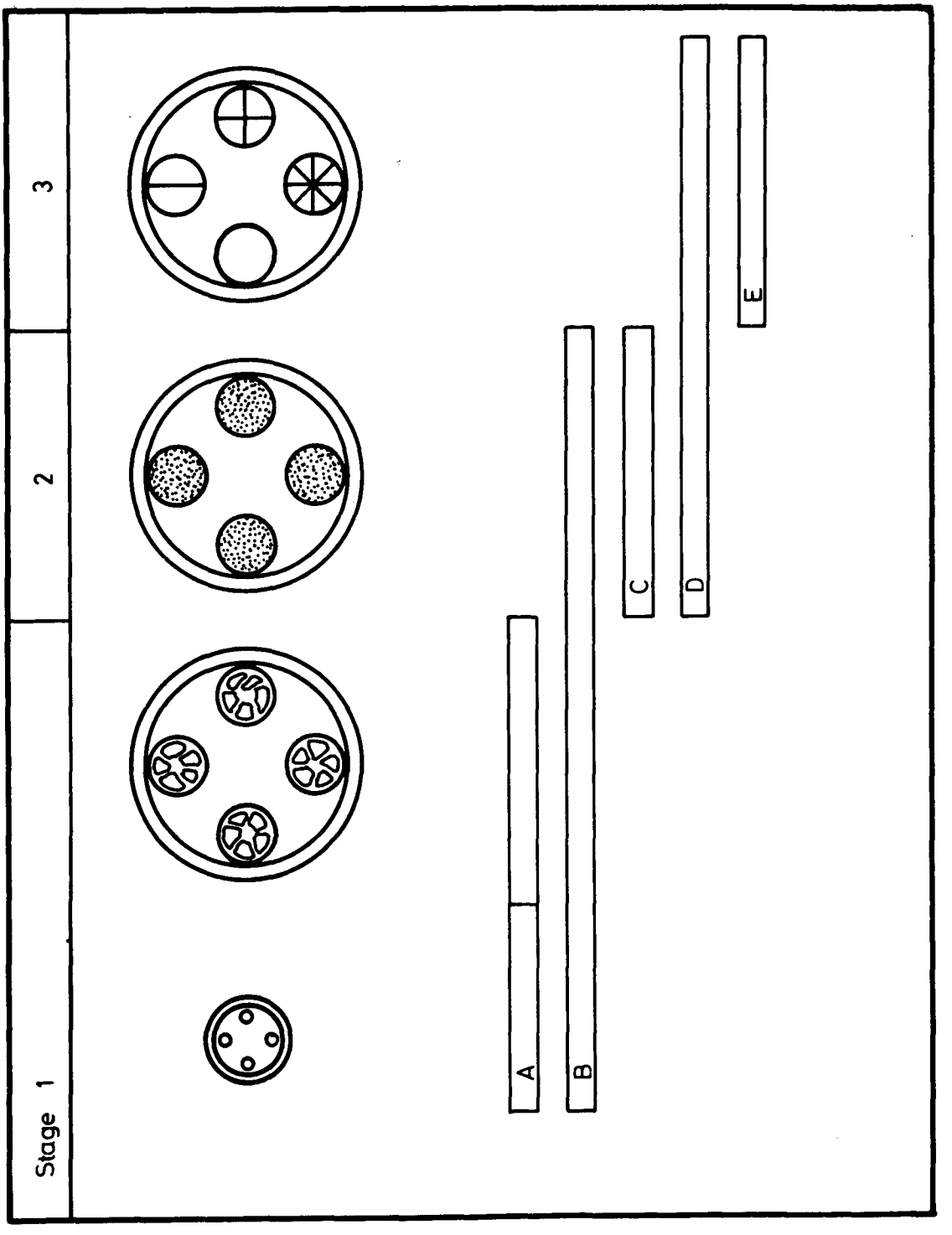

Stage 1
2
3
A
B
C
D
E

Figure 13. DNA synthesis in the presence and absence of Δ9 THC or carrier: Preparative CsCl density profiles of DNA from radioactively labeled HK-10 spheroids.

Triplicate, newly innoculated asynchronous 5 ml cultures of strain HK-10 (SVM, pH 7) were incubated in the presence of 2-^{3}H-adenine (20 uCi/ml) alone, or 20 uCi/ml 2-^{3}H-adenine and carrier (40 ul/ml) or THC (400 ug/ml) for 18 hr under asynchronous growth conditions as described for figure 7. Lysates were prepared as described below, centrifuged in preparative CsCl density gradients (19C, 64 hr) as described. Solid circles represent cpm ^{3}H (Volvox DNA) and vertical bars indicate the banding position of ^{14}C E. coli DNA (10 ul lysate) included in the gradients as markers.

A. Control (no carrier or THC): Radioisotope was added at time 0, and after 18 hr the spheroids were dissociated (1 hr, 0.1 ml tween; 30 min protease) and lysed (60C, 40 min) as described for figure 7B.

B. Carrier Control: Carrier and radioisotope were simultaneously added at time 0, and after 18 hr the spheroids were dissociated (no tween, 20 min protease) and lysed as described in A above.

C. THC Experimental: THC and radioisotope were simultaneously added at time 0 and after 18 hr the spheroids were dissociated and lysed as in B above.

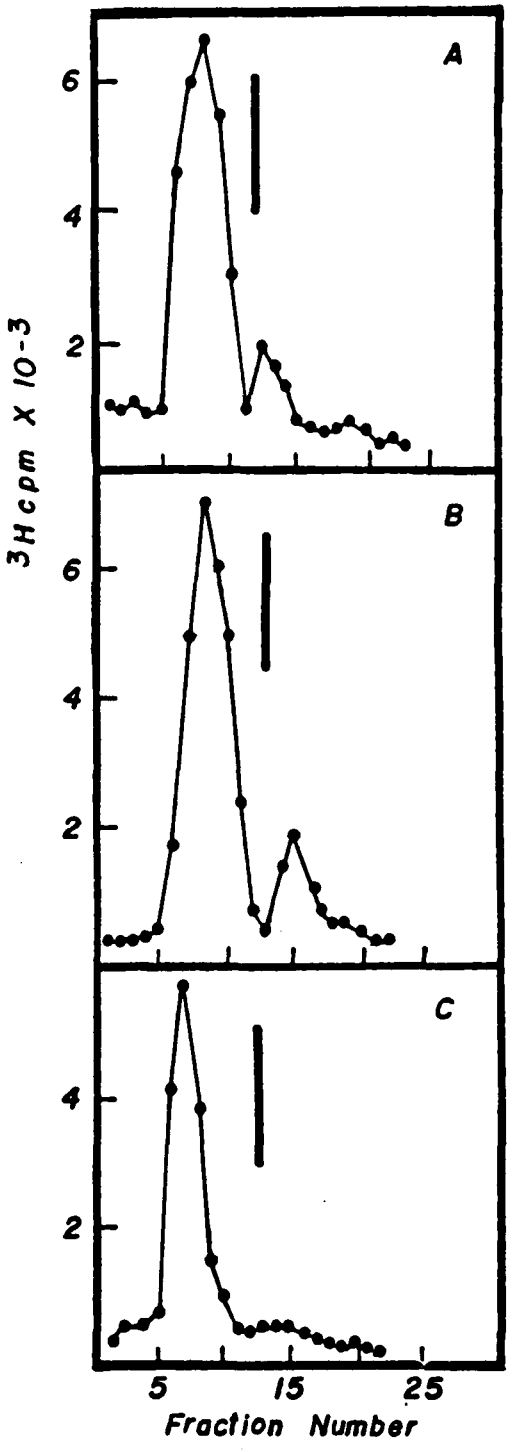

A
B
C
6
4
2
6
4
2
4
2
3H cpm X 10-3
5
15
25
Fraction Number

Figure 14. Uptake of 2-^{3}H-adenine by HK-10 spheroids in the presence and absence of Δ^{9} THC or carrier; media depletion analysis.

