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Templeman, Malka Naomi, Ph.D.

City University of New York, 1987

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THE EXTRACELLULAR MATRIX OF VOLVOX CARTERI

by

MALKA NAOMI TEMPLEMAN

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

1987

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ABSTRACTTHE EXTRACELLULAR MATRIX OF VOLVOX CARTERI

by

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Advisor: Professor Roy E. McGowan

The extracellular matrix of Volvox carteri f. nagariensis has been isolated and its composition correlated with morphological changes of the normal developmental cycle. Radioactive and non-radioactive matrix components of two strains of Volvox (HK10 and NB7) were extracted and fractionated by column chromatography. The matrix was found to consist of two major fractions, a complex polysaccharide and a lower molecular weight glycoprotein.

Matrix extracted from Volvox at specific stages of the life cycle exhibited altered ratios of carbohydrate to protein as a function of development. The carbohydrate/protein ratio increases from ~1:1 to ~3:1 as the newly released spheroid grows to the fully expanded pre-release stage. Pulse labeling of the matrix during specific

developmental intervals revealed that the increase in the polysaccharide fraction was due to de novo synthesis during pre-release expansion. Matrix extracted from strain NB7 was very similar in biochemical composition and elution profile to the matrix of strain HK10.

DIS 111, a mutant of strain HK10 defective in the synthesis and deposition of extracellular matrix, was compared cytologically to the normal spheroid by means of electron microscopy. Electron dense regions surrounding both the individual cells of the normal spheroid and lining the outer surface of the spheroid were absent from DIS 111. Extracellular material synthesized by DIS 111 was recovered from the medium, concentrated, and separated by exclusion gel chromatography. This analysis revealed diminished polysaccharide and protein "matrix like" components.

Extracellular material was extracted from the related genus Chlamydomonas during the stress induced "palmelloid" phase wherein individual cells are embedded in a matrix like material. Palmelloid extracellular material was fractionated and found to compare closely to normal Volvox matrix.

The glycoprotein fraction of the matrix, extracted from heterogeneous spheroids of HK10 and NB7, was compared on 10% SDS-polyacrylamide gel slabs and on 5%-15% gradient gels. Approximately seven proteins were homologous to

both strains and each strain contained its own specific polypeptide. Matrix glycoproteins from HK10 spheroids at specific developmental stages of the life cycle were electrophoresed on gradient gels. Certain polypeptide bands were found to be stage specific, whereas others were common throughout the life cycle.

This thesis is dedicated to the memory of my
grandmother

מערע לבקה בת ר' אפרים יוסף ז"ל

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INTRODUCTION

I. Molecules of the cell surface.

Preface: " The periplast.....which has hitherto passed under the name of cell wall, contents, and intercellular substance, is the subject of all the most important metamorphic processes, whether morphological or chemical, in the animal or the plant. By its differentiation every variety of tissue is produced."

- T. H. Huxley (1853).

The old concept that the extracellular matrix, the structurally stable material that surrounds the cell, functions as an inert supporting material is now recognized as obsolete. Cells actively interact with their own matrix and with the extracellular matrix of other cells. A structural and functional continuum is formed between cell interfaces (cell membranes, cell walls) and the molecules of the matrix. The metabolism, fate of the cell, its shape, and many of its properties, are continuously related to and depend on the composition and organization of the matrix (Roseman, 1973).

Grobstein (1974) defined the extracellular matrix as "a labile intercellular continuum, locally alterable in penetrability and other properties by physiological shifts....a complex bio-physico-chemical matrix." While describing the influence of the extracellular matrix on gene expression Grobstein identified five essential aspects of involvement. The matrix is a complex macromolecular product of the embedded cells. Matrix materials not only provide mechanical cohesiveness but act upon and alter the activities of the embedded cells. Among the influences exerted by matrix is modification of gene expression in associated cells. This is especially striking when groups of cells having different developmental histories associate. Cell-extracellular matrix interactions can "turn-on" new gene expressions and maintain these expressions in the adult organism.

The surface of eukaryotic cells plays an extremely important role in diverse developmental and physiological phenomena. The surface glycocalyx may be involved in and responsible for the phenomenon of intercellular adhesion. This is thought to be a primary process in the development of embryonic organs, the normal growth of tissues, the advent of malignancy, and more (Roseman, 1973). Elucidating the composition of the cell surface and the extracellular matrix is critical to understanding the processes in which they are involved.

The extracellular matrix of both animal and plant tissue is a heterogeneous assemblage of several classes of macromolecules. The composition of the macromolecules can be a function of source, time of development, and the external environment. The three classes of macromolecules prominent in extracellular matrices are glycoproteins, collagen, and glycosaminoglycans. Glycosaminoglycans are branched carbohydrates that almost always exist in situ as covalently bound side chains of proteins forming complexes termed proteoglycans (Toole, 1981). Toole, cites alterations in the composition of extracellular glycosaminoglycans during morphogenesis as support for the concept that these glycosaminoglycans interact with other cell surface macromolecules. This formation of pericellular coats may be of central importance in the regulation of cell behavior.

II. Cell walls and surface macromolecules of plants.

A. Higher plants.

The growing plant cell wall is a biphasic structure, consisting of a rigid skeleton of cellulose microfibrils cross-linked with a gel-like matrix. The matrix is composed of non-cellulosic polysaccharides and

glycoproteins (Northcote, 1972). Extensibility, digestibility, and adherence are important properties of cell walls, dictated by the cross linking of non-cellulosic components (Fry, 1986). Two of the matrix components, extensin and arabinogalactans, are glycoprotein in nature. Extensin is an insoluble, highly basic glycoprotein, making up 1-10% of the wall matrix, with at least two polypeptide backbones rich in hydroxyproline and bearing short polysaccharide side chains (Albersheim, 1972). Arabinogalactan proteins are soluble, acidic proteoglycans with long carbohydrate side chains covalently bound to the polypeptide core (Fincher et al., 1983).

Lamport (1967, 1970; Lamport and Miller, 1971) reported the occurrence of the hydroxyproline rich glycoprotein extensin in flowering plants, and demonstrated that the carbohydrate protein linkage, which involves the hydroxyproline hydroxyl group in a glycosidic linkage with arabinose, is stable to alkaline degradation (Lamport, 1967). The hydroxyproline-O-glycosidic linkage was shown by Lamport to be widely distributed in the plant kingdom, occurring in green algae, liverworts, horsetails, mosses, and ferns, as well as in higher plants. The only sugars found to be involved in the linkage are arabinose and very rarely galactose (Miller and Lamport, 1972). There appears to be a trend towards a

longer arabinose oligosaccharide substituent in more advanced groups, and a correlation between the extent of hydroxyproline glycosylation and phylogenetic position.

A precursor of extensin has been isolated from carrot disks and found to be associated with the cytoplasmic organelles prior to incorporation into the cell wall (Brysk and Chrispeels, 1972). This finding correlates with the contention of Pickett-Heaps based on electron micrographs, that the Golgi apparatus is the site of synthesis of cell surface polysaccharides (Northcote and Pickett-Heaps, 1966). In addition to being localized in situ, extracellular hydroxyproline rich glycoproteins have been isolated from suspension cultured tobacco plant cells (Hori and Sato, 1977) and sycamore cells (Heath and Northcote, 1971).

B. Algae.

The cell walls of algae differ from those of higher plants in many respects. The proportion of fibrillar material is less than in higher plants, and that of the matrix and intercellular material greater than in higher plants. In addition, many species exude polysaccharide material onto their outer surfaces (Percival and McDowall, 1981). The extracellular material present on the surfaces is a feature of nearly all algal cells,

attaining industrial importance in some forms, e.g. kelps. The Golgi apparatus has been implicated in the production of algal extracellular matrix, including red algal mucilage and slime polysaccharide secretion of the brown algae (McCandless and Craigie, 1973).

The cell wall may be regarded in a broad sense as any material which may be transiently associated with the cell surface (Lamport and Catt, 1984). Proteins are invariably associated with the complex polysaccharides of algal cell walls (Percival and McDowall, 1981). The culture medium of growing Chlamydomonas cells contains protein components that are liberated not only during release of the zoospores from the mother-cell walls, but also during the cell enlargement phase of the vegetative cell cycle (Schlosser, 1981).

The most common cell surface glycoproteins contain hydroxyproline and occur throughout the algae. There is histochemical evidence of these glycoproteins by light and electron microscopy in Pandorina (Fulton, 1978, I and II), and by co-fractionation of protein and carbohydrate in Chlamydomonas. Earlier, Roberts (1974) had shown the cell wall of Chlamydomonas contained glycoprotein subunits. He confirmed that hydroxyproline containing glycoproteins are the predominant structural components of the cell wall, as they constitute the bulk of the wall's dry weight. Miller and Roberts (1976) extracted

polysaccharide bound hydroxyproline from cell wall preparations of Chlamydomonas. Lang and Chrispeels (1976), using radioactive tracer studies, demonstrated the spontaneous release of hydroxyproline rich glycoproteins of the cell wall during the vegetative cell cycle of the algae.

Lamport conducted a comparative investigation to determine the prevalence of hydroxyproline heterooligosaccharides among the algae. The results of the study confirmed the existence of a hydroxyproline arabinose linkage in all green algae investigated and the absence of the glycoprotein from red algae, which as a group is devoid of hydroxyproline. Lamport further demonstrated that these proteoglycan components that are extracellular components of the Volvocales differ in their composition from the hydroxyproline oligosaccharides common to higher plants and other algae and may be related to the lower tensile strength of the cell walls of the Volvocales (Lamport, 1967, 1970; Lamport and Miller, 1971).

The preponderance of hydroxyproline rich glycoproteins as extracellular components has thus been demonstrated throughout the plant kingdom, and the presence of these macromolecules has been confirmed in algae as well as the higher plants. The function of these components is still a subject for speculation, but their occurrence in such a

wide range of genera implies that their role is a significant one.

C. Metabolism / catabolism of matrix components.

The existence of glycoprotein/ proteoglycan extracellular components in unicellular or colonial algal forms implies the necessity for proteolytic enzymes to dissolve the matrix in order to allow for the release of the individual cells or the maturing daughter organisms during vegetative reproduction, as well as for liberation of spermatogonia and penetration of the gametes through the sheath material. The finding of such proteolytic enzymes would therefore be additional, although indirect, evidence of the existence of proteinaceous extracellular components in the algae.

Schlosser (Schlosser et al., 1976; Schlosser, 1981) isolated a species specific autolysine from Chlamydomonas and utilized the enzyme as a means of isolating protoplasts of the organism. Jaenicke has isolated and extensively studied the "release enzyme" of Volvox (Jaenicke and Waffenschmidt, 1979). Jaenicke and Waffenschmidt have compared the proteolytic processes involved in the liberation of zoospores in Chlamydomonas and the release of mature daughter spheroids in Volvox (Jaenicke and Waffenschmidt, 1981). They determined there

is no cross lysis in the Volvocales, and the proteoglycan walls or matrix material of Chlamydomonas is not attacked by the Volvox sheath lysine and vice versa. These investigators did find a strong biochemical similarity between the two enzymes; both were found to be tryptic serine proteases, with a similar pH optimum of just slightly basic (pH 7.5-8.5), single chain proteins with molecular weights of 37,000-40,000 D, and are heat labile. The sperm packets and spermatozooids of Volvox have also been found to have proteolytic activity (Jaenicke and Waffenschmidt, 1981). Snell recently reviewed cell-cell interactions in Chlamydomonas (Snell, 1985), and he cites evidence by other investigators indicating that the adhesiveness of either intact cells or isolated flagella involves hydroxyproline containing proteinaceous components of the matrix.

III. Volvox as an experimental organism.

Volvox represents multicellular development in its most fundamental form, two cell types comprising a single organism. The vegetative somatic cells and the reproductive gonidia are embedded in and communicate via the gelatinous extracellular matrix. The organism undergoes a well defined developmental program during its

life cycle including morphological growth and embryogenesis. These attributes present Volvox to the researcher as an ideal model for the study of multicellular differentiation and development.

The Volvox life cycle occurs entirely within and is uniquely linked to events occurring in the extracellular matrix. The extracellular matrix is the ground material in which cellular communication, development, maturation, and finally the emergence of the new generation, occurs. Cellular growth, predominantly in the gonidia, occurs in Volvox. The dramatic increase in spheroid diameter during the life cycle is attributable to two distinct expansion phases of the spheroid, whereby the component cells are separated by the deposition of new extracellular matrix material. The two expansion phases are separated in time and stage of the Volvox life cycle. Post-release expansion, refers to spheroid growth during the maturation of juvenile spheroids prior to gonidial cleavage and embryogenesis. Pre-release expansion, refers to the more dramatic expansion phase occurring in the maternal matrix after cleavage and inversion have been completed; and, prior to the release of nascent juvenile spheroids.

Aberrant patterns of matrix development occur in some mutant forms of Volvox. Certain mutants fail to expand fully whereas others, such as DIS 111, fail to synthesize

functional matrix components and have no spheroid integrity, dissociating immediately after cleavage and inversion to yield suspensions of the two cell types.

A. Morphology:

Volvox is probably the earliest recorded green flagellate. It is mentioned in a letter from Leewenhoek to the Royal Society of London in 1700. In 1753 Linnaeus in The Species Plantarum recognized Volvox as a green algae but classified the organism as an animal. The Volvocales, traditionally referred to as motile colonies, upon closer examination appear to be truly multicellular organisms. In Gonium, Pandorina, and Volvulina, all cells of the colony have the potential to function both as asexual reproductive cells and as isogamous gametes. Certain species of Eudorina show the first morphological differentiation into somatic and reproductive cells. The genus Volvox represents the ultimate expression of "colonial" development in the volvocine line of development. Blackman and Tansley proposed (in 1902) that Volvox retained the primitive chlamydomonad type cell while attaining an advanced stage in evolutionary development. This organism realizes the utmost potentialities of the construction plan for a motile spherical coenobium (Systematics of the Green Algae,

Irvine and John eds., 1984).

The Volvox colonies, large enough to be seen with the naked eye, are beautiful microscopic organisms, actively motile, with some species containing as many as 20,000 cells. Each individual organism contains only two cell types, somatic and reproductive, embedded in a proteinaceous mucilage. The colony is spherical (referred to as a Volvox "spheroid") with a single layer of small, regularly spaced flagellated somatic cells positioned externally. Asexual reproduction is brought about by mitotic division of the single celled gonidia that are housed inside the spheroid and are considerably larger than the somatic cells.

The life cycle of Volvox strain NB7 was studied by Kochert and recorded in detail in a series of light micrographs (Kochert, 1968). Ultrastructural studies of the Volvocales were first described by Lang in 1965. She contends that the ultrastructure of the colonial organisms is basically that of Chlamydomonas, with the apparent substitution of a fibrillar layer of colonial matrix for the discrete cell wall. The ultrastructure of the asexual colony of Volvox carteri was described in detail by Kochert and Olson (1970,I; Olson and Kochert, 1970,II), who also were the first to show the existence of endosymbiotic bacteria in a male strain of the species, KA-1 (Kochert and Olson, 1970).

The early systematic study and use of Volvox as an experimental organism may be accredited largely to the contributions of Richard Starr. Starr described in detail the isolation of Volvox strains from soil samples, the transfer of germlings to axenic cultures, and the growth and maintenance of the asexual spheroid (Starr, 1969). He recorded a detailed light microscopical investigation of the development and embryogenesis of the asexual spheroid including enlargement, gonidial cleavage, and cell movements (Starr, 1970).