Triplicate cultures containing 400 asynchronous spheroids/ml in 7.5 ml SVM (pH 7), were incubated with 15 uCi/ml 2-^{3}H-adenine in the presence or absence of THC (300 uCi/ml) or carrier (30 ul/ml) for 25 hr under asynchronous growth conditions. Three 20 ul samples of supernatant growth media, withdrawn from each culture at time intervals indicated (as described under Materials and Methods), were assayed for radioactivity as described. % uptake values represent the ratio of the average of cpms at time 0 to those at time intervals indicated.

a. Control: 2-^{3}H-adenine added at time 0. (Solid circles.)

b. Carrier Control: 2-^{3}H-adenine and carrier added simultaneously at time 0. (Open circles.)

c. THC Experimental: 2-^{3}H-adenine and THC added simultaneously at time 0. (Triangles.)

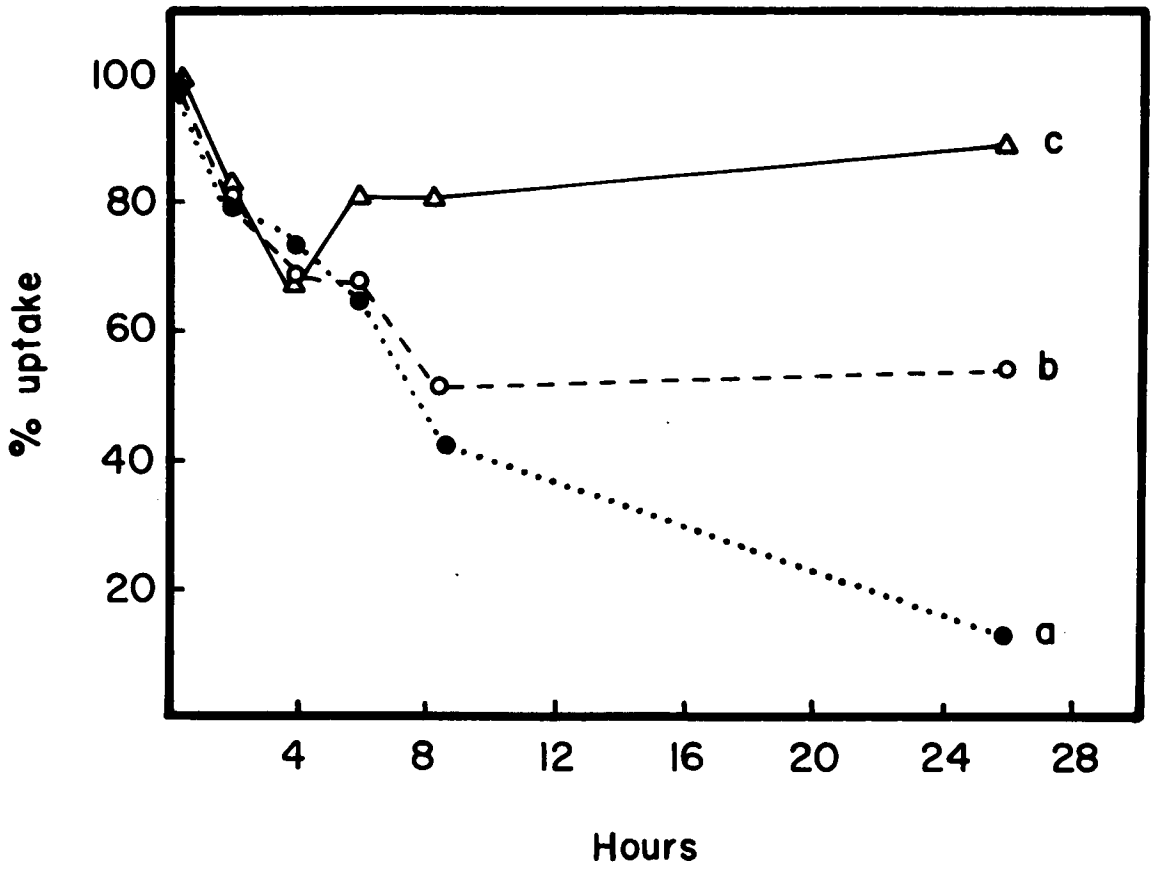

100
80
60
40
20
% uptake
4
8
12
16
20
24
28
Hours
a
b
c

Figure 15. The effect of different concentrations of Δ^9 THC on DNA synthesis: Preparative CsCl density gradient profiles of DNA from radioactively labeled HK-10 spheroids.

Four equivalent newly innoculated 5 ml cultures (SVM, pH 7) of strain HK-10 (enriched with postinversion, prerelease spheroids as described in text) were incubated in the presence of 2-^{3}H-adenine (20 uCi/ml), and carrier or various concentrations of THC for 19 hr under asynchronous conditions (16 hr light/8 hr dark, constant agitation). Lysates were prepared as described for figure 13 and centrifuged in preparative CsCl density gradients (19C, 48 hr). Solid circles represent cpm $^3H_{14}C$ (Volvox DNA) and vertical bars indicate banding position of marker ^{14}C *E. coli* DNA included in gradients.