B. Asexual Development:

The process of asexual reproduction in Volvox is accomplished by cleavage of gonidia in the asexual organism to form young spheroids. A series of synchronous cell divisions, cleavage, leads to the formation of a hollow ball of cells, much like blastula formation in vertebrates. Unequal division (at the 16 or 32 cell stage in V. carteri) results in the differentiation of the gonidial initials from somatic cells during juvenile spheroid formation.

At this point the somatic cells are in the interior portion of the sphere with their anterior, flagellated ends directed toward the inside of the sphere, and the gonidia are on the outer surface of the embryo. The

Figure 1.

The life cycle of Volvox carteri: A diagrammatic representation (courtesy of Dr. J. Blamire).

The life cycle depicted here normally spans a period of 48 hours under conditions of 31 hours light/ 17 hours dark occurring optimally in cultures of up to 500 ml SVM pH 8.0.

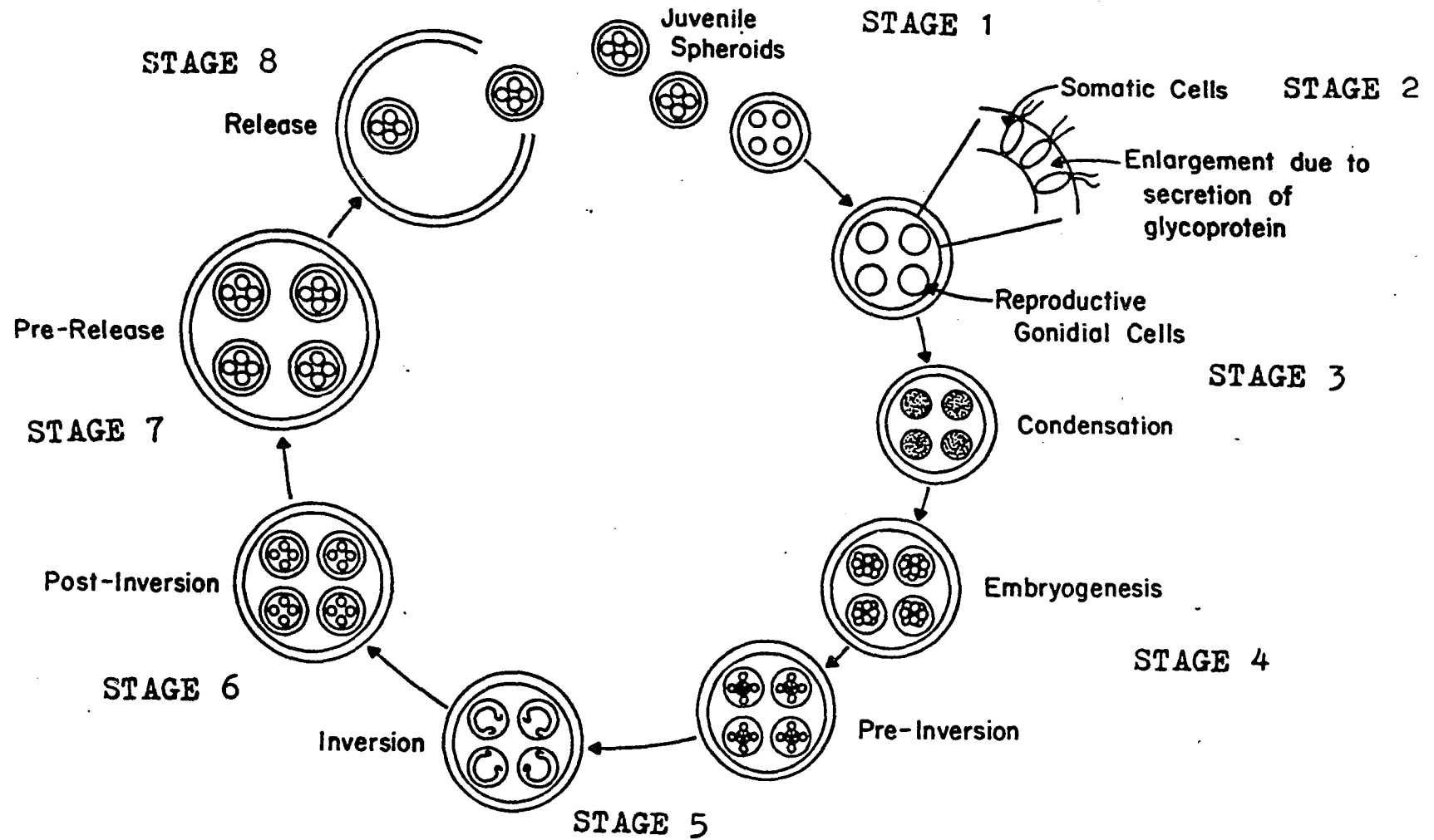
The newly released juvenile spheroids are ~100 um in diameter immediately upon release and grow rapidly to 150-200 um while still in the vacuolated stage. This phase is referred to in this study as "post release expansion". The size of the gonidia during this stage increases from ~30-50 um, and the somatic cells are <10 um in diameter.

The gonidia of the spheroid are visibly more dense as they pass through the condensation stage in preparation for cleavage, and no further expansion occurs at this point. The gonidia undergo a series of cleavage stages, which do not occur synchronously in all the gonidia of the spheroid, optimally forming a ball of sixteen gonidia (in V. carteri f. nagariensis strain HK10) or eight gonidia (in V. carteri f. weismania strain NB7) surrounding a cluster of ~5,000 smaller somatic cells, pre-inversion. The cells of the spheroid undergo an inversion process during which the inner somatic cells

pass through a phialopore described in detail by Viamontes (1977), producing a spheroid with the normal configuration of somatic cells surrounding gonidia.

At post-inversion, the spheroid starts a significant expansion phase, "pre-release expansion", with the diameter of the spheroid increasing from ~200 μm to 350 μm -400 μm before the newly formed juveniles are finally released from the maternal spheroid.

LIFE CYCLE OF *VOLVOX CARTERI*



embryo undergoes a complex series of morphogenetic movements termed "inversion", closely resembling gastrulation, which result in the spheroid turning itself inside out by the movement of the cells through a phialopore. The gonidia now occupy the posterior, interior portion of the spheroid, with the flagellated somatic cells at the periphery.

During the post inversion maturation period the somatic cells synthesize and secrete extracellular matrix material. The somatic cells move apart from one another as the matrix material is deposited and the young spheroid grows in size, "expansion". The juveniles are enclosed in a vesicle derived from the gonidial cell wall within the parental spheroid. When mature, a dissolution of the parental matrix material occurs and the juvenile spheroids are "released". A proteolytic release enzyme has been isolated from V. carteri (Jaenicke and Waffenschmidt, 1979, 1981; Jaenicke, 1982). The somatic cell sheaths, "ghosts", dissociate after the release of the juveniles. The individual somatic cells are terminally differentiated and, although they swim and are viable for ~144 hours after cell division, undergo senescence and die (Pommerville and Kochert, 1982). The somatic cells of the newly released juvenile spheroids secrete matrix material and the spheroids enlarge. The reproductive gonidia mature and undergo cell division to

produce another asexual generation.

In addition to describing the morphology of Volvox, the early investigations of the organism indicated the importance of cytoplasmic factors in the gonidia for the normal development of the spheroid, much along the line of reasoning of August Weismann and his "germ plasm" theory. By irradiating mature gonidia of V. carteri f. weismania (named after Weismann for this reason) with UV light prior to cleavage, spheroids are produced which lack one or more gonidia (Kochert and Yates, 1970). Centrifuging mature gonidia displaces the cytoplasm and subsequent cleavage produces spheroids with altered distributions of gonidia. Kochert explained these phenomena by a cytoplasmic localization theory, which claimed that cytoplasmic patterning was determined during the period of gonidial growth and enlargement preceeding cleavage.

Glycoproteins of the extracellular matrix have been implicated in the control of differentiation in Volvox. Sumper proposed a model explaining gonidial positioning and pattern formation during embryogenesis in V. carteri (Sumper, 1979) based on experimental findings that chemical modification of extracellular glycoproteins by lectins such as concanavalin A (Kurn and Sela, 1979), sugar complexing chemicals such as borate, or proteases (Wenzel and Sumper, 1979), disturb developmental control.

These investigators identified a sulfated cell surface glycoprotein with a molecular weight of ~185,000 D (SSG 185) synthesized by Volvox and implicated it in the control of embryogenesis (Sumper and Wenzl, 1980; Wenzl and Sumper, 1981). The sulfated glycoprotein, SSG 185, is short lived with a half life of ~30 minutes and appears only during embryogenesis. It serves as a precursor for a high molecular weight (polymeric) component of the extracellular matrix. SSG 185 is a developmentally regulated molecule, with the molecular weight altered as a result of specific developmental events, having a lower molecular weight during early embryogenesis and increasing thereafter.

Although the synthesis of this component and its developmental modification correlates with embryonic development occurring in the gonidia, it has been isolated in association with the extracellular matrix surrounding the somatic cell layer (Wenzl et al., 1984). This somatic cell association is in agreement with similar findings by Kirk (Kirk and Kirk, 1983). The Golgi bodies of the somatic cells have been implicated in the synthesis of the components of the extracellular matrix (Dauwalder et al., 1980).

Willadsen and Sumper (1982) demonstrated ATP dependent incorporation of sulfate into SSG 185 in vitro using sonicates of Volvox spheroids. SSG 185 is further thought

to be a modified form of an extracellular matrix component involved in the process of sexual induction, since at least two sulphated glycoproteins (F-SG and M-SG) are produced in response to the sexual inducer. Volvox extracellular matrix glycoproteins have thus been implicated in a key role in the asexual development as well as the sexual induction of the organism.

Of all aspects of embryogenesis in Volvox the most striking and well studied is the phenomenon of inversion. A cytoplasmic bridge system links all cells of a Volvox embryo producing a regularly spaced cytoskeleton that plays a crucial role in maintaining cell-cell relationships during embryonic development. Using electron microscopy, Bisalputra and Stein first demonstrated the existence of cytoplasmic bridges in V. aureus (Bisalputra and Stein, 1966). Green, Viamontes, and Kirk, studied the development of the bridge network in V. carteri, and determined they are formed as a result of incomplete cytokinesis localized along the cleavage furrow of dividing cells (Green and Kirk, 1981; Green et al., 1981). The only place in the developing embryo where cytoplasmic bridges are not present is in the region of the phialopore, controlling inversion. Inversion requires microtubule driven elongation of cells to produce flask shaped cells (resembling Holtfreter's bottle cells of amphibian gastrulation) and cytochalasin sensitive

migration of cytoplasmic bridges to the periphery of the flask cells (Viamontes and Kirk, 1977; Viamontes et al., 1979).

It has been determined, that besides the intracellular cytoskeleton, a number of extracellular matrix macromolecules function in the control of the inversion process. Wenzl and Sumper characterized a sulfated glycoprotein (I-SG) whose appearance correlates with the occurrence of inversion (Wenzl and Sumper, 1982). They have recently reported the isolation of a glycosphingolipid from the matrix and have implicated it in the regulation of inversion. They found that antibodies specific for this antigen were able to block inversion in vivo when introduced to a population of synchronously dividing spheroids. No other aspect of reproduction or viability was affected (Wenzl and Sumper, 1986).

C. Sexual Reproduction:

The asexual cell cycle may be repeated indefinitely or until sexual reproduction and gametogeny is induced. Volvox is heterogamous, forming female and male gametes either in one spheroid (monoecious) or in separate strains (dioecious). The male and female are morphologically indistinguishable in the vegetative

state. Generally, sexuality is favored as cultures age. William Darden, a graduate student of Dr. Starr's, initiated research in the control of the sexual process in Volvox aureus and the use of the genus for the study of development with his observation and study of the induction of sexual phases of the life cycle (Darden, 1966).

Kochert studied the development, both asexual as well as sexual, of Volvox carteri f. weismania. His results showed that male sexuality was spontaneously induced in this strain. A sexual inducer secreted by the male strain was isolated and found to cause sexual differentiation of the female (Kochert, 1968). Starr distinguishes the non-inducibility of the male of V. carteri f. weismania from the behavior of a related heterothallic strain he isolated in 1967 (V. carteri f. nagariensis strains HK 9, male, and HK 10, female). In this form of the species, the sexual inducer produced by the male has the potential to induce both androgonidia and egg formation in asexual colonies (Starr, 1960).

Volvox exists basically as a haploid organism. The only diploid stage is the zygote, with meiosis occurring during zygote germination and only one product of meiosis surviving. Both male and female spheroids are formed by successive divisions of haploid gonidia of asexual spheroids of either male or female strains.

Sexually induced spheroids of the male strains have both vegetative somatic cells and reproductive cells, androgonidia, in a ratio of 1:1 (in V. carteri f. nagariensis strain HK 9), totalling about 500 cells. The androgonidia in male spheroids enlarge, undergo division to form packets of 64-128 biflagellate sperm. The mature sperm packets are released through a pore formed in the somatic cell layer of the parent spheroid (Kochert, 1975; Starr, 1969, 1970).

Sexual spheroids of female strains resemble the asexual spheroid in the low ratio of reproductive cells to somatic cells, with about 30-45 eggs per spheroid (in V. carteri f. nagariensis strain HK10). Fertilization occurs when freely swimming sperm packets encounter female colonies of a compatible strain. Contact and binding is made between the flagella of a sperm packet and the flagella of the somatic cells of the female spheroid. A glycoprotein receptor is thought to be involved in this attraction (Kochert, 1975). Within minutes after binding to the female colonies, the sperm packets undergo dissolution to release individual sperm which penetrate the female spheroid through a fertilization pore in the somatic cell layer. This process involves a solubilization of the extracellular matrix of the female spheroid. The fertilized zygote develops into a thick walled, orange zygospore, resistant

to dessication and responsible for Volvox being able to survive unfavorable environmental conditions (Kochert, 1975).

The fluid from a culture containing sperm packets contains a substance capable of inducing the production of male spheroids in strain HK9, and necessary for the induction of female spheroids. The induction of sexuality in all strains of Volvox is brought about by the action of glycoprotein "hormone like" inducers (Starr, 1969). Investigators have isolated the sexual inducer (Kochert and Yates, 1974; Pall, 1974; Starr and Jaenicke, 1974) and have identified the biologically active principle as a glycoprotein on the basis of its binding to concanavalin A, and positive assays for both sugar and protein. The molecular weight is estimated at ~30,000 daltons, and the hormone is thermostable. An attempt has been made to quantitate the action of the inducer, which has been estimated to be effective at the concentration of two molecules of inducer for the sexual induction of a gonidium (Pall, 1973).

Recently, Kirk and Kirk (1986) determined that in response to heat shock (the elevation of temperature to 42.5°C for 1-3 hours) both male and female spheroids of Volvox carteri underwent "autoinduction" of sexuality, and produced and released into the medium a substance identical to the sexual inducer. They surmise this

phenomenon to be advantageous in the natural habitat of Volvox, producing drought resistant zygotes during the summer's heat, to enable the organism to survive the drying up of the ponds.

The phenomena of sexuality and the inducer of Volvox are both interesting and relevant to the study of the extracellular matrix of the organism, and lend further support to the evidence of the proteinaceous nature of the matrix. Dissolution of the parental sheath material must occur to effect the release of the sperm packets from mature male spheroids, and the fertilization reaction involves proteolysis of the extracellular matrix of the female during the formation of the fertilization pore. Kochert has shown that inhibitors of protein synthesis and soybean trypsin inhibitor will block fertilization pore formation and penetration of the female spheroid (Kochert, 1975).