A. Carrier Control: Radioisotope and carrier (40 ul/ml) were added simultaneously at time 0.

B. 100 ug/ml THC: Radioisotope and THC were added at time 0.

C. 200 ug/ml THC: Radioisotope and THC were added at time 0.

D. 400 ug/ml THC: Radioisotope and THC were added at time 0.

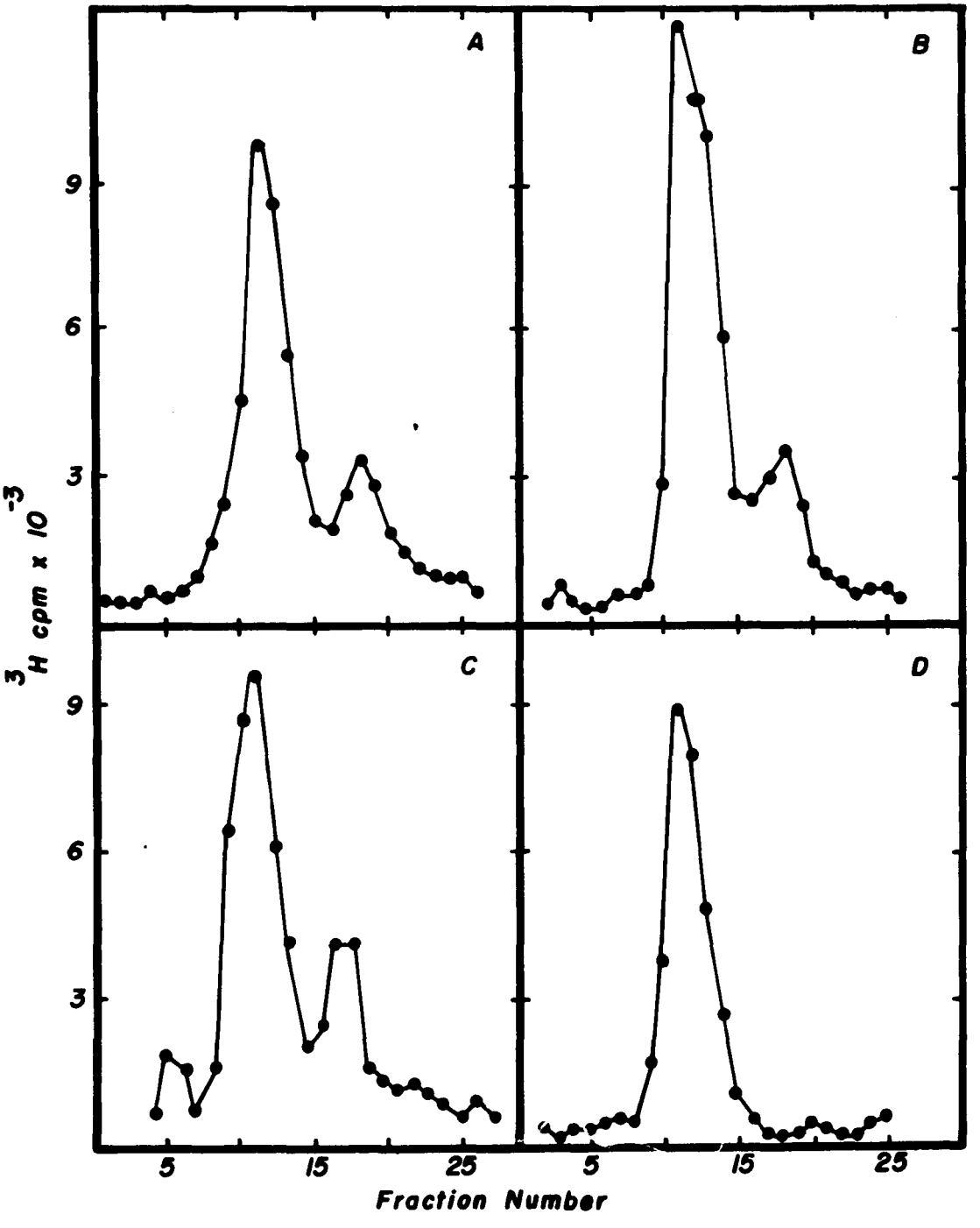

A
B
C
D
9
6
3
9
6
3
^3H cpm x 10^{-3}
5
15
25
5
15
25
Fraction Number

Figure 16. The effect of Δ^9 THC on the patterns of nuclear and cytoplasmic DNA synthesis occurring during the asexual life cycle of strain HK-10: Preparative CsCl density gradient profiles of DNA radioactively labeled during various stages of the life cycle, in synchronous cultures, in the presence of THC or carrier.

Equivalent 5 ml samples were axenically removed from aerated 1 l flasks containing spheroids (in 500 ml SVM, pH 8), maintained under synchronous growth conditions as described. (At each stage indicated below, triplicate cultures were removed from the same sample flask; profiles of controls, receiving radioactivity alone, are not presented in this figure but resemble those of the carrier controls presented.) Radioactivity and carrier or THC were added simultaneously at time 0 and the cultures incubated under synchronous growth conditions for the times specified below. Lysates were prepared as described for figure 13 ((a) and (b) 40 min protease, (c) and (d) 30 min protease, (e) and (f) 60 min protease) and centrifuged in CsCl density gradients as described (48 hr, 19C). Solid circles represent cpm ^{3}H (*Volvox* DNA) and vertical bars indicate banding position of marker ^{14}C *E. coli* DNA included in gradients.

a. Early expansion in the presence of carrier: Newly inverted spheroids were incubated in the presence of 2-^{3}H-adenine (20 uCi/ml) and carrier (40 ul/ml). Lysates were prepared after 17 hr when 20% of the spheroids had released.

b. Early expansion in the presence of THC: Newly inverted spheroids were incubated in the presence of 2-^{3}H-adenine (20 uCi/ml) and THC (400 ug/ml). Lysates were prepared after 17 hr when 20% of the spheroids had released.

c. Late expansion in the presence of carrier: Newly released spheroids were incubated in the presence of 2-^{3}H-adenine (20 uCi/ml) and carrier (40 ul/ml). Lysates were prepared after 18 hr when most spheroids were in the condensation stage and 20% in early cleavage.

d. Late expansion in the presence of THC: Newly released spheroids were incubated in the presence of 2-^{3}H-adenine (20 uCi/ml) and THC (400 ug/ml). Lysates were prepared after 18 hr when most spheroids were in the condensation stage and 20% in early cleavage.

e. Cleavage in the presence of carrier: Spheroids in the condensation stage were incubated in the presence of 2-^{3}H-adenine (40 ul/ml) and carrier (40 ul/ml). Lysates were prepared after 13 hr when 90% of the spheroids were in the postcleavage, preinversion form and 10% had inverted.

f. Cleavage in the presence of THC: Spheroids in the condensation stage were incubated in the presence of 2-^{3}H-adenine (40 ul/ml) and THC (400 ug/ml). Lysates were prepared after 13 hr when 90% of the spheroids were in the postcleavage, preinversion form and 10% had inverted. At the time of lysis (after 13 hr) most gonidia appeared to have been arrested in early cleavage.