Recently Gilles, Gilles, and Jaenicke (Gilles et al., 1983, 1984, 1985) have implicated components of the extracellular matrix in binding the sexual inducer. Sulfation of surface glycoproteins of female gonidia, and phosphorylation of matrix components produced by somatic cells occurs within 0.5-1 hour after addition of the sexual inducer (Wenzl and Sumper, 1982; Gilles et al., 1983). No binding of fluorescent anti-inducer antibodies was observed by the gonidial membrane, whereas many

binding sites are located throughout the spheroid. The strongly acidic matrix which contains polymer bound sugar acids as well as sulfated and phosphorylated sugars is a logical candidate for interaction with the strongly basic (pI=10.5) sexual inducer (Gilles et al., 1984).

Since the ultimate target of the sexual inducer is the gonidia, it was important to determine how the message was transmitted from the matrix acceptor molecules to the target cells. As soon as the organism responds to the inducer, the somatic cells synthesize and secrete a phosphoprotein into the matrix, which then utilizes matrix cyclic AMP as a secondary messenger (Gilles et al., 1985). High levels of cAMP have been shown to inhibit sexual induction. Interestingly, a non-inducible sterile male strain has been shown to have a 10-20 X higher concentration of cAMP levels, whereas the sexual constitutive sex^C mutant has a very low level of cAMP (Weisshaar et al., 1984). Calmodulin has been implicated in the control of development and morphogenesis in Volvox (Jaenicke, 1982). Calmodulin dependent protein kinases are thought to be the missing link between external Ca⁺⁺/ cAMP levels and phosphorylation of extracellular matrix glycoproteins. These matrix glycoproteins then trigger internal messages for the sexual transformation of gonidial cells.

D. Genetics:

Volvox, with its haploid genome and only two cell types, is especially suitable for the study of the genetic control of development. Sessoms and Huskey induced morphogenetic mutant formation in Volvox carteri by chemical mutagenesis using the mutagens EMS (ethyl methane sulfonate) and MNNG (nitrosoguanidine) (Sessoms and Huskey, 1973). The phenotypes produced affected the asexual life cycle and many were temperature sensitive.

Two types of mutations affected the development of the extracellular matrix. A non-expander mutant was isolated where the cells remain close together and very little matrix is synthesized, with the result that almost no increase in the diameter of the spheroid occurs. An unequal expander mutant was also isolated where cells in the anterior portion of the spheroid are greatly separated with more than the normal amount of matrix between them, and cells in the posterior portion of the spheroid remain close together. Another type of mutation produced Volvox with no spheroid integrity, apparently due to a deficiency in the production of functional matrix. These dissolver mutations, similar to the one described in this thesis (DIS 111), result in spheroids that fall apart after inversion, producing free swimming somatic cells and functional gonidia that fall to the

bottom of the culture vessel. Spheroids with a mutation termed "delayed dissolver" retain their integrity until after the juveniles begin cleavage, whereupon the parental colony dissociates.

A spontaneous mutation, which is also frequently observed as a result of mutagenesis, is the somatic regenerator. This mutation causes the normally terminally differentiated somatic cells to enlarge, lose their flagella and dedifferentiate as functional gonidia into new individuals of like phenotype. This mutation has been the object of investigation by a number of different research teams. Margolis-Kazan and Blamire have shown that the phenomenon of the somatic regenerator is associated with abnormally high levels of cytoplasmic DNA synthesis. Using the cannabanoid THC, they have shown that selective inhibition of cytoplasmic DNA synthesis by the drug inhibits the dedifferentiation of somatic cells in the mutant (Margolis-Kazan and Blamire, 1979).

Huskey and Griffen (1979) isolated "reg" (somatic regenerator) strains and demonstrated that the reg A locus is one of the key loci maintaining germ-soma dichotomy. They found that the reg A gene product exerts its action on somatic cells after embryogenesis is completed and when cytodifferentiation of the two cell types begins in the juvenile spheroids. Kirk et al., (1987) investigated the mechanism by which the gene is

normally inactivated, and presumptive somatic cells terminally differentiated, during asexual development. They demonstrated that hypermutability of the reg A locus occurs in the gonidia, in which the gene is normally not expressed, during a brief period prior to cytodifferentiation at which time the reg A gene acts in somatic cells. They conclude that the mutational properties of the reg A locus imply regulatory properties of the gene in the control of asexual development and cell differentiation in Volvox.

E. Biochemical studies; synthesis of cellular macromolecules.

1) DNA synthesis:

Kochert and Jaworski (1972) isolated and characterized the DNA of V. carteri f. weismania. Two main DNA species were identified; a major component is nuclear DNA and a minor component is satellite DNA, assumed to be chloroplast (and mitochondrial) DNA. Yates studied DNA synthesis in spheroids of synchronous cultures, in isolated somatic cells, and in gonidia isolated from intact spheroids by protease treatment (Kochert, 1975; Yates et al., 1975). The DNA profile of somatic cells closely resembles that of Chlamydomonas both in quantity

and in buoyant density. They found the gonidia contain ~25 times as much DNA as do somatic cells, and the relative proportion of gonidial chloroplast DNA to total gonidial DNA is 30% as compared to 6.5% for the somatic cells. DNA synthesis was determined to be maximal during the period of gonidial cleavage with an additional synthetic period occurring in the early stages of pre-release expansion.

Margolis-Kazan and Blamire (1979, Margolis-Kazan, 1978) compared the biophysical and biosynthetic properties of DNA extracted from three strains of Volvox. They confirmed the existence of two distinct DNA species corresponding to nuclear and cytoplasmic (predominantly chloroplast) DNA. They followed the synthesis of cellular DNA extracted from synchronous cultures of V. carteri f. nagariensis strain HK10 during the asexual life cycle, and determined that whereas nuclear DNA appeared to be synthesized throughout the life cycle, cytoplasmic DNA is synthesized predominantly during the post embryonic period of pre-release expansion. Using two agents which alter DNA metabolism, THC (tetrahydrocannabinol) and EB (ethidium bromide), these investigators determined that THC selectively inhibits cytoplasmic DNA synthesis (Margolis-Kazan and Blamire, 1979) whereas (although eventually depressing all DNA synthesis) the immediate effect of EB is to inhibit the synthesis of nuclear DNA

while allowing some cytoplasmic DNA synthesis to continue. Morphological aberrations accompany the inhibition of DNA synthesis. THC at concentrations >125 ug/ml causes the arrest of gonidia at the condensation (pre-cleavage) stage. EB at concentrations of 25-30 ug/ml causes the cessation of cell division between the 4 and 32 cell stage, and at concentrations >30 ug/ml causes the degeneration of all gonidia. These results indicate that changes in the normal pattern of embryogenesis mediated by these drugs are a result of interference with the natural changes in the proportions of nuclear and cytoplasmic DNA, thereby implicating DNA metabolism in the normal regulation of development in Volvox.

2) RNA synthesis:

Ribosomal RNA's have been isolated and characterized in V. carteri strain NB7 (Kochert, 1971). Four high molecular weight rRNA components have been identified; two from cytoplasmic ribosomes and two from chloroplast ribosomes. Gonidia contain much more rRNA than the somatic cells, presumably enough to supply all the cells of the developing embryo with ribosomes during the cleavage period. The ratio of chloroplast rRNA to cytoplasmic rRNA was also found to be higher in gonidia than in somatic cells. Yates found the most active period

of rRNA synthesis in both somatic cells and gonidia was during the period of early spheroid enlargement (post-release expansion) (Kochert, 1975).

Caplan and Blamire (1980) isolated and characterized polyadenylated RNA extracted from V. carteri f. nagariensis strain HK10. They found the terminally differentiated somatic cells contain uniformly larger poly(A) segments than the gonidia or developing embryos, which display the greatest degree of size heterogeneity. They concluded that changes in poly(A) RNA metabolism accompanies development and cellular differentiation in Volvox.

3) Protein synthesis:

Kochert (1975) found that all stages of the Volvox life cycle require protein synthesis, as cyclohexamide (1 ug/ml) blocks further development at all stages of the life cycle. Yates et al., (1975) determined that the most prominent phase of total spheroid protein synthesis occurred after gonidial cleavage during the stage of daughter spheroid growth (pre-release expansion). They also found an increase in total spheroid polysaccharide content during the pre-release expansion phase, followed by a decrease during spheroid release which they attributed to solubilization of the parent spheroid

matrix, a polysaccharide containing material.

Kirk and Kirk (1976), utilizing a technique they devised for the separation of somatic cells and gonidia (personal communication, see Appendix), investigated and found reproducible cell type and stage specific differences in protein synthetic patterns in cells free of interfering extracellular matrix components. They showed that arginine is preferentially incorporated into the reproductive gonidia, which have four times as high an arginine to lysine ratio as the vegetative somatic cells. These investigators found that arginine is incorporated 500 to 1,000 times more rapidly than other amino acids by V. carteri f. nagariensis strain HK10 (Kirk and Kirk, 1976). They subsequently determined that the uptake of the basic amino acid arginine (and urea) is mediated by a specific carrier in Volvox (Kirk, D.L. and Kirk, M.M., 1978) and Chlamydomonas (Kirk, M.M. and Kirk, D.L., 1978).

Using radioactive sulfate Kirk followed the polypeptide labeling patterns of somatic cells, gonidia, embryos, and juvenile spheroids of V. carteri f. nagariensis strain HK10 isolated from synchronous cultures. Hagen and Kochert (1980) had previously demonstrated the existence of differences in the polypeptide labeling patterns between young somatic cells and gonidia of a related strain of Volvox, V. carteri f. weismania strain NB7, and

between somatic cells extracted from spheroids at different developmental stages. Kirk and Kirk found that shortly after the completion of embryogenesis the polypeptide labeling pattern of the two cell types is nearly identical. The synthetic patterns of the two cell types begin to diverge after a few hours, which they correlate with the breakdown of the numerous cytoplasmic bridges involved with cellular migration during embryogenesis (see Introduction, Asexual Development). It appears that the breakdown of cytoplasmic bridges restricts the intercellular transport of cytoplasmic messages, thereby localizing protein synthetic patterns to the individual cell types. By the time the gonidia are mature there are more differences than similarities in the proteins synthesized by somatic cells and gonidia. As the gonidia progress in development through embryogenesis, stage specific changes in polypeptide synthesis are numerous.

Kirk et al., (1983) demonstrated that two extracellular high molecular weight glycoproteins implicated in the control of cellular differentiation during cleavage are not synthesized by the gonidia or the developing embryo as was suggested (Sumper et al., 1980), but instead are labelled in, and therefore presumably synthesized by, the somatic cells of the parental spheroid. Evidence suggests that extracellular matrix proteins are in fact

synthesized by the somatic cells, glycosylated by the Golgi bodies therein, and later discharged by vesicles to their extracellular destinations.

In addition to distinguishing stage specific differences in protein synthetic patterns of the two cell types of Volvox, Kirk and Kirk (1985) noted marked, reversible changes in the pattern of labeling of major polypeptides when cultures were transferred from light to dark or vice versa. The response to light was most noticeable in cells completing embryogenesis (which in light/dark synchrony occurs in the dark). They pursued this finding with the intent of determining whether the proteins synthesized in response to light induction were the products of newly transcribed messengers, or the results of translational control. Proteins produced by the in vitro translation of RNA's isolated from unilluminated and illuminated juveniles were not distinguishable by electrophoresis, nor did actinomycin have an inhibitory effect on the synthesis of these proteins. They conclude that light exerts its activation effect on protein synthesis in juvenile spheroids at the translational level, and implicate this translational regulation as the means by which light/ dark synchronization of the Volvox life cycle operates on a molecular basis.

It should be noted that no doubt transcriptional

control of cellular differentiation exists in Volvox. Histones, the basic chromosomal proteins implicated in genetic regulation, have been isolated from Volvox carteri f. nagariensis strain HK10 (Bradley et al., 1974). Darden cites the formation of a complex between a nucleic acid containing extract of V. aureus and calf thymus histones that induces differentiation of male spheroids in asexual individuals of the species (Darden, 1973).

F. The extracellular matrix.

The matrix is composed of protein and carbohydrate. Treatment of Volvox spheroids with proteases will solubilize the matrix and yield a suspension of isolated cells (personal observation). Ethanol precipitation of the suspension yields a white precipitate consisting of one third protein and two thirds carbohydrate. The principal sugars present are arabinose and galactose. Amino acid analysis reveals that 21% of the residues are hydroxyproline, thus resembling the glycoprotein "extensin" described by Lamport as an important component of higher plant cell walls (Kochert, 1975).

As was discussed in detail with respect to the various aspects of Volvox development, morphogenesis and differentiation of asexual spheroids, as well as the

normal functioning of the sexual life cycle, have been intimately linked to the synthesis and deposition of the extracellular matrix. Growth of Volvox spheroids is dependent on the deposition of the gelatinous proteoglycan matrix surrounding the individual vegetative and reproductive cells during the expansion phases of the asexual life cycle. The mature daughter spheroids, formed within the reproductive gonidia, are released from the parent when the matrix is enzymatically dissolved (Jaenicke and Waffenschmidt, 1979). At maturity a proteolytic enzyme is activated locally and lyses the somatic sheath so that the progeny are liberated and become mobile. When the offspring have been released, the empty somatic sphere disintegrates and the somatic cells undergo senescence (Pommerville and Kochert, 1982). The enzyme is excreted into the medium and has been purified and identified as a neutral protease which requires Ca^{++} ions for activity. The enzyme seems to be regulated by an inhibitor which is destroyed when the algae mature. The Volvox release enzyme was found to resemble the cell wall lysine produced by mating Chlamydomonas for the liberation of zoospores both in its mode of action and in its biochemical properties (Jaenicke and Waffenschmidt, 1981).

The developmental and morphogenetic significance of the matrix is demonstrated by the isolation of induced

morphological mutants. These mutants dissociate at an early stage of embryogenesis, releasing free gonidia and somatic cells, due to the absence of a normal matrix (Sessoms and Huskey, 1973).

The process of glycoprotein synthesis in Volvox matrix requires membrane bound glycosyl transferase as does the mammalian system, and is inhibited by tunicamycin (Muller et al., 1981; Jaenicke, 1982). The inhibition of protein glycosylation by tunicamycin has been shown to interfere with normal developmental processes linked to matrix synthesis in Volvox including morphogenesis and sexual induction (Kurn and Duksin, 1982).

Matrix components have recently been implicated in numerous aspects of Volvox embryogenesis. Sumper investigated the appearance of a high molecular weight sulfated glycoprotein during embryogenic cell divisions and implicated this matrix component in the determination of cleavage patterns in the juvenile spheroids (Sumper, 1979). A short lived hydroxyproline-rich matrix glycoprotein that appears in inverting Volvox embryos has been characterized (Schlipfenbacher, 1986).

Matrix components have been implicated in the sexual induction of Volvox spheroids by binding sexual inducer hormone molecules (Gilles et al., 1983, 1984, 1985). The mating reaction itself involves the enzymatic dissolution of the extracellular matrix by the sperm bundles, a

Ca^{++} activated process that resembles the acrosin reaction of mammalian sperm. The sperm bundles dissociate and the individual sperm move in the gelatinous matrix, which becomes more fluid.

Early investigations of the extracellular matrix of Volvox were of a histochemical and ultrastructural nature. Using histochemical techniques Burr and McCracken (1973) identified a sulfated polysaccharide component in the surface layer of Volvox spheroids. McCracken and Barcello (1976) studied the ultrastructure of the intact sheath of Volvox rousseletii within which are embedded the flagellated somatic cells. They found that the sheath is composed of fibrillar material arranged in a characteristic pattern. The outer boundary is composed of a tripartite structure consisting of a two electron dense layers separated by an electron transparent layer. Internal to the tripartite structure is fibrous material which becomes disperse as it nears the protoplasts. Based on ruthenium red staining the internal fibrillar material has been identified as containing acid mucosaccharide. Periodic acid Schiff stain revealed the presence of glycoproteins in the internal matrix. The various staining techniques utilized indicated that carbohydrate containing materials are integral components of the Volvox matrix, just as they are important surface components of many types of cells, implicated in

cell-cell adhesion and cell surface specificity.