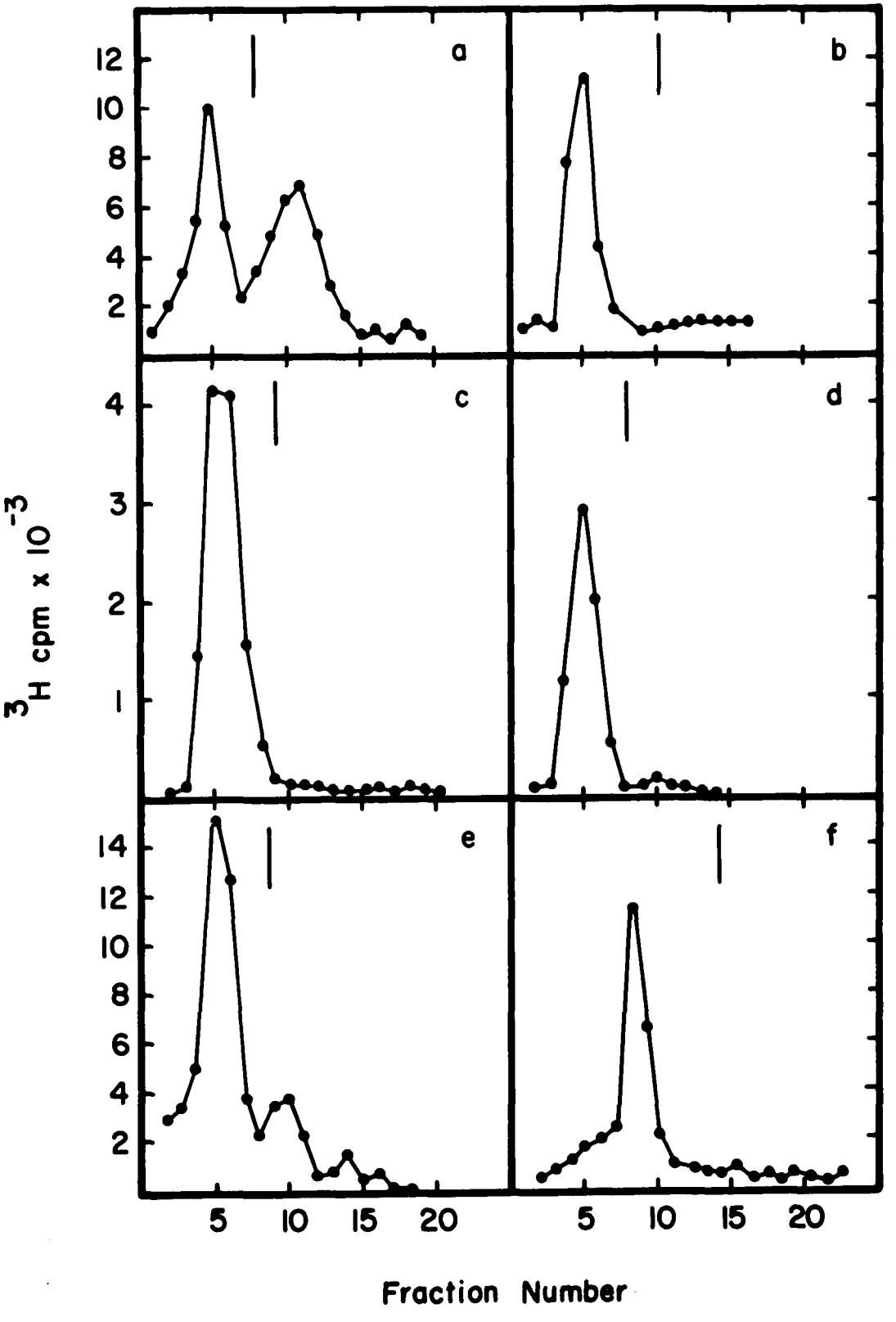

a
b
c
d
e
f
^{3}H cpm x 10^{-3}
Fraction Number
5
10
15
20

Figure 17. Degradation of prelabeled cytoplasmic DNA by Δ^9 THC: Preparative CsCl density gradient profiles of DNA from HK-10 spheroids, radioactively labeled with 2-^{3}H-adenine and subsequently treated with THC or carrier.

A newly innoculated 5 ml (SVM, pH 7) culture of strain HK-10 was incubated under asynchronous conditions (16 hr light, 8 hr dark; agitation) in the presence of 2-^{3}H-adenine (20 uCi/ml). After 48 hr, 2.5 ml aliquots of the culture were steriley transferred to new tubes containing 2.5 ml SVM, pH 7 and additional 2-^{3}H-adenine (10 uCi/ml) was added to each tube. After incubating for 48 hr, most media was withdrawn from each culture and the spheroids resuspended in a final volume of 10 ml (SVM, pH 7). After the addition of carrier (30 ul/ml) or THC (300 ug/ml) the cultures were incubated for 18 more hr. Following dissociation and lysis, as described for figure 13, the material was centrifuged in a CsCl density gradient (19C, 46 hr), as described. Solid circles represent cpm ^{3}H, ^{14}C (Volvox DNA) and vertical bars indicate banding position of marker ^{14}C E. coli DNA.

A. Carrier Control: 30 ul/ml carrier treatment for 18 hr after labeling for 96 hr.

B. THC experimental: 300 ug/ml THC treatment for 18 hr after labeling for 96 hr.

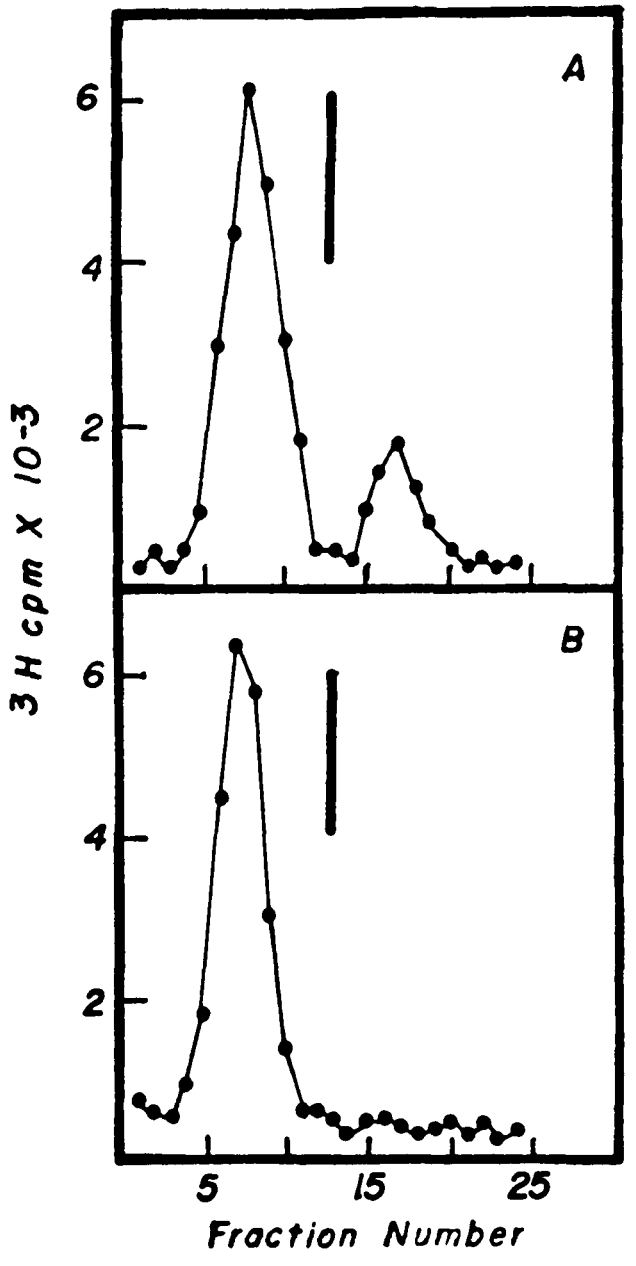

A
B
6
4
2
6
4
2
3H cpm X 10-3
5
15
25
Fraction Number

Figure 18. Degradation of prelabeled cytoplasmic DNA by ethidium bromide (EB): Preparative CsCl density gradient profiles of DNA from HK-10 spheroids radioactively labeled with 2-^{3}H-adenine for 24 hr and subsequently treated with EB.

Two 5 ml cultures of HK-10 spheroids, prelabeled with 2-^{3}H-adenine were prepared as described for figure 17. Following the dilution of media, as described (10 ml SVM, pH 7), one tube was innoculated with EB (25 ug/ml). The two cultures were then incubated for 24 hr and their lysates prepared and centrifuged in preparative CsCl density gradients (19C, 47 hr) as described. Solid circles represent cpm ^{3}H (Volvox DNA) and vertical bars indicate the banding position of marker ^{14}C E. coli DNA.