The site of synthesis of the carbohydrate containing matrix macromolecules has been localized to the Golgi vesicles of the somatic cells (Dauwalder et al., 1980). Comparing the ultrastructure of the normal HK10 strain and a matrix dissociator mutant, Dauwalder, Whaley, and Starr demonstrated the transport of materials from the rough endoplasmic reticulum to the Golgi apparatus, and assembly therein of a fibrillar material which is then transported to the cell surface in secretory vesicles. The mutant was found to lack formation of fibrillar material, however increases in the number of Golgi stacks were seen, indicating that the assembly of membrane components by the Golgi vesicles continued even though sheath materials were not secreted.

Kirk, Birchem, and King, (1986) have recently reported a comparative electron microscopic investigation of the structure of the extracellular matrix of different species of the genus Volvox. They describe the existence of intricate variations of matrix architecture within the genus with the conservation of many morphological features. They devised a nomenclature to describe and define four specific regions of the matrix; the flagellar zone (FZ), consisting of attachments of the extracellular matrix (ECM) around the flagella; the boundary zone (BZ), consisting of portions of the ECM that are continuous

over the outer surface of the organism (excluding the flagellar areas); the cellular zone (CZ), consisting of specializations of the ECM surrounding the individual cells; and the deep zone (DZ), consisting of the components of the matrix that fill the central region of the organism, internal to the cellular region.

The variations of compartment architecture and morphological composition exhibited by the various members of the Volvocales, and the different species of Volvox were compared. The most striking differences were observable in the modifications of the periflagellar zones and in the relative amount of total spheroid volume occupied by the deep, amorphous region lying interior to the gonidial cells. Both of these regions of the extracellular matrix have been implicated as regions of evolutionary significance.

The flagellar region is involved in the mating reaction, with flagellar interaction being a sexual recognition mechanism in the Volvocales (Coggin et al., 1979). Sexual agglutinins of Chlamydomonas resemble components of the extracellular matrix in both composition (by containing hydroxyproline rich glycoproteins) (Cooper, 1983; Snell, 1985) and in three dimensional structure (Goodenough et al., 1985). Two very closely related strains of Volvox, V. carteri f. nagariensis and V. carteri f. weismania, that do not mate

nor have cross inducibility, are found to differ significantly in the morphology of their periflagellar regions.

The existence of the cellular zone distinguishes multicellular Volvocales from unicellular forms, and the existence of a distinct deep zone within the spheroid matrix was found to distinguish higher from lower multicellular forms. There appears to be an evolutionary transition from almost no central lumen in small colonial forms such as Pandorina, to Volvox, where the deep zone occupies as much as 90% of the spheroid volume in some species. This may be of evolutionary significance (Goodenough, 1985) especially in view of developmental control mechanisms recently attributed to proteoglycans, glycoproteins, and cyclic AMP localized in the extracellular matrix of Volvox.

Kirk et al. (1985) found the most conserved morphological feature of the Volvocalean extracellular matrix to be the external tripartite layer that surrounds every individual in the order, whether the organism is unicellular (e.g. Chlamydomonas), colonial (Gonium, Pandorina, Eudorina), or truly multicellular (Volvox). The external layer of Chlamydomonas has been studied in detail and found to contain hydroxyproline rich glycoproteins. Fulton (1978, I and II) compared the ultrastructural composition of the colonial matrix of

Pandorina to the outer surface of Chlamydomonas and found many similarities, and the ultrastructure of various species of Volvox has been analyzed (Lang, 1965; Crayton, 1982).

When combined with biochemical and genetic analyses, the histochemical and ultrastructural studies of the extracellular matrices of the Volvocales will no doubt enlighten us as to the evolutionary phylogeny of extracellular architecture in this ancient and diverse lineage.

RATIONALE AND PLAN

To fully comprehend development in Volvox, it is essential to understand the composition of the extracellular matrix and how the matrix changes during growth and maturation. It is also advantageous to understand normal versus abnormal patterns of Volvox development and the concomitant biochemical changes in the matrix.

Studying the extracellular matrix of Volvox requires isolating the matrix away from cellular material. This necessitates the creation of methods for; extraction of the matrix, fractionating the matrix, assaying matrix components, as well as electrophoretic techniques for distinguishing specific matrix polypeptides.

The above experimental protocols allows for identification of changes in the extracellular matrix during development, and for determination of whether the dramatic increase in size that occurs during expansion is due to deposition of new or different matrix components, or merely to the imbibition of water. The first goal is to characterize the extracellular matrix and follow the matrix composition throughout the life cycle, observing

any stage specific changes that may occur. The use of radioactive isotopes was necessary in order to pin point the time of synthesis of the identifiable matrix components. Comparison of the normal matrix of two strains of Volvox, HK10 and NB7, which are morphologically and developmentally very similar, yet which react with differing sensitivity to protease treatment might reveal critical polypeptide differences. Abnormal development in the matrix mutant, DIS 111, should also reveal critical matrix constituents when its extracellular components are compared to the matrix of the normal HK10 spheroid.

Since the intention of this thesis is to address the issue of the extracellular matrix as a function of development, it is therefore an attempt to identify key proteins present in the matrix of normal Volvox. Electrophoresis was used to compare polypeptide banding patterns of matrix proteins isolated from different strains of Volvox, from the normal HK10 spheroid at different stages in the developmental cycle, and from the spheroid with a defective pattern of matrix synthesis.

Lastly, the phenomenon of palmelloid "matrix" formation in Chlamydomonas was approached to see whether any homology exists between the matrix of the palmelloid cluster and the extracellular matrix of the related Volvocales, Volvox.

MATERIALS AND METHODS

I. Maintenance of cultures, growth, and synchronization.

A. Strains:

Volvox carteri f. nagariensis strain HK10 (female) and Volvox carteri f. weismania strain NB7 (female) (Volvox strains HK10 and NB7, kindly supplied by Dr. R. Starr) were used in all studies of normal development. For the purpose of comparison with the normal extracellular matrix, DIS 111 (kindly supplied by Dr. R. Huskey), a mutant of strain HK10 that has an abnormal pattern of extracellular matrix deposition, was utilized. DIS 111 dissociates immediately after inversion and fails to exist as a mature spheroid.

B. Culture medium and growth conditions:

Axenic cultures of Volvox were maintained in SVM (synthetic Volvox medium, Appendix section II). Stocks were kept at room temperature under constant

illumination of 350-400 foot candles provided by a cool white fluorescent light source. Working cultures were maintained with constant aeration under conditions of 24 or 48 hour illumination cycles (16 hours light/ 8 hours darkness or 31 hours light/ 17 hours darkness).

Volvox grows normally at pH 7.0-8.0 and temperatures of 25⁰C-32⁰C. At pH 7.0 and lower temperatures (<30⁰C) cultures grow more slowly and are heterogeneous. At pH 8.0 and higher temperatures the growth cycle is accelerated to 48 hours and growth remains synchronous even in 10 liter cultures. Working cultures were maintained at pH 8.0 and 30⁰C whenever yields of synchronous organisms were necessary.

C. Culture vessels:

Stock cultures were maintained in loosely capped 15x150mm culture tubes containing 10 ml SVM.

D. Preparation of innoculum for heterogeneous growth of Volvox.

Tubes containing ~750 spheroids/ 10 ml were transferred into 1 liter Ehrlenmeyer flasks containing 500 ml SVM pH 8.0. After four days growth the flasks were inoculated directly into 10 gallon carboys containing 10 liters of

SVM.

E. Preparation of innoculum for synchronous growth of Volvox.

Stock cultures were grown well into the stationary phase, ensuring a large population of spheroids whose individuals accumulated at the pre-cleavage stage. The smallest spheroids were collected by phototaxis. The densely populated culture was exposed to a directed light source in an enclosed, darkened, sterile area and the spheroids allowed to concentrate at the mouth of the culture vessel. Aliquots of 5 ml were transferred into large culture tubes ("banana tubes") containing 300 ml SVM pH 8.0.

The cultures were aerated using electronic air pumps with the air filtered through bacterial air vents (Gelman Sciences, Ann Arbor, MI).

F. Radioactive cultures.

Aliquots of 5 ml were withdrawn from an exponentially growing culture of Volvox and transferred into 25 ml of sterile SVM, pH 8.0, to give a final concentration of 750 spheroids/ tube. Radioactive isotope was added to the cultures which were loosely capped and aerated by

constant shaking at room temperature. Aliquots were removed, and the spheroids washed, counted, and frozen at specified times.

In order to pulse label Volvox at specific stages of their life cycle, spheroids were withdrawn from synchronous cultures and treated as described.

Radioisotopes used were; $\text{NaH}^{14}\text{CO}_3$ (released from $\text{Ba}^{14}\text{CO}_3$, 0.05-0.1uCi/ml), ^3H -arginine (5 uCi/ml), ^3H -proline (1 mCi/ml), ^{14}C -proline (50 uCi/ml).

G. Maintenance of axenic cultures.

Stocks and cultures were monitored for bacterial contamination by sterily transferring aliquots onto nutrient agar plates and monitoring for bacterial growth.

II. Harvesting of cultures.

A. Non-radioactive cultures.

Non-radioactive cultures were harvested by collecting spheroids at the desired stages on either Miracloth (All Purpose Cloth, Chicopee Mills, Inc. Milltown, N.J.) or Nitex monofilament cloth (Tetko, Inc. Precision Woven Screening Media, Elmsford, N.Y.). Depending on the

volume, cultures were either poured, pipetted directly, or siphoned onto the cloth.

Volvox to be resuspended for counting or determination of viability were washed with SVM. Spheroids used for matrix extraction were washed with distilled water to prevent interference with the biochemical assays. Samples were removed for microscopic examination, measured with an ocular micrometer in order to determine size and stage of the life cycle, and frozen until used.

Synchronous cultures were sequentially filtered through 250 μ m Nitex which retained the large pre-release spheroids, 125 μ m Nitex which retained the condensed pre-cleavage spheroids, and 80 μ m Nitex, through which passed the very small Volvox (nascent spheroids). Although light/ dark induced synchrony was effective in producing stage specific Volvox, size selective filtration ensured optimal homogeneity of Volvox at specific stages (see Appendix, section X).

B. Radioactive cultures.

Radioactive cultures were filtered through Nitex monofilament cloth and the filtrate monitored for radioactivity. The spheroids were then washed exhaustively and the filtrate reexamined to determine that no background radioactivity remained. Samples

containing 20 μ l of spheroid suspension were removed in triplicate using a Sigma micropipette, dried onto Whatman 2.4 cm glass fiber circles (Whatman GF/A, W & R Balson Ltd., England), inserted into scintillation vials, 4 ml scintillant (containing 0.4 g PPO and 50 mg POPOP/liter toluene, NEN) added, and the radioactivity measured in a Beckman liquid scintillation counter.

III. Isolation of Volvox cells and extraction of cellular protein.

It was necessary to separate the Volvox extracellular matrix from the two cell types in order to analyze cellular and extracellular components individually. The problem of separation was approached from two aspects, chemical and mechanical.

A. Chemical separation:

Volvox spheroids (strain HK10) were incubated in the presence of protease (Protease type V, Sigma), 100 μ g/ml, for four hours at 24°C with shaking. Microscopic examination showed the matrix to have been dissolved, individual cells released, and no intact spheroids present. Isolated cells were washed repeatedly with SVM

pH 8.0 in the presence of the protease inhibitor PMSF, centrifuged gently in a Sorvall superspeed RC2-B automatic refrigerated centrifuge at 6,000 x g for 10 minutes, and resuspended in extraction buffer (0.05 M Tris-Cl, pH 7.2, 0.05 M sucrose, 0.01 M MgCl_2 , 0.005 M B-mercaptoethanol).

Cells were disrupted by breakage at 0°C in the omnimixer with glass beads at top speed, and centrifuged at 15,000 x g for 25 minutes. The supernat was used as a crude protein extract as described later (see Methods, section VIII; preparation of samples for electrophoresis).

Cell lysates were diluted 1:1 with electrophoresis sample solvent, placed in a boiling water bath for two minutes, cooled, and layered into wells of 7.5% or 10% SDS-polyacrylamide slab gels (Electrophoresis - see Methods, section VIII).

B. Mechanical separation:

Dounce Tissue Grinder:

The most effective means of disrupting spheroids and obtaining maximal yields of cells and matrix is by gentle mechanical breakage using a glass homogenizer fitted with a ground glass pestle.

A 10 l culture of Volvox was harvested by filtration

through two layers of Miracloth. The collected spheroids were resuspended in 100 ml SVM pH 8.0 and spheroid density determined by microscopic examination. 0.2 ml samples were transferred to depression slides, stained with IKI, and counted. Spheroids were totally disrupted by seven full strokes with the loose fitting "A" ball. This method is gentle enough to leave cells intact and viable as determined by observation.

Disrupted spheroids were passed through 300 um, 125 um, and 80 um Nitex to separate cells from the matrix sheaths and washed repeatedly with SVM to unclog pores. Gonidia and somatic cells pass through with the filtrate, with any intact spheroids and the large sheaths retained.

To separate the cell types successive size filtration may be utilized. Kirk and Kirk (personal communication) have devised a technique using Ludox density gradients to separate the gonidia and somatic cells (see Appendix, section VI).

C. Extraction of cellular protein.

Sonication (Bronson Sonifier - Cell Disruptor):

Acetone extraction followed by sonication for five minutes at high speed was found to be the most efficient means of cell lysis for protein extraction once the individual Volvox cells were isolated .

The Volvox cells that passed through 125 um nitex were concentrated by centrifugation at 15,000 x g for 10 minutes. The pellets were extracted with four volumes of 90% acetone to remove chlorophyll, air dried, and resuspended in extraction buffer, (see Methods, section III, B). The cell suspension was placed in small plastic centrifuge tubes and sonicated at 0°C using the microtip at high speed for five minutes. The sonicate was incubated with 200 ug/ml DNase (Sigma DN-100) and centrifuged at high speed (15,000 x g) for 20 minutes. The supernat was used as the cell lysate for the determination of cellular protein by the microbiuret method, and for analysis on SDS-polyacrylamide gel discs or slabs. When small quantities of spheroids were used and diluted excessively, an Amicon filtering apparatus was used to concentrate the protein so that the final protein concentration was 3 mg/ml.

IV. Matrix extraction and isolation.

A. Chemical:

1) Characterization of extracted matrix.

The non-ionic detergents Tween 40 and Tween 80 (Sigma) were found to solubilize the extracellular matrix of

Volvox and facilitate cell separation for the purpose of studying cellular DNA (Margolis-Kazan, 1978). In this study, a mixture of these detergents was used as an agent for the extraction of matrix components from the intact spheroids.

Volvox carteri f. nagariensis strain HK10 cultures were grown for five days in 100 ml SVM, pH 8.0/ 250 ml Ehrlenmeyer flasks to a spheroid density of 200 spheroids/ ml, and concentrated to a final volume of 10 ml. A 50% mixture of Tween 40-Tween 80 was added to give a final concentration of 0.05%-1.0% Tween mixture, and the tubes incubated at 24°C with shaking. The medium turned cloudy with the extraction of extracellular matrix components. Spheroids and any free cells were removed by filtration through fine nylon cloth (7 um nitex). Spheroids were then washed and resuspended in fresh SVM, pH 8.0. The spheroids remained intact, viable, and were allowed to continue to develop and reproduce.

The extracted material (the filtrate) was precipitated by $(\text{NH}_4)_2\text{SO}_4$ or TCA added dropwise to a final concentration of 5%. The precipitate was concentrated by centrifugation at 15,000 x g for ten minutes and the supernat discarded. The resulting pellet of the precipitate was neutralized with 1N NaOH, resuspended in distilled water to a total volume of 5 ml, and protein

concentration determined by the microbiuret method. Carbohydrate was determined by staining with PAS (periodic acid Schiff reagent) (see Appendix, section IV).

2) Time course of matrix extraction.

A heterogeneous culture of Volvox with a density of 200 spheroids/ ml in a 250 ml Ehrlenmeyer flask containing 100 ml SVM, pH 8.0, was incubated in the presence of 0.8% Tween mixture (50% Tween 40-50% Tween 80) in a 24°C water bath with shaking. 5 ml aliquots were withdrawn sterilly at 15 minute intervals, filtered through glass wool plugs into 13 x 100 mm test tubes to remove spheroids and free cells, and chilled to 0°C. The samples were assayed for protein content with microbiuret reagent.

3) Time course extraction of ^{14}C labeled matrix.

A 10 ml heterogeneous culture of Volvox carteri f. nagariensis strain HK10 in SVM, pH 8.0., with a spheroid density of 200 spheroids/ ml was made radioactive for 18 hours with $\text{NaH}^{14}\text{CO}_3$ (2×10^7 cpm/ ml). Spheroids were filtered through two layers of Miracloth, washed three times with non-radioactive SVM, resuspended in 5 ml of the same (SVM, pH 8.0), and incubated in the presence of 1.0% Tween mixture in a 24°C water bath

with shaking. Aliquots of 0.5 ml were withdrawn at the indicated time intervals, the spheroids removed by filtering through glass wool, and 20 μ l aliquots of filtrate transferred in triplicate onto Whatman paper filter discs. The filters were dried under an infra red lamp, inserted into scintillation vials, and 4 ml of scintillation cocktail (containing 0.4 g PPO and 50 mg POPOP/ liter toluene, NEN) added. Radioactivity was measured in a Beckman liquid scintillation counter.

4) Electrophoretic analysis of Tween extracted matrix components.

Disposable culture tubes containing heterogeneous spheroids of Volvox carteri f. nagariensis strain HK10 in 10 ml SVM, pH 8.0, at a spheroid density of 200 spheroids/ ml were incubated with appropriate radioactive metabolites for 18 hours (^3H -arginine (5uci/ml, NEN) or $\text{NaH}^{14}\text{CO}_3$ (4×10^5 cpm/ml, released from $\text{Ba}^{14}\text{CO}_3$, ICN, see Appendix, section I). Spheroids were washed and resuspended in non-radioactive medium containing 1% Tween mixture. After three hours of incubation in a 24°C water bath with shaking, the medium was filtered through glass wool and the extracted matrix components precipitated with cold TCA at a final concentration of 5% for one hour at 4°C . The precipitate was prepared for electrophoresis as follows.

The precipitate was neutralized by the addition of 1N NaOH with the use of the indicator dye bromocresol purple, redissolved in sample buffer (1% SDS, 10% sucrose, 1% mercaptoethanol, and 0.05M tris-Cl, pH 6.8), and placed in a boiling water bath for two minutes. Samples were kept in plastic microfuge tubes for immediate use or frozen for later use.

SDS-polyacrylamide gel slabs (1.5 mm thick x 11 cm long) were prepared according to the method of Laemmli (1970). The separating gel was 10% acrylamide-bisacrylamide, tris-Cl pH 8.8, overlaid with a stacking gel of 5% acrylamide, tris-Cl pH 6.8, and was pre-electrophoresed in electrode buffer (6.0 g/l tris, 28.8 g/l glycine, 1% SDS) at 50V overnight (using a Heathkit power supply). Samples of 30-60 ul (containing 5-10 ug protein and 20 ul 0.05% bromophenol blue as a tracking dye) were layered onto each of thirteen sample wells. Electrophoresis was performed for five hours (50V in the stacking gel, 100V in the separating gel) or until the tracking dye reached within one centimeter of the bottom of the gel.

The gel slab was removed from the glass plates, the proteins fixed in 50% TCA for one hour at 4°C and visualized by staining with Coomassie Brilliant Blue (0.1% in 40% TCA at 37°C for thirty minutes followed by destaining in 7% glacial acetic acid at room temperature

overnight, and/ or by scintillation autoradiography. The fluorographic procedure used was that devised by Bonner and Lasky (1974).

The gel slab was immersed twice in twenty volumes dimethylsulfoxide (DMSO) for thirty minutes to remove all water, followed by treatment with a mixture of 10% W/W PPO in DMSO (four times the volume of the gel slab) for three hours. The gel was then soaked in twenty volumes of water for one hour to precipitate the PPO. The PPO impregnated gel was dried onto filter paper (Whatman 3MM chromatography paper) and exposed to Kodak PP royal X-omat film (5x7 sheets) for eighteen hours to visualize ^{14}C labeled proteins or forty eight hours to visualize ^3H labeled proteins.

B. Mechanical extraction:

Physical separation of the extracellular matrix was accomplished by thawing frozen Volvox spheroids and disrupting spheroids using the Dounce tissue grinder (see Methods, section III). Thawed Volvox were kept on ice and one third volume distilled water added when necessary. Aliquots of ~7 ml were subjected to seven full strokes of the Dounce homogenizer with the loose fitting "A" ball, or until microscopic examination showed the sheaths to be disrupted and no intact spheroids

present. Cellular breakage was avoided at all times. If any given homogenization of an aliquot of Volvox proved to be too severe, and cellular breakage occurred, that portion (i.e. those spheroids) were discarded.

The resulting mixture of cells, sheaths, and matrix ("crude spheroid lysate") was filtered through a Buchner funnel fitted with a circle of 80 um Nitex overlaid upon a circle of Miracloth cut to fit the inner diameter of the funnel. A vacuum was attached and the viscous liquid pulled through, all the while gently lifting clogging material (cells and sheaths) from side to side with a blunt spatula. Evacuation was continued until all the liquid was removed. This step was repeated using washed filters until all the thawed spheroids had been disrupted and the matrix extracted.

The filtrate was then passed slowly, one aliquot at a time, through a millipore scintered glass filter, fitted with Whatman glass fiber paper. The glass fiber paper was replaced with a fresh circle for each aliquot of lysate. The vacuum was retained until the GF/A was dry. The resulting clear, highly viscous filtrate was termed the "crude matrix prep".

The crude matrix prep was transferred to a 250 ml round bottom flask, quick frozen at -70°C , and lyophilized. The dried matrix material was weighed and resuspended in distilled water or an appropriate buffer.

Aliquots of 0.5 ml were assayed for protein and carbohydrate using the methods of Lowry (Appendix, section III) and Dubois (Appendix, section IV) respectively. The extracted matrix was frozen until used.

V. Protein purification.

A. Exclusion gel chromatography:

1) Sepharose 4B column chromatography.

Sepharose 4B (Pharmacia) beads were hydrated in buffer (0.02 M tris-Cl, 0.01 M NaCl, pH 8.0 or 0.1 M sodium phosphate, pH 7.8, see Appendix), with 0.02% sodium azide added to prevent bacterial contamination, for about five hours at room temperature. A 2.5 x 56 cm chromoflex chromatography column was packed with a 5% slurry, allowed to settle, and small particles removed. The column was equilibrated with the above buffer. A 55 ml void volume was determined using Blue Dextran in the elution buffer. 2-2.5 ml of the crude matrix preparation resuspended in buffer were layered onto the column. The column was eluted with two volumes of buffer at room temperature and 4.5 ml fractions collected.

Fractions were assayed for protein according to the method of Lowry, and for carbohydrate following the

method of Dubois as described. Absorbancy was measured at 750 nm (for protein) and 480 nm (for sugar) in a Gilford spectrophotometer using glass cuvettes.

The fractions comprising the protein peaks were pooled and dialyzed exhaustively against distilled water at 4°C to remove all salts (Spectrapor membrane tubing). This desalting step was essential to any further resolution of matrix proteins. A first change was made 1-2 hours after dialysis was initiated, with four more changes of distilled water over 48 hours. The dialysate was then transferred to 25-50 ml round bottom flasks, quick frozen by gently swirling in liquid propanol at -70°C, and concentrated by lyophilization. The resulting powder was weighed and frozen for storage.

2) Sephadex G-200 column chromatography.

Sephadex G-200 gel filtration was used to determine whether the glycoprotein component resolved by fractionation of the Volvox extracellular matrix was a single entity or a comigration of non-covalently linked entities.

a. Non-radioactive matrix glycoproteins were isolated from spheroids by fractionation on a column of Sepharose 4B as described. Glycoprotein containing fractions were concentrated by lyophilization, resuspended in buffer (0.02 M tris-Cl pH 8.0, 0.1 M NaCl, 0.01% sodium azide)

to a total volume of 2 ml, and layered onto a 2.5 cm x 45 cm column of Sephadex G-200 beads (Pharmacia) equilibrated with the same buffer. The column was then eluted with two volumes of buffer and 4.5 ml fractions were collected. Samples were then assayed for protein and carbohydrate according to the methods of Lowry and Dubois respectively.

b. Glycoproteins made radioactive with $\text{NaH}^{14}\text{CO}_3$ were isolated on minicolumns of Sepharose 4B as described. The radioactive glycoproteins were concentrated by lyophilization, resuspended in buffer as in "a", and chromatographed on mini columns of Sephadex G-200. Fractions of 0.5 ml were collected and monitored for radioactivity as described.

B. Ion exchange chromatography: DEAE cellulose.

A 1 ml sample of thawed extracellular matrix extracted from spheroids of strain HK10 was equilibrated with 1 ml of starting buffer (0.05M tris-Cl pH 8.5, 0.01% sodium azide). The sample was applied to a column of DEAE cellulose (Whatman) which had also been equilibrated with starting buffer. The column was eluted at room temperature with a linear gradient of 0-0.5 M NaCl dissolved in the same buffer. Fractions were assayed for

the presence of protein and carbohydrate. The column was re-equilibrated after each use.

VI. Matrix isolation and protein purification from V. carteri f. nagariensis strain HK10 mutant DIS 111, V. carteri f. weismania strain NB7, and synchronous cultures of strain HK10.

A. DIS 111.

Cells of V. carteri f. nagariensis strain HK10 dissociator mutant DIS 111 (isolated by Griffin and Husky) were transferred from stock tubes containing 10 ml SVM into 125 ml Ehrlenmeyer flasks containing 75 ml SVM pH 8.0. Cultures were maintained under conditions of 16 hours light/ 8 hours dark. After growth of five days - one week cultures of DIS 111 were transferred by aliquots to the Dounce tissue grinder, gently homogenized 1-2 strokes to avoid cellular damage, and filtered through glass fiber circles fitted over a millipore filter. The filtrate was dialyzed against distilled water at 4°C to remove salts, lyophilized, and the resulting powder resuspended in chromatography buffer as above. Protein and carbohydrate content were determined by the methods of Lowry and Dubois as described.

Sepharose 4B columns were equilibrated with buffer as above and 2.5 ml of the DIS 111 preparation were layered onto a column. A 60 ml void volume was collected and then the column connected to the fraction collector. Fractions containing 4.5 ml were collected and assayed for the presence of protein and carbohydrate as above. The glycoprotein containing fractions were combined, dialyzed, and lyophilized.

B. NB7.

Samples of V. carteri f. weismania strain NB7 were grown and harvested as described in Methods sections I and II. The extracellular matrix was isolated essentially the same way as described for normal V. carteri f. nagariensis strain HK10. The only critical difference was found to be in the increased sensitivity of the NB7 spheroid to homogenization. It was therefore essential to modify the extraction procedure for cultures of NB7 by diluting the thawed, concentrated Volvox with distilled water, and limiting the number of strokes using the Dounce tissue grinder. Only the "A" ball could be used, and it was essential to examine the spheroids microscopically after each stroke.

NB7 crude matrix lysate was then treated as described in section V for the preparation of crude matrix and

protein purification from cultures of strain HK10.

C. Protein purification from synchronous cultures of V. carteri f. nagariensis strain HK10.

Volumes of 500 ml SVM pH 8.0 in large culture tubes with 1 liter capacity were inoculated with overgrown stock tubes of HK10 and maintained in constant temperature water baths at 30°C with a light/ dark cycle of 31 hours light/ 17 hours dark. Transfers were made after the release of juvenile spheroids from the maternal organism. Young spheroids were concentrated by phototaxis and 10 ml transferred to fresh medium. Transfers were made every two days (i.e. 1X life cycle).

1) Post release:

Newly released Volvox (see figure 1) were harvested immediately after release. The release process was controlled by transferring cultures with spheroids beginning to release (determined by microscopic observation) to a dark enclosure and stopping aeration until 80%-90% of the maternal spheroids released their juveniles.

Cultures were filtered through 80 um Nitex as described, and the filtrate with the nascent spheroids was pipetted through 7 um Nitex. The nascent spheroids were trapped on

the Nitex, gently scraped off with a spatula, and the filter washed. The spheroids were washed with distilled water and frozen.

2) Pre-release:

Fully expanded Volvox were grown as above and harvested after cleavage and inversion were completed and as the first spheroids in the culture were beginning to release.

The fully expanded Volvox were trapped on 300 um Nitex. Spheroids at the pre-release expansion stage are extremely viscous, and the culture must be diluted considerably with distilled water to remove adhering material (i.e. smaller spheroids and sheath material). The spheroids are examined microscopically, measured with an ocular micrometer, and frozen in 20 x 150 mm culture tubes.

VII. Preparation of radioactive matrix proteins.

A. Labeling:

Spheroids were made radioactive by incubation with ^3H -proline, ^{14}C -proline, or $\text{NaH}^{14}\text{CO}_3$, as follows. Five ml aliquots of Volvox at the appropriate stage were withdrawn from a heterogeneous or synchronous

culture grown in a 1 liter culture tube, and transferred into sterile culture tubes containing 5 ml sterile SVM to give a final spheroid density of ~750 spheroids/ tube. Radioactive isotope was added and the cultures shaken at low speed at room temperature in the light for 18 hours or pulse labeled accordingly.

The spheroids were harvested by pipetting the radioactive culture onto 7 μ m Nitex monofilament cloth and washing repeatedly with nonradioactive SVM or distilled water. The spheroids were then monitored for specific activity by removing 20 μ l samples in triplicate, drying them onto 2.4 cm GF/A discs, counting the number of spheroids, adding scintillant, and measuring the radioactivity in a Beckman scintillation counter. The labeled spheroids were frozen until used.

B. Extraction of radioactive matrix:

Radioactive spheroids were thawed, resuspended in distilled water, and homogenized using the Dounce tissue grinder until microscopic examination showed the spheroids to be disrupted. The homogenate was filtered through GF/A circles fitted onto a mini-millipore filter. The filtrate was concentrated by lyophilization, and the resulting powder weighed and frozen.

C. Exclusion gel chromatography:

The radioactive matrix preparation was resuspended in chromatography buffer and 20 ul samples transferred onto GF/A discs, dried, and radioactivity measured. A 0.5ml sample of radioactive matrix was layered onto a mini-column (1 cm x 56 cm) of Sepharose 4B equilibrated with phosphate buffer as described. The column was then eluted with buffer, a 15 ml void volume was determined, and 0.5 ml fractions were collected.

Fractions were assayed for carbohydrate and protein using microassays of the methods used for non-radioactive matrix. 20 ul of each fraction were then transferred onto Whatman GF/A filters (2.4cm diameter). The filters were dried, inserted into scintillation vials, and 4 ml of toluene based scintillation cocktail (Liquiflour, NEN) added. Radioactivity was measured with a Beckman LS-150 Liquid Scintillation Counter.