A. Control: Diluted culture incubated for 18 hr after labeling for 96 hr.

B. EB Experimental: Diluted culture incubated with 25 ug/ml EB for 18 hr after labeling for 96 hr.

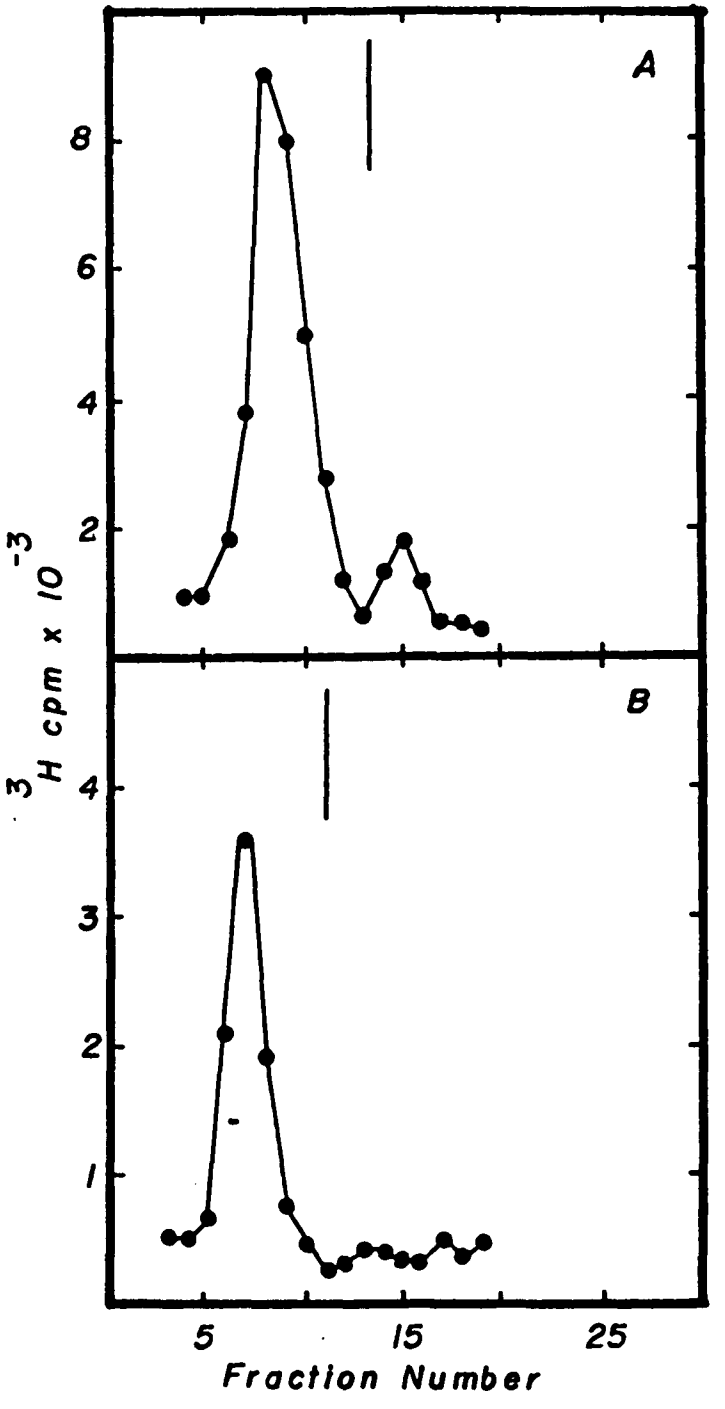

A
B
8
6
4
2
4
3
2
1
^3H cpm x 10^{-3}
5
15
25
Fraction Number

Figure 19. The effect of *in vivo* ethidium bromide treatment on existing cytoplasmic DNA: Microdensitometer tracings of purified DNA from HK-10 spheroids treated with ethidium bromide and centrifuged to equilibrium in analytical CsCl density gradients.

Purified DNA (obtained from 10 l cultures of HK-10 spheroids) was prepared for and centrifuged to equilibrium in analytical CsCl density gradients (containing *M. lysodeikticus* marker DNA) as described for figure 2.

a. Markers, *E. coli* DNA (p=1.710) and *M. lysodeikticus* DNA (p=1.731).

b. DNA from untreated HK-10 spheroids plus marker.

c. DNA from HK-10 spheroids incubated in the presence of 25 ug/ml EB for 20 hr prior to harvesting plus marker.

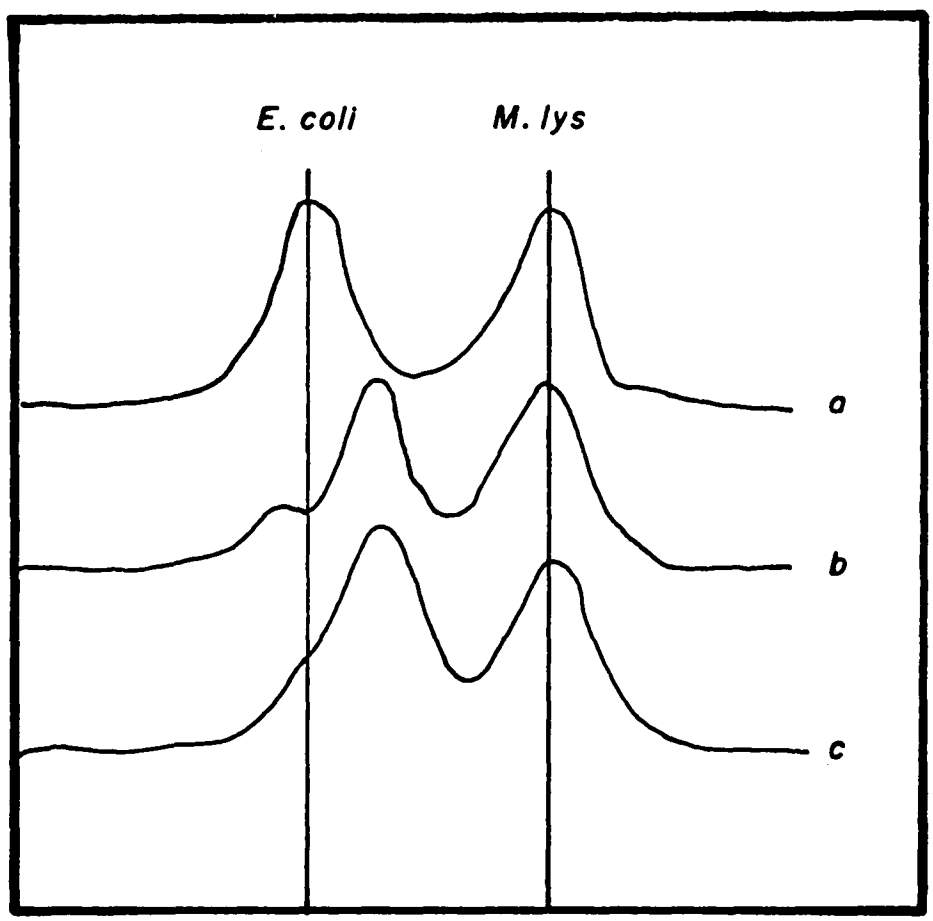

E. coli
M. lys
a
b
c

Figure 20. Diagrammatic representation of the asexual life cycle of a somatic cell regenerator mutant of V. carteri f. nagariensis, (HMK-01 reg).

Stage 1: A juvenile spheroid expands by the somatic cell synthesis of sheath glycoprotein material. Gonidial cells (reproductive), internal to the sphere, enlarge and mature. At maximal point of growth the gonidia 'condense' and begin cleavage, embryogenesis and finally give rise to new juvenile spheroids within the parental sphere.

Stage 2: A segment of the parental sphere showing the differentiated somatic cells.

Stage 3: The somatic cells lose their differentiated form and begin to enlarge.

Stage 4: The cells grow in size and are seen to contain vacuoles.

Stage 5: The contents of the cells 'condense' and become granular.

Stage 6: Cell division.

Stage 7: The release of groups of newly inverted spheroids formed from regenerated somatic cells.