The fractions containing radioactivity were combined and dialyzed exhaustively against distilled water. The dialysate was lyophilized, weighed, and frozen for storage.

VIII. Electrophoretic analysis of matrix proteins.

A. SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel slabs (7.5% or 10% polyacrylamide) were cast according to a modification of the discontinuous buffer system of Laemmli (1970) using a concentration of acrylamide: methylenebisacrylamide of 30:0.8 (Eastman Kodak) with a separating gel buffer of tris-Cl pH 8.3. The separating gel was overlaid with a 5% stacking gel in a buffer of tris-Cl pH 6.8. TEMED was included in the buffers, the solutions degassed, and ammonium persulfate added to polymerize the gels. The gels were cast by pouring the acrylamide solutions between glass plates (16 cm x 20 cm, Bob's Machine Shop, Aquebog, N.Y.) held together by lucite spacers (1.5 mm thick) with a thin coating of petroleum jelly to create a seal when compressed with binder clips. After the separating gel solidified, the surface was washed free of unpolymerized acrylamide, and the stacking gel cast. A lucite well forming comb was inserted and the gel, overlaid with distilled water, allowed to polymerize overnight. Running buffer was prepared and samples were prepared according to a modification of the method of Weber and Osborn (1975).

Samples of 25 ul-75 ul were layered into the wells

using a 50 ul Hamilton syringe. A 25 ul sample of a mixture of standards including fetuin, transferrin, BSA, and lysozyme (with bromophenol blue added as a tracking dye) was layered into one or both of the outermost wells. Electrodes were attached to a Heathkit power supply and gels were electrophoresed at 100V until the tracking dye reached the bottom of the gel.

Gels were stained as follows:

- 1) Coomassie Blue - Gels were fixed overnight in 100-200 ml CBB (Coomassie Brilliant Blue) fixative (50% methanol, 5% glacial acetic acid, 45% distilled water) and stained for 30-45 minutes in sufficient CBB solution to cover the gels. The gels were destained with 3-4 changes of 100-200 ml destaining solution and allowed to further destain overnight.

- 2) Periodic Acid Schiff (PAS) reagent - Gels were fixed overnight in 100-200 ml PAS fixative solution. The gels were then covered with a 0.7% PAS solution for 2-3 hours followed by treatment with 0.2% sodium metabisulfite for 2-3 hours with one solution change after 30 minutes. After clearing, Schiff reagent color developed in 12-18 hours at room temperature, and the gels stored at 4°C. To intensify the dye reaction, the gels were transferred to 0.2% K-metabisulfite in 5% acetic acid.

3) Concanavalin A was conjugated to LDH and precipitated the glycoproteins in the gels. The glycoproteins were then visualized by the detection of the oxidoreductase activity of the LDH using lactate, NADH, and nitroblue-TTC and obtaining a formazan precipitate (according to the method of Avigad, 1978).

B. SDS-polyacrylamide gradient gels.

5%-15% gradient gels were cast using the same buffer system as described above. 12.5 ml 15% acrylamide solution was layered onto the bottom between the glass plates, followed by 30 ml of a continuous gradient of acrylamide (prepared by mixing equal volumes of 5% and 15% acrylamide solutions in a gradient maker), and overlaid with 5 ml of a 5% acrylamide solution into which the well former was inserted.

1) Preparation of samples:

3-50 μ l of sample in concentrations of 0.5-10 mg protein/ ml in sample solvent were layered into the wells. Lyophilized glycoprotein samples were resuspended in distilled water to a final concentration of 100 μ g/ μ l for later use to be diluted as follows;

a. 10 μ l concentrate (i.e. 100 μ g protein) was dissolved in 90 μ l sample solvent to give a total volume of 100 μ l

with a protein concentration of 10 ug/ul

b. 1/10 dilutions were made in sample solvent to give 1, 2, and 5 ug/ul samples.

3-50 ul of sample in concentrations of 0.5-10 ug protein/ ul in sample solvent were layered into the wells. 3 ul standards in sample solvent with bromophenol blue as a tracking dye were layered into one or both outer wells. Electrophoresis was performed at 150V (25ma --> 10ma) for 3.5 hours and then for 0.75 hours after the tracking dye reached the bottom of the gel. The gel was removed with utmost care so as not to touch any surface of the gel with metal.

2) Silver staining :

Gels were fixed by soaking in 50% methanol in water containing 0.1% of a 38% formaldehyde solution in a pyrex dish for one hour and then changed and soaked in the same overnight. Gels were silver stained according to the method included in the Polysciences, Inc. Silver Stain Kit (1983) which is a modification of Wray and Boulikas (1981).

The fixed gel was agitated for five minutes in 0.1%-38% formaldehyde in water and then washed and swelled five minutes by agitating in distilled water. Solutions were gently aspirated away with a disposable capillary pipette tipped with nylon tubing. The gel was soaked

for 30 minutes in 5 mg DTT/ liter water (made up fresh) or enough to cover the gel.

While the gel was soaking, silver stain and developer were prepared as follows:

Silver stain;

1.4 ml concentrated ammonia was added with stirring to 21 ml 0.36% NaOH in a 250 ml beaker. 0.8 g silver nitrate (Sigma) was inserted into a test tube and dissolved in 4 ml water. One pipetteful of silver nitrate solution at a time was added with stirring to the beaker, followed by 74 ml water. If necessary, the solution was titrated with concentrated ammonia until clear.

Developer;

2.5 ml 1% citric acid and 0.25 ml formaldehyde were added to distilled water in a 500 ml graduated cylinder. The solution was shaken, water added to 500 ml, and shaken again.

After 30 minutes the DTT was decanted, the silver stain added, and the gel agitated constantly for 15 minutes. The silver nitrate stain was decanted into a clean beaker. The gel was rinsed 3X with distilled water, and allowed to sit for 5 minutes in water.

The gel was transferred to developer and incubated until the polypeptide bands were observable (a maximum of 15

minutes). To minimize and slow background stain development, methanol was added to the developer to a final concentration of 1%.

The developer was decanted, stop bath (3% acetic acid in 50% methanol) added, and the gel allowed to soak for at least one hour. To prepare for drying, the gel was transferred to 5% acetic acid in 20% methanol.

Gels were dried onto Whatman chromatography paper using a sintered nylon support over a grooved lucite frame attached to a vacuum. Gels were dried overnight and photographed.

IX. Concanavalin A-agarose affinity columns.

Lyophilized matrix preparations or glycoproteins of V. carteri f. nagariensis strain HK10 were dissolved in adsorbing buffer (0.02M tris-Cl pH 8.0, 3mM Ca++, 3mM Mg++, 1mM Mn++) and layered onto a 1.5 cm x 20 cm chromatography column packed with concanavalin A-agarose and equilibrated with adsorbing buffer. Fractions of 1 ml were manually collected and monitored for protein content by measuring absorbancy at 280 nm.

The column was then eluted with the addition of 0.02 M or 0.05 M sodium borate buffer. Fractions of 1 ml were collected manually and monitored for protein by measuring

absorbancy at 280 nm. After optical density readings returned to background levels, the column was eluted with saturated borate buffer.

All fractions were assayed for protein using the method of Lowry and for carbohydrate using the method of Dubois.

X. Electron microscopy of wild type spheroids of V. carteri f. nagariensis strain HK10 and the dissociator mutant DIS 111.

Normal HK10 spheroids and cell suspensions of the dissociator mutant DIS 111 were prefixed in 1.5% glutaraldehyde buffered in 0.1 M sodium cacodylate (pH 7.2), followed by fixation in fresh 1.5% glutaraldehyde for one half hour at room temperature, and one half hour chilled on ice. Volvox were post-fixed in 1% OsO₄, pH 7.0, with 0.08% or 0.16% ruthenium red stain in 0.1 M sodium cacodylate buffer for three hours at 0°C.

Specimens were washed in 0.1 M sodium cacodylate buffer for ten minutes and dehydrated in a series of ethanol changes to propylene oxide; 50% ethanol (10 minutes), 70% ethanol (overnight), 95% ethanol (10 minutes), 100% ethanol (3 X 10 minutes), propylene oxide (2 x 5 minutes).

Specimens were infiltrated with a 1:1 mixture of Spurr/

propylene oxide for three hours, and then in pure Spurr overnight. The specimens were transferred to beam capsules and embedded in pure Spurr. Polymerization was in a 70°C oven overnight.

The specimens were sectioned with a glass knife, transferred to carbon coated copper grids, and stained with Reynold's (lead citrate) stain. The sections were viewed and filmed using a Zeiss electron microscope (9S-2).

XI. Growth of palmelloid Chlamydomonas and extraction of extracellular components.

Cells of Chlamydomonas reinhardi (wild type strain, + mating type, kindly supplied by Dr. C. Forest) were maintained in stock culture tubes each containing 10 ml "normal" medium for Chlamydomonas (see Appendix, section IX). Stocks were transferred to 35 mm x 300 mm culture tubes containing 150 ml each of the normal medium. After growth of four days in normal medium, under constant illumination and aeration by bubbling, transfers were made into large test tubes containing either 1/8000 M EDTA (12.5×10^{-5} M) or 1/300 M Potassium citrate.

The algal cells were then cultured for two days with continuous illumination in the above medium to induce

palmelloid formation. The cultures were examined microscopically to determine palmelloid formation. The palmelloid Chlamydomonas were harvested by centrifugation at 12,000 x g/ 10 minutes. The pellet was washed and resuspended in distilled water. The suspension was homogenized with the "B" ball of the Dounce tissue grinder until the palmelloid clusters were dislodged and dissociated. The suspension was then filtered through Whatman GF/A circles fitted onto a millipore scintered glass filter and the filtrate transferred to a round bottom flask, quick frozen, and lyophilized to a powder.

The palmelloid preparation was resuspended in phosphate buffer as described, assayed for protein and carbohydrate using the methods of Lowry and Dubois, and layered onto a chromatography column of Sepharose 4B. After elution of the 55 ml void volume, 4.5 ml fractions were collected.

The fractions were assayed for the presence of protein and carbohydrate as described. Fractions containing glycoprotein were combined, transferred into dialysis tubing, and dialyzed, with a minimum of four changes, against distilled water. The dialyzate was transferred to Corex tubes, quick frozen, and lyophilized. The powder was weighed and frozen until use, when it was resuspended in sample buffer, and electrophoresed on SDS-polyacrylamide slab gels.

RESULTS

I. General characterization of the extracellular matrix.

A. The Volvox spheroid; normal development.

Two female strains of Volvox were used in this study; Volvox carteri f. nagariensis strain HK10 and Volvox carteri f. weismania strain NB7.

The extracellular matrix of Volvox is an ideal subject for the study of protein synthesis throughout the Volvox life cycle. The matrix of this multicellular alga is an integral component of the organism and is closely linked to the normal pattern of development (figure 1). Microscopic examination clearly shows that much of the volume of the mature, normal Volvox consists of the extracellular matrix. The two stages of spheroid expansion (figure 1) are causally related to increased matrix deposition. Upon comparing the mass of the intact spheroids to the mass of the free gonidia and somatic cells, it was determined that much of the spheroid mass is noncellular. Protease treatment of Volvox spheroids

Table 1.

A comparison of cell mass/ spheroid mass in Volvox carteri f. nagariensis strain HK10.

Heterogeneous cultures of Volvox were grown in 1 l flasks containing 500 ml SVM, pH 8.0 to a final density of ~200 spheroids/ ml. Spheroids were harvested by filtration through Miracloth, transferred to a pre-weighed beaker, and spheroid mass (wet weight) determined. The spheroids were resuspended in SVM, pH 8.0, containing protease (100 ug/ml, type V, Sigma) for four hours at 24°C with constant agitation, until microscopic examination showed that no intact spheroids remained.

Free gonidia and somatic cells were washed repeatedly in the presence of the protease inhibitor PMSF, centrifuged at 6,000 x g in a pre-weighed centrifuge tube, the supernatant decanted, and the cell mass (wet weight) determined.

RATIO OF CELL MASS/ SPHEROID MASS IN VOLVOX, STRAIN HK10

<u>Spheroids/ ml</u> <u>(500 ml SVM)</u>	<u>Spheroid mass</u> <u>(wet weight)</u>	<u>Cell mass</u> <u>(wet weight)</u>	<u>Total cell mass/</u> <u>Total spheroid mass</u>
200 $\pm$ 5	0.985 g	0.454 g	0.46
200 $\pm$ 5	1.717 g	0.920 g	0.54

causes dissolution of the matrix, freeing the individual cells. Volvox were weighed before and after protease treatment. It can be seen (table 1) that the cells themselves constitute only ~50% of the total spheroid mass.

The normal growth of Volvox is affected by the age and density of the culture. This effect is manifested by the number of spheroids arrested or retarded in the expansion stages or by the rapid growth and development of the spheroids through embryogenesis and the release process (table 2). It may be seen that a greater percentage of the population is engaged in embryogenesis in newly innoculated cultures and in cultures that are less densely populated than in older, denser cultures. The results of table 2 indicate that the greatest number of spheroids in all cultures except the least dense is engaged in the process of post release expansion.

B. The molecular composition of the extracellular matrix.

The extracellular matrix is a gelatinous sheath that surrounds the individual Volvox cells. It forms the communication network between the two cell types, the gonidia and the somatic cells. The developing, nascent spheroids are buffered by the matrix, and it serves as

Table 2.

The Volvox growth cycle: The spheroid population at specific stages of the life cycle as a function of age and density of the culture.

Heterogeneous cultures of Volvox carteri f. nagariensis strain HK10 were grown in 1 liter Ehrlenmeyer flasks containing 500 ml SVM pH 8.0 under illumination conditions of 17 hours light/ 8 hours dark. At the indicated number of days growth, three - five 0.1 ml aliquots were withdrawn, transferred to a depression slide, stained with IKI, and spheroid density determined. A 10 ml sample was withdrawn from the culture, transferred in small aliquots to a slide with an etched grid, stained with IKI, and the spheroids examined microscopically and catagorized according to the developmental stage of the Volvox life cycle.

Age of Culture (# days growth) vs. % Population in Stage of Life Cycle:

Developmental Stage	# Days Growth						
	1	2	3	4	5	6	7
post release expansion	13.3	68.6	70.9	58.0	85.6	65.9	53.5
embryogenesis	33.3	18.1	13.2	33.0	9.9	3.9	18.1
inversion	22.7	1.2	0.9	2.0	9.0	---	2.2
pre-release expansion	26.7	2.4	8.2	6.0	9.0	20.8	19.1
release	4.0	9.6	1.8	1.0	2.7	2.8	4.1

Spheroid Density (# spheroids/ ml) vs. % Population in Stage of Life Cycle

Developmental Stage	<u># spheroids/ ml</u>										
	40	100	116	193	213	313	363	500	560	1,310	1,420
post release expansion	13.3	70.9	68.6	66.7	49.5	86.0	58.0	85.6	70.7	57.5	65.0
embryogenesis	33.3	18.2	18.1	14.9	7.4	11.0	33.0	9.9	1.1	24.8	6.0
inversion	22.7	0.9	1.2	---	0.9	1.0	2.0	9.0	---	3.5	---
pre-release expansion	26.7	8.2	2.4	17.7	25.7	2.0	6.0	9.0	25.8	12.4	24.0
release	4.0	1.8	9.6	0.7	64.0	---	1.0	2.7	2.4	1.8	5.0

Table 3.

A comparison of the percentage of spheroid protein in the extracellular matrix of Volvox carteri f. nagariensis strain HK10 and Volvox carteri f. weismania strain NB7.

Heterogeneous cultures of HK10 and NB7 were grown in 500 ml SVM pH 8.0 for one week. The spheroids were harvested by filtration through double Miracloth and the wet weight determined. The spheroids of each strain were resuspended in 25 ml SVM pH 8.0 and divided into two aliquots each of 12.5 ml. One sample of each culture was treated with 1 mg/ml protease (HK10 in a 36°C water bath with agitation, NB7 at room temperature) for < 1 hour, until microscopic examination showed the spheroids were disrupted. PMSF in fresh SVM was added to each culture and the cells centrifuged at 10,000 x g for 10 minutes. All four cultures were bleached with 3 volumes of acetone and centrifuged at 6,000 x g for 10 minutes. The pellets were resuspended in 3 ml of 10% NaOH at room temperature overnight.

A 0.4 ml aliquot of 1% CuSO₄ was added to each suspension, mixed vigorously, allowed to stand for 10 minutes, and centrifuged at 10,000 x g for 10 minutes. The protein content of the cultures was determined by measuring the absorbancy of each supernat at 560 nm.

VOLVOX : STRAINS HK10 AND NB7

% Total Spheroid Protein in the Extracellular Matrix

<u>Sample Volvox</u>	<u>wet weight</u>	<u>protein/ ml</u>	<u>matrix protein/ spheroid protein</u>
HK10 intact	2.03 g	5.42 mg	1.58/5.42=29%
HK10 pronase	1.015 g	3.84 mg	
NB7 intact	2.84 g	3.53 mg	1.41/3.53=39%
NB7 pronase.	1.42 g	2.12 mg	

Table 4.

A comparison of the protein content of mechanically extracted extracellular matrix to the protein content of intact spheroids of Volvox carteri f. nagariensis strain HK10 at specific developmental stages of the life cycle.

Synchronous cultures of HK10 in 500 ml SVM, pH 8.0, were filtered through Nitex cloth, examined microscopically to determine stage homogeneity, and resuspended in 10 ml distilled water. Aliquots of 0.1 ml were removed, stained with IKI, and the spheroid density determined. The spheroids were divided into two culture tubes in volumes of 5 ml:

- 1) Chlorophyll was extracted with three volumes of acetone chilled to 0°C, centrifuged at 10,000 x g/ 10 minutes, and the pellet air dried, resuspended, and frozen in 0.1 ml distilled water. Spheroids were resuspended in distilled water and lysed by sonication.
- 2) Spheroids were concentrated by filtration, resuspended in 2.5 ml distilled water, and disrupted using the Dounce tissue grinder. The matrix was filtered through glass wool and frozen.

All volumes were brought to 2.5 ml with distilled water. Protein content was determined by the method of Lowry.

Table 4 (ctd.)

A₁, A₂ = Pre-release expansion;

1 x 10⁴ spheroids/ml.

B₁, B₂ = Post release expansion;

2 x 10⁴ spheroids/ml.

C₁, C₂ = Pre-release expansion;

6 x 10³ spheroids/ml.

D₁, D₂ = Embryogenesis, (18 hours after release,

dark stage); 1.5 x 10⁴ spheroids/ml.

H₁, H₂ = Heterogeneous culture;

4 x 10³ spheroids/ml.

RATIO OF MECHANICALLY EXTRACTED MATRIX PROTEIN TO TOTAL SPHEROID PROTEIN

Sample	Spheroid Density	Stage	Source of Protein	ug protein/ml	matrix protein/ spheroid protein
A ₁	1 x 10 ⁴ spher/ml	pre-release	spheroid lysate	203.5	A ₂ /A ₁ =0.36
A ₂	1 x 10 ⁴ spher/ml	pre-release	matrix extract	74.2	
B ₁	2 x 10 ⁴ spher/ml	post release	spheroid lysate	570.5	B ₂ /B ₁ =0.19
B ₂	2 x 10 ⁴ spher/ml	post release	matrix extract	107.9	
C ₁	6 x 10 ³ spher/ml	pre-release	spheroid lysate	131.5	C ₂ /C ₁ =0.48
C ₂	6 x 10 ³ spher/ml	pre-release	matrix extract	62.5	
D ₁	1.5 x 10 ⁴ spher/ml	embryo-genesis	spheroid lysate	847.0	D ₂ /D ₁ =0.26
D ₂	1.5 x 10 ⁴ spher/ml	embryo-genesis	matrix extract	218.6	
H ₁	4 x 10 ³ spher/ml	hetero-geneous	spheroid lysate	468.0	H ₂ /H ₁ =0.39
H ₂	4 x 10 ³ spher/ml	hetero-geneous	matrix extract	187.0	

the cytoskeleton of the mature organism.

The percentage of total spheroid protein present in the matrix was determined for both strains HK10 and NB7 by comparing total spheroid protein to cellular protein after dissolution of the matrix by protease treatment (table 3). Matrix protein constitutes ~30% of total spheroid protein in strain HK10, and ~40% in strain NB7.

It may be seen (table 4) that the percentage of total spheroid protein present in the extracellular matrix varies as a function of the developmental stage of the organism. As the spheroid expands there is an increase in the relative amount of assayable extracellular matrix protein present in the spheroid, compared to total spheroid protein. The results of table 4 show that extracellular matrix protein, mechanically extracted from newly released spheroids arrested during post release expansion, comprises less than 20% of total spheroid protein. The protein content of the matrix relative to total spheroid protein increases through embryogenesis. The mechanically extracted matrix of the fully expanded spheroid is seen to comprise >40% of the total protein present in the Volvox spheroid at this stage of development.

Table 5 indicates that the molecular composition of the extracellular matrix, as determined by the ratio of carbohydrate to protein in the matrix extracted at

Table 5.

Ratios of carbohydrate/ protein of extracellular matrix mechanically extracted from spheroids of V. carteri f. nagariensis strain HK10 and V. carteri f. weismania strain NB7 at specific developmental stages of the life cycle.

HK10 and NB7 spheroids were grown heterogeneously or in synchronous cultures, harvested by filtration, and frozen. The cultures were thawed, disrupted using the Dounce tissue grinder, and the matrix concentrated by lyophilization. The crude matrix preparation was resuspended in the appropriate volume of 0.1 M phosphate buffer, pH 7.8, with 0.02% sodium azide added, for fractionation on exclusion columns of Sepharose 4B. Aliquots of 0.05 ml were withdrawn and assayed for protein and carbohydrate content according to the methods of Lowry and Dubois respectively.

RATIO OF CARBOHYDRATE TO PROTEIN IN VOLVOX MATRIX

<u>Strain</u>	<u>Stage and Size</u>	<u>ug CHO/ml</u>	<u>ug protein/ml</u>	<u>Ratio CHO/protein</u>
HK10	heterogeneous	125	62	2.02
HK10	heterogeneous	155	82	1.89
HK10	heterogeneous	80	40	2.0
HK10	heterogeneous	105	52	2.02
HK10	heterogeneous	110	63	1.74
HK10	newly released	22	20	1.1
HK10	newly released, 150um	60	58	1.03
HK10	newly released, 100-150um	40	40	1.0
HK10	vacuolated, 200-250um	48	25	1.92
HK10	pre-cleavage, 200um	120	100	1.2
HK10	pre-cleavage, 200um	45	42	1.07
HK10	pre-cleavage, 200um	25	20	1.25
HK10	pre-release, 350um	160	70	2.29
HK10	pre-release, 350um	160	55	2.99
HK10	pre-release, 350-400um	150	48	3.12
NB7	heterogeneous	95	40	2.38
NB7	heterogeneous	78	47	1.66

* CHO/ ml matrix extract; protein/ ml matrix extract
 0.05 ml matrix preparation in 1 ml distilled water
 Assayed for CHO by the method of Dubois
 Assayed for protein by the method of Lowry.

specific stages of the life cycle, increases as a function of spheroid development. There is a 1:1 ratio of carbohydrate to protein present in matrix mechanically extracted from newly released spheroids. The carbohydrate content of the matrix is not observed to increase significantly as the spheroid expands prior to embryogenesis. The most prominent increase in the ratio of matrix carbohydrate to protein occurs during the stage of pre-release expansion. Here increased synthesis of polysaccharide components of the matrix (figures 21 and 26) produce a matrix carbohydrate to protein ratio of ~3:1.

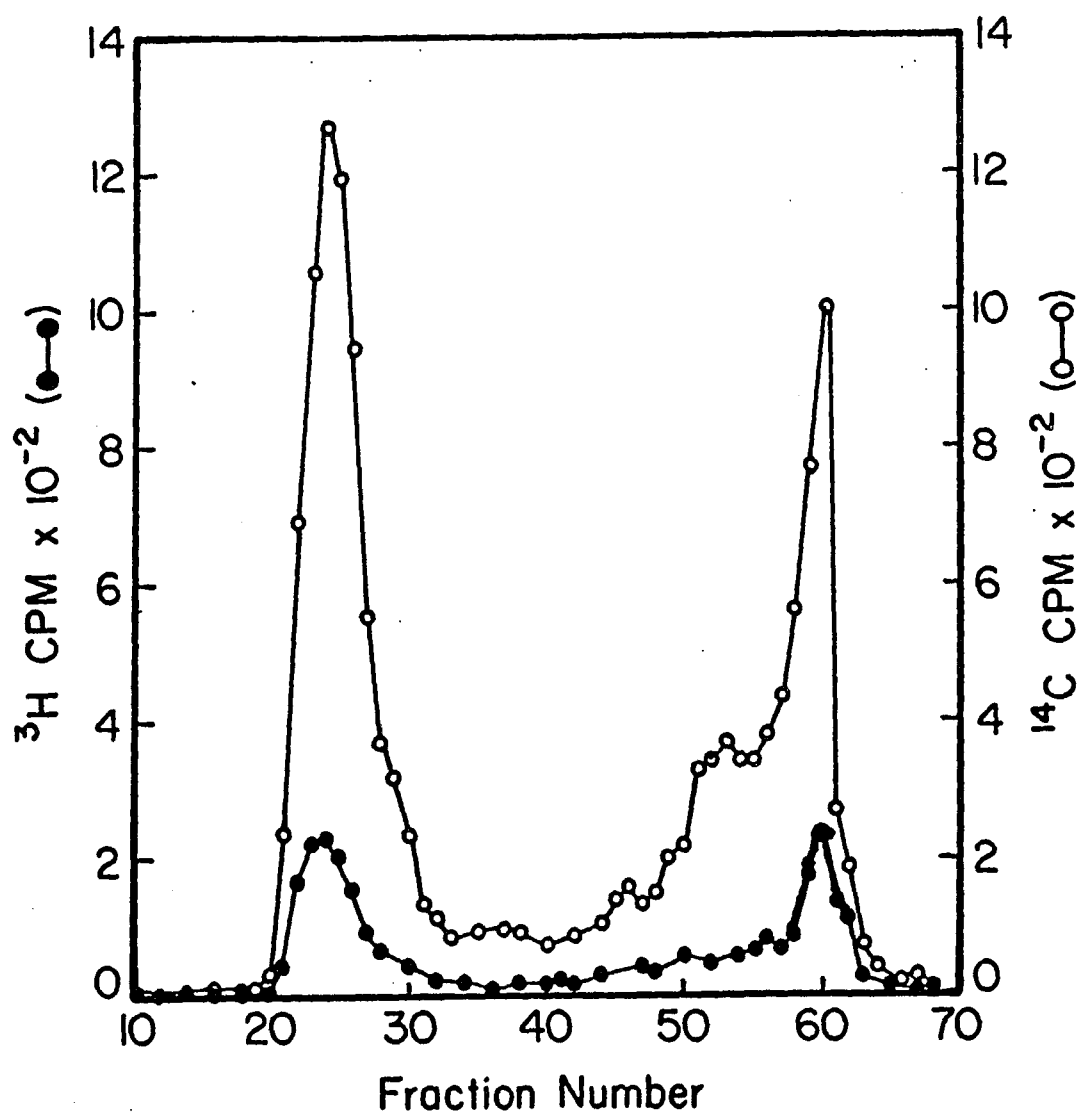
C. Radioactive isotope incorporation into the extracellular matrix.

^3H -arginine and ^3H -proline are incorporated readily by Volvox (Kirk and Kirk, 1978). Although arginine has been determined to be the amino acid most efficiently incorporated into Volvox and Chlamydomonas proteins, arginine comprises <2% of Volvox matrix amino acids whereas hydroxylated proline comprises 21% of the amino acid residues of the matrix (Kochert, 1975). Proline is therefore the amino acid of choice for the incorporation of radioactive amino acids into Volvox matrix. $\text{NaH}^{14}\text{CO}_3$ may also be utilized successfully in studies

Figure 2.

Radioactive isotope incorporation into the extracellular matrix of heterogeneous spheroids of V. carteri f. nagariensis strain HK10: $\text{NaH}^{14}\text{CO}_3$ and ^3H -proline.

A 10 ml heterogeneous culture of HK10 was made radioactive with 0.3 ml $\text{NaH}^{14}\text{CO}_3$ stock, and 75 μl ^3H -proline. The culture was incubated at room temperature overnight with constant agitation. The spheroids were harvested by filtration through 7 μm Nitex cloth, washed with 10 volumes of distilled water, and disrupted using the Dounce tissue grinder. The extracellular matrix was filtered through glass fiber filters and concentrated by lyophilization. The radioactive matrix was resuspended in 0.75 ml of 0.1 M sodium phosphate buffer, pH 7.8, with 0.02% sodium azide added, and 10 μl aliquots were withdrawn from each matrix preparation in triplicate to measure radioactivity. A 0.7 ml sample of resuspended matrix (2.3×10^4 cpm $^{14}\text{C}/10\mu\text{l}$, 5.1×10^3 cpm $^3\text{H}/10 \mu\text{l}$) was layered onto a Sepharose 4B mini column equilibrated with 0.1 M sodium phosphate buffer. The column was eluted with two volumes of 0.1 M sodium phosphate buffer and seventy fractions of 0.5 ml were collected. Aliquots of 20 μl were withdrawn from each fraction, transferred to GF/A filter discs, dried, scintillant added, and radioactivity measured in a Beckman liquid scintillation counter.



of radioactive isotope incorporation, since Volvox, being a photoautotroph, readily utilizes bicarbonate.

It was necessary to determine whether ^3H -proline and $\text{NaH}^{14}\text{CO}_3$ were incorporated into the extracellular matrix of Volvox and that incorporation of these radioisotopes was not limited to cellular components. HK10 spheroids were made radioactive with both ^3H -proline and $\text{NaH}^{14}\text{CO}_3$ and the matrix isolated by mechanical extraction using the Dounce tissue grinder. The isolated matrix was fractionated on a chromatography column of Sepharose 4B. It was determined that both ^3H -proline and $\text{NaH}^{14}\text{CO}_3$ are incorporated into extracellular components (figure 2).

Radioactive isotope incorporation experiments were performed on both NB7 and HK10 using $\text{NaH}^{14}\text{CO}_3$, ^{14}C -proline, and ^3H -proline, in order to determine the relative efficiency of these three radioactive isotopes for the preparation of radioactive extracellular matrix to be extracted from both strains of Volvox.

The results of the radioactive isotope incorporation experiments seen in figure 3, led to the conclusion that $\text{NaH}^{14}\text{CO}_3$ was the radioactive isotope of choice for the most efficient means of preparing radioactive extracellular matrix to be extracted from Volvox spheroids. It may be seen that both $\text{NaH}^{14}\text{CO}_3$ and ^{14}C -proline are incorporated into extracellular

Figure 3.

Radioactive isotope incorporation into the extracellular matrix of V. carteri f. nagariensis strain HK10 and V. carteri f. weismania strain NB7.

Heterogeneous cultures of HK10 and NB7 were grown in 1 liter culture tubes for 4 days and 10 ml aliquots sterilly transferred to eight culture tubes. The cultures were made radioactive with;

Tube 1 = HK10, 0.5 ml $\text{NaH}^{14}\text{CO}_3$ stock.