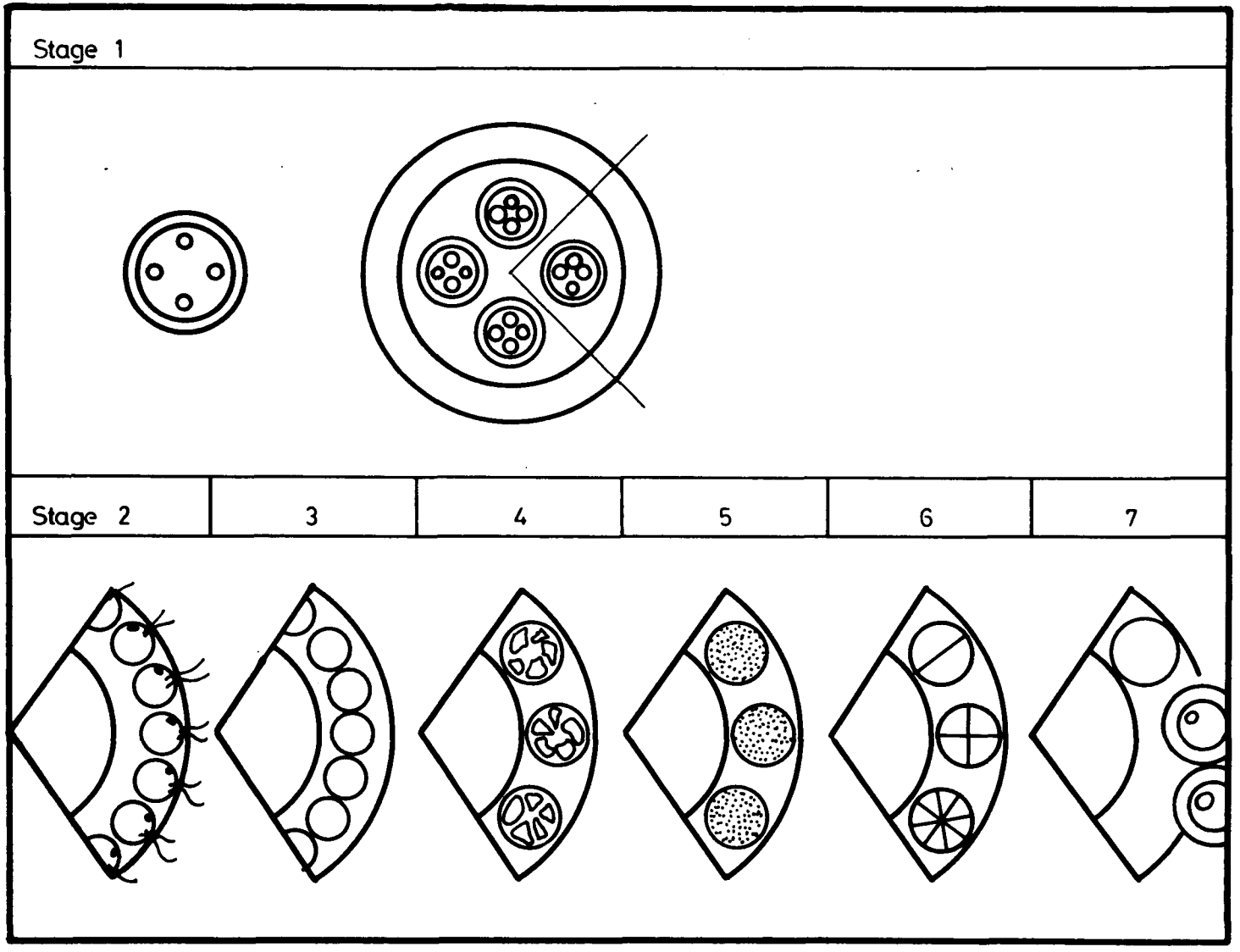

Stage 1
Stage 2
3
4
5
6
7

Figure 21. DNA synthesis in the somatic cell regenerator mutant, HMK-01 reg: Preparative CsCl density gradient profile of radioactivity labeled DNA.

A newly innoculated 5 ml culture of mutant HMK-01 reg (SVM, pH 7) was treated with 2-^{3}H-adenine (20 uCi/ml) at time 0 and incubated under asynchronous growth conditions as described. Additional SVM (2.5 ml, pH 7) and 2-^{3}H-adenine (15 uCi/ml) were added to the culture after 48 hr and after 5 days lysates were prepared as described for figure 7 (1 hr tween, 40 min protease dissociation; 60 min lysis at 60C). The sample was then centrifuged in a preparative CsCl density gradient (20C, 68 hr) as described. Solid circles represent cpm ^{3}H (Volvox DNA) and vertical bar indicates the banding position of ^{14}C E. coli DNA included in the same gradient as a marker.

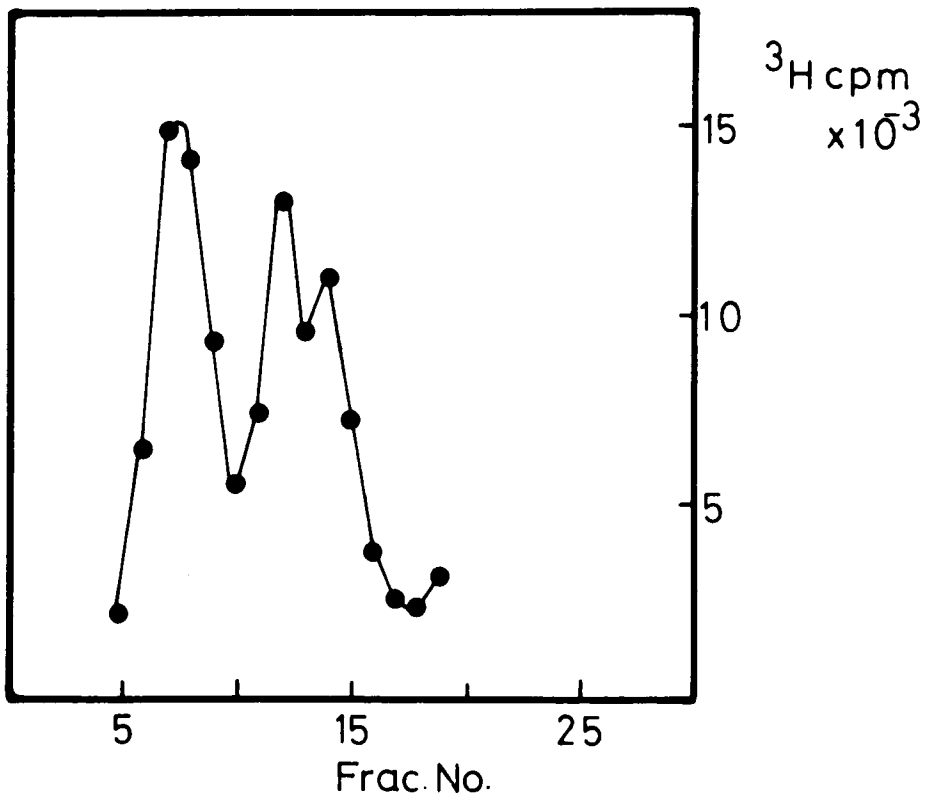

^{3}H cpm
x10^{-3}
15
10
5
5
15
25
Frac. No.

Figure 22. DNA synthesis in mutant HMK-01 reg in the presence of carrier and/or Δ^9 THC: Preparative CsCl density gradient profiles of radioactively labeled DNA.

Duplicate, newly innoculated 5 ml cultures (SVM, pH 7) of mutant HMK-01 reg were incubated in the presence of 2-^{3}H-adenine (20 uCi/ml) and carrier (40 ul/ml) or THC (400 ug/ml) under asynchronous growth conditions for 18 hr. Lysates were prepared as described for figure 13 (40 min protease dissociation, 60 min lysis at 60C) and the samples centrifuged in preparative CsCl density gradients (20C, 63 hr) as described. Solid circles represent cpm ^{3}H (Volvox DNA) and vertical bars indicate the position of marker ^{14}C E. coli DNA.

A. Carrier Control: Carrier and radioisotope were added simultaneously at time 0.

B. THC Experimental: THC and radioisotope were added simultaneously at time 0.

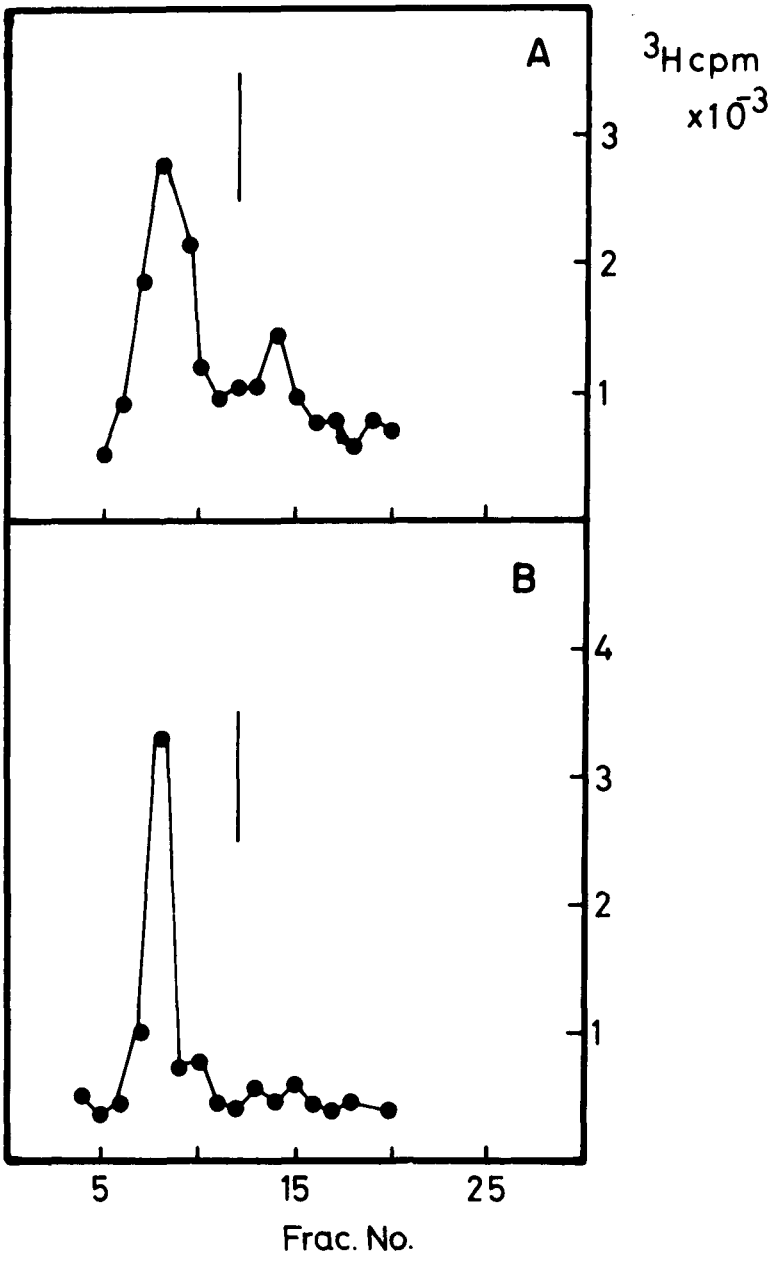

A
B
^{3}H cpm
x10^{-3}
3
2
1
4
3
2
1
5
15
25
Frac. No.

Plate 1. Stages during the asexual life cycle of Volvox carteri f. nagariensis (HK-10).

A. Late Expansion: Maturing spheroid with vacuolated gonidia.

B. Gonidial Condensation: Mature spheroid containing vacuolated, condensed and dividing gonidia. Arrow points to a "condensation stage" gonidium.

C. Early Expansion: Postinversion juvenile spheroids within parent.

For details and descriptions see text.

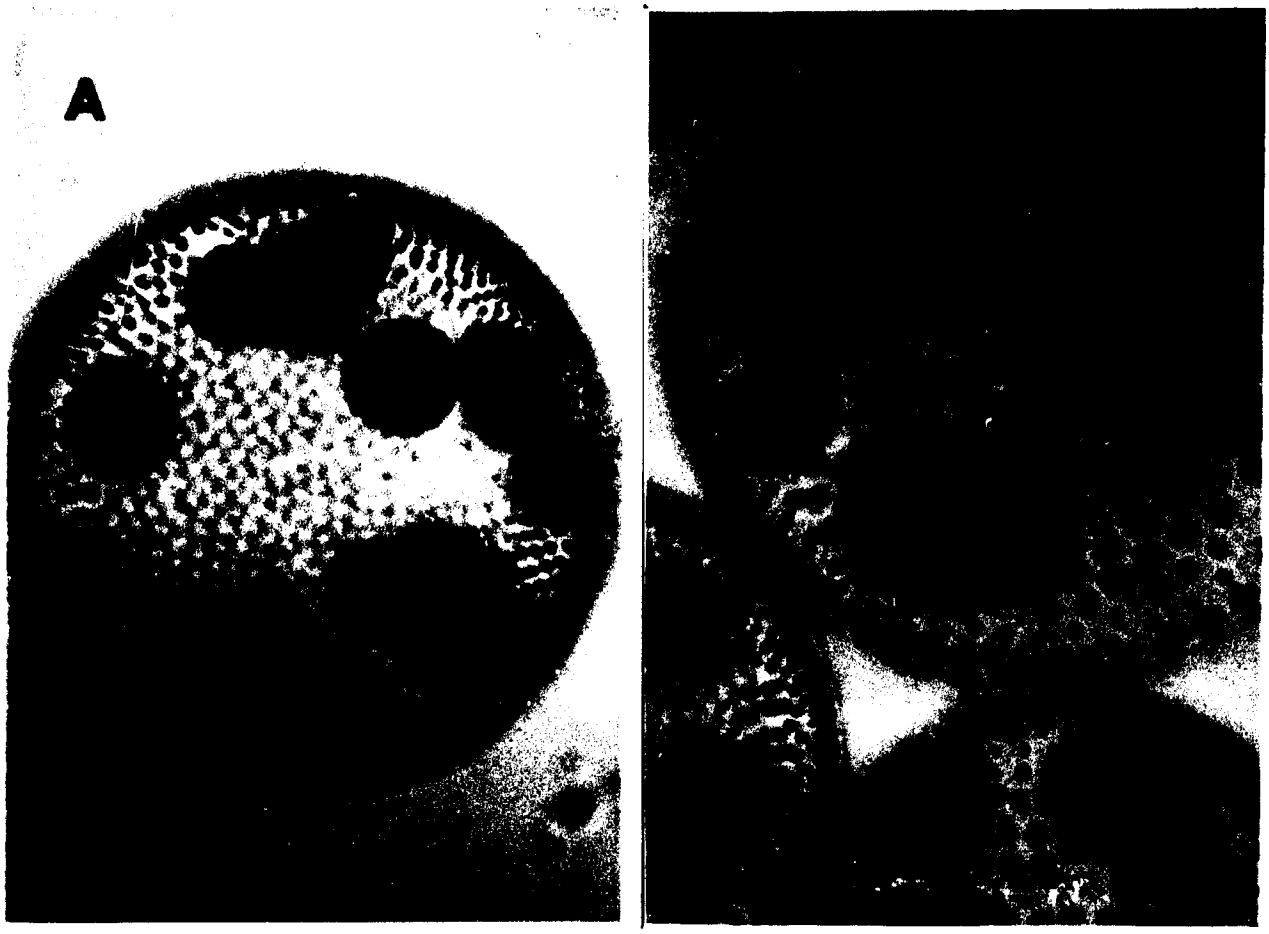

A

Plate 2. Effects of treatment with delta-9-tetrahydrocannabinol.

A. 300 ug/ml THC: Induced "condensation" of immature and enlarged gonidia (20 hr treatment).

B. 50 ug/ml THC ("in vitro" gonidium): Unequal division and cell separation (18 hr treatment).

C. 50 ug/ml THC ("in vitro" gonidium): Aberrant cleavage (18 hr treatment).

See text for explanations and experimental details.

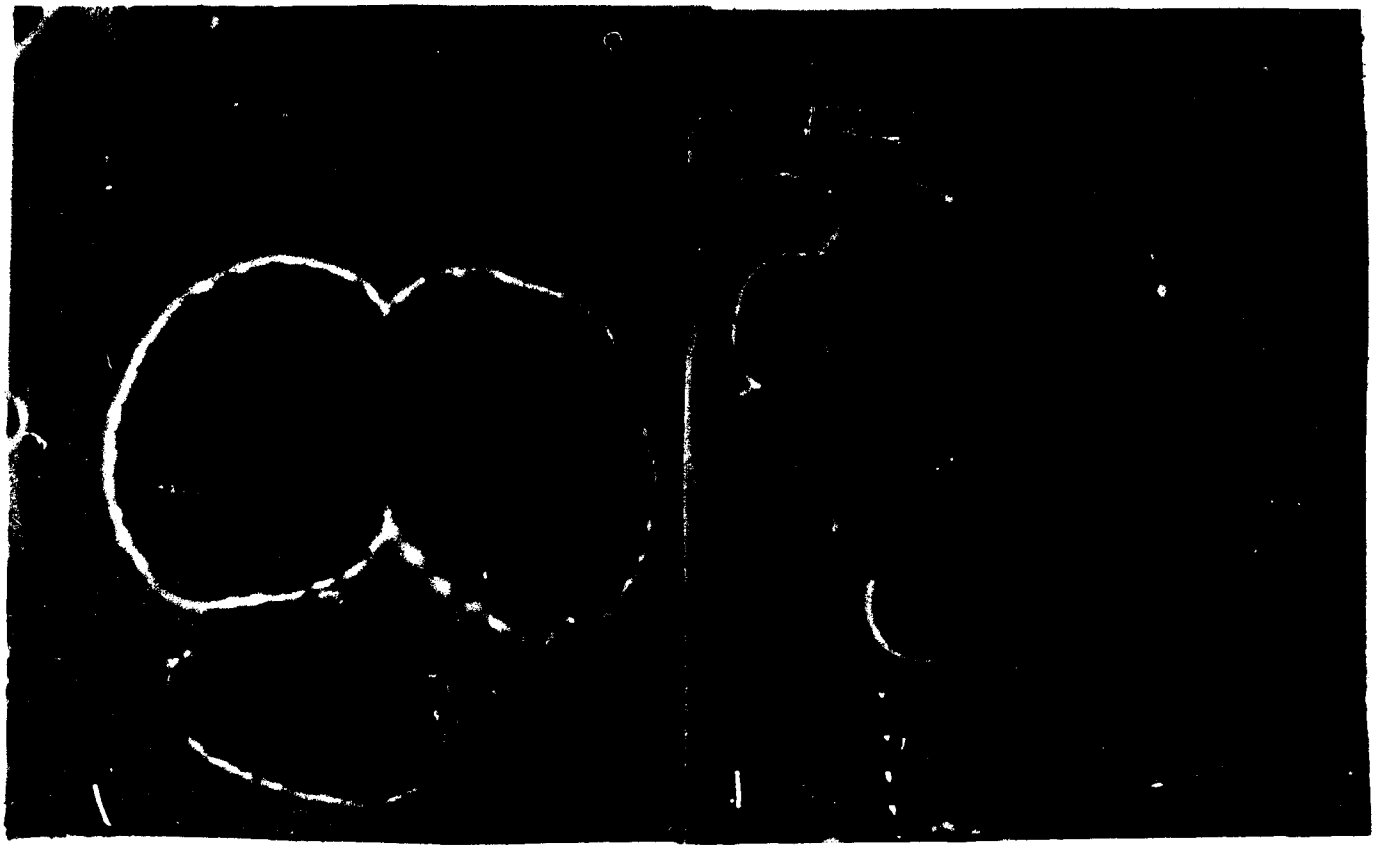


Plate 3. Effects of treatment with ethidium bromide.

Arrest in "cleavage" observed after 24 hr treatment with 25 ug/ml EB.

See text for experimental details.

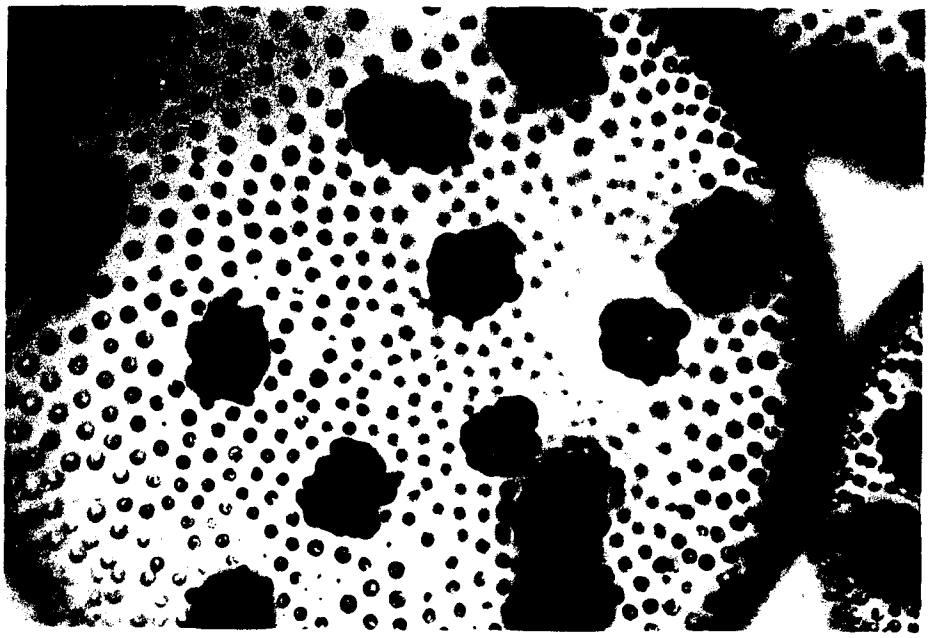


Plate 4. Somatic cell regenerator mutant, HMK-01 reg.

HMK-01 mutant releasing juvenile spheroids: Somatic cells of newly released spheroids appear "normal" while those of parent are in various stages of "regeneration" and "gonidial" maturation.

See text and figure 20 for mutant life cycle details.

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