Tube 2 = HK10, 50 μl ^3H -proline.

Tube 3 = HK10, 50 μl ^{14}C -proline.

Tube 4 = HK10, no radioactive isotope.

Tube 5 = NB7, 0.5 ml $\text{NaH}^{14}\text{CO}_3$.

Tube 6 = NB7, 50 μl ^3H -proline.

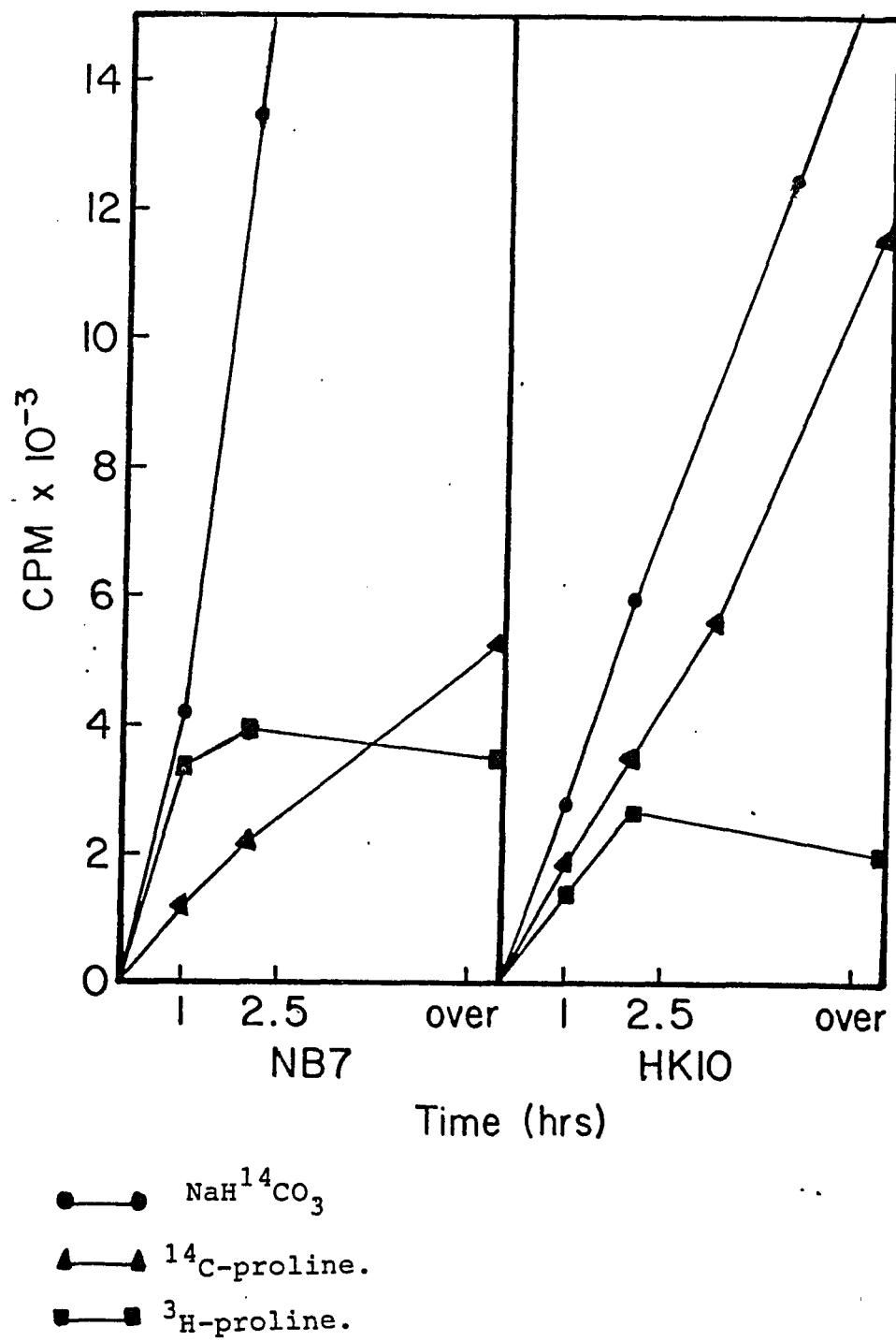
Tube 7 = NB7, 50 μl ^{14}C -proline.

Tube 8 = NB7, no radioactive isotope.

The spheroids were incubated for a total of 24 hours at room temperature, with constant illumination and agitation. Samples containing 2 ml were withdrawn from each non-radioactive culture and spheroid density was determined by staining with IKI; HK10 = 727 spheroids/ 2 ml, NB7 = 707 spheroids/ 2 ml.

At the indicated times 2 ml were withdrawn from each radioactive culture, spheroids were washed with 10 volumes of non-radioactive SVM, and frozen. When all the

samples had been collected, spheroids were thawed and the volumes of all tubes brought to 2 ml with distilled water. The spheroids were disrupted with the Dounce tissue grinder, the extracellular matrix filtered through glass fiber filters, and the matrix chilled. Aliquots of 10 ul were withdrawn from each tube, transferred onto GF/A filter discs, dried, scintillant added, and the radioactivity measured with a Beckman liquid scintillation counter.



components of both HK10 and NB7 linearly with time. The incorporation of ^3H -proline was observed to decrease after 1.5 hours of increasing incorporation, for both strains studied. Although increasing incorporation of both $\text{NaH}^{14}\text{CO}_3$ and ^{14}C -proline into matrix components occurred throughout the 24 hour labeling period, radioactive bicarbonate was consistently incorporated with greater efficiency into the matrices of both HK10 and NB7 (as measured by the extraction of radioactive matrix over the time period followed).

II. Extraction of Volvox extracellular matrix.

A. Chemical extraction:

In order to characterize the matrix components it was necessary to isolate the matrix from the cells of the spheroid. Volvox extracellular matrix may be extracted by various solubilizing agents.

Non-ionic detergents.

The use of non-ionic detergents is well documented as a method for extracting membrane and cell surface proteins (Helenius and Simons, 1975) because of their non-denaturing mode of interaction with the extracted

extracellular components. Margolis-Kazan (1978) pretreated Volvox with a mixture of the non-ionic detergents Tween 40 and Tween 80 to facilitate matrix solubilization for the extraction of Volvox DNA.

In the study reported here, Volvox spheroids were treated with a 1:1 mixture of Tween 40/ Tween 80. Microscopic observation showed that the spheroids remained intact and viable following Tween treatment as determined by their motility, normal development, and ability to reproduce when transferred to fresh SVM. It was therefore concluded that Tween extracted "solubilized" components were non-essential to the viability of the organism.

Overgrown cultures of Volvox (personal observation) and other algae appear cloudy with much non-essential extracellular material released into the growth medium (Voigt, 1985). Volvox proteins spontaneously released into the medium may be isolated and quantitated (table 6). Tween treated cultures have a similar cloudy appearance, with extracted matrix components released into the medium. The non-ionic detergents Tween 40 and Tween 80 were therefore used to facilitate the extraction of matrix components from the Volvox spheroids.

Tween extracted matrix components were precipitated with 5% TCA at 4°C, centrifuged, and the supernat discarded. The Tween extracted matrix was determined to

Table 6.

Proteinaceous material released into the medium by cultures of Volvox carteri f. nagariensis strain HK10.

Overgrown cultures of V. carteri strain HK10, free of contamination, were checked for spheroid density by removing 0.10 ml samples in triplicate and staining with IKI. A 10 ml sample with spheroid density of 340 spheroids/ ml was then filtered through glass wool. The filtrate was assayed using the microbiuret method to determine the presence of protein in the medium.

0.25 ml = 0.081 mg = 324 ug/ml

0.50 ml = 0.223 mg = 446 ug/ml

1.00 ml = 0.358 mg = 358 ug/ml

It was determined that 376 ug protein/ ml were present in the medium.

be glycoprotein in nature on the basis of biochemical and histochemical analysis. The pellet was neutralized in 1 N NaOH and resuspended in SVM to a total volume of 5 ml. The "pellet" tested positive for both protein (3 mg/ml using microbiuret reagent) and carbohydrate (stained with PAS - periodic acid Schiff reagent) (see Appendix, sections III and IV).

After 24 hours in 1% Tween mixture lipid components were removed, as seen by the accumulation of lipid droplets in the extracted material. Spheroid viability decreased, as determined by the inability of the organism to recover and resume motility and normal development when transferred to fresh medium.

Ionic detergents.

Volvox cells were penetrated readily by the ionic detergent SDS (sodium dodecyl sulfate). After five minutes of incubation with 0.01% SDS at room temperature chlorophyll was extracted and spheroid viability, as determined by recovery upon transfer to fresh medium, decreased. Tubes containing SDS treated Volvox were observed to have bleached cellular debris precipitated from a green solution.

1) Extraction of Volvox extracellular matrix using Tween.

Volvox HK10 spheroids were incubated for two hours in

0.8% Tween mixture and 5 ml aliquots withdrawn at 15 minute intervals. The samples were filtered through either 7 um Nitex or glass wool in order to remove spheroids and isolated cells from the solubilized matrix components.

Tubes containing the extracted matrix components were assayed for protein content using microbiuret reagent. Under these conditions of incubation with the non-ionic detergent Tween, a period of rapid extraction of proteinaceous material is followed by a period of extraction at a reduced rate. It may be seen from the results of figure 4 that 75% of the total extracted matrix protein was extracted in <45 minutes. Only 25% additional matrix protein was extracted over the remaining 1.25 hours of incubation with Tween. After 1.5-2 hours the total concentration of protein extracted was ~1 mg/ml (~5 ug protein/spheroid).

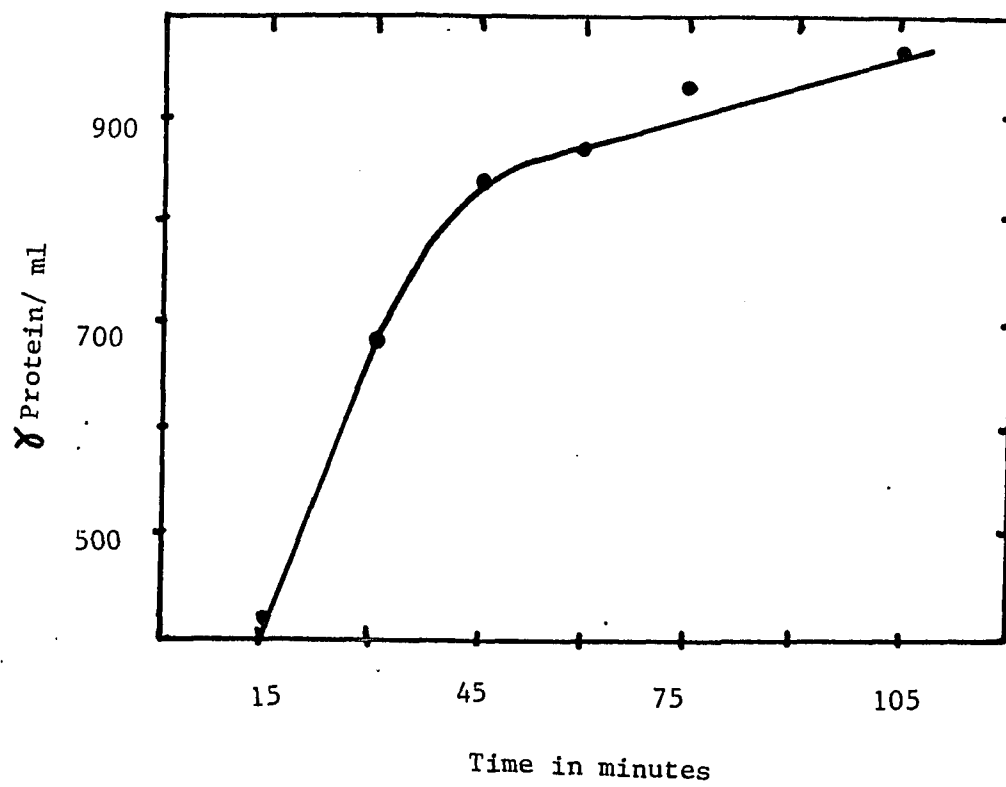
2) Extraction of radioactive matrix components from Volvox spheroids of strain HK10 using Tween.

Spheroids withdrawn from a heterogeneous culture of HK10 were made radioactive with $\text{NaH}^{14}\text{CO}_3$ for 18 hours, washed and resuspended in non-radioactive SVM. Matrix material was extracted by a 1.5% concentration of a 1:1 mixture of the non-ionic detergents Tween 40/ Tween 80 over a period of 1.5-3 hours with samples withdrawn

Figure 4.

Time course of extracellular matrix extracted from a heterogeneous culture of Volvox carteri f. nagariensis strain HK10 using a 1:1 mixture of the non-ionic detergents Tween 40/ Tween 80.

A heterogeneous culture of HK10 was transferred to a 250 ml ehrlenmeyer flask containing 100 ml SVM, pH 8.0, to yield a spheroid density of 200 HK10 spheroids/ ml. A 1:1 mixture of Tween 40/ Tween 80 was added to the culture to a final concentration of 0.8%. The culture was incubated in a 24°C water bath with constant agitation. aliquots of 5 ml were withdrawn sterilly at 15 minute intervals and filtered through glass wool to remove spheroids and free cells. The samples were assayed for protein content with microbiuret reagent using BSA as a standard (1 o.d. unit A 290 = 362 mg protein).



and radioactivity measured at the indicated time intervals.

The results obtained under these conditions for the extraction of radioactive matrix seen in figures 5 and 6 showed a positive correlation with the extraction of non-radioactive matrix by Tween treatment seen in figure 2. There is a period of rapid extraction followed by a leveling off and extraction at a constant rate. The results of figure 6 show that 75% of the total amount of radioactive matrix extracted was extracted during the first 0.75 hours. The rate of extraction leveled off over the next 1.25 hours as an additional 25% of the total radioactive matrix was extracted, at a rate of ~10% total extractible CPM/ hour. After 2 hours of incubation of the spheroids with the detergent, no additional extraction of radioactive matrix occurred.

3) Relative extraction of radioactive matrix from two strains of Volvox, HK10 and NB7.

Samples of spheroids from heterogeneous cultures of HK10 and NB7 were each made radioactive by incubation with $\text{NaH}^{14}\text{CO}_3$ for 18 hours. After resuspension in non-radioactive SVM, the spheroids were incubated separately with a 1:1 mixture of Tween 40/ Tween 80 to a final concentration of either 1% or 2% Tween. Samples were withdrawn at the indicated time intervals and the

Figure 5.

Time course of matrix extraction from radiocative cultures of V. carteri f. nagariensis strain HK10 using a 1:1 mixture of the non-ionic detergents Tween 40/ Tween 80.

A 5 ml sample of spheroids were removed from a heterogeneous culture of Volvox HK10 and transferred to a culture tube containing 5 ml SVM pH 8.0. Each culture was made radioactive with the addition of 0.03 ml ^{14}C -bicarbonate stock (2×10^8 cpm/ml) (total radioactivity 6×10^5 cpm/ml) and the spheroids incubated for 24 hours in a 24°C water bath with agitation. Spheroids were filtered through 7 um Nitex, washed in non radioactive SVM 3X, and resuspended in 5 ml SVM pH 8.0. Tween mixture was added to yield a final concentration of 1.5% and incubated in a 24°C water bath with agitation. Samples of 0.5 ml were removed and filtered through glass wool. Aliquots of 20 ul were withdrawn in triplicate and transferred to Whatman 2.4 cm paper filter discs. Samples were dried, scintillant added, and radioactivity measured using a Beckman liquid scintillation counter.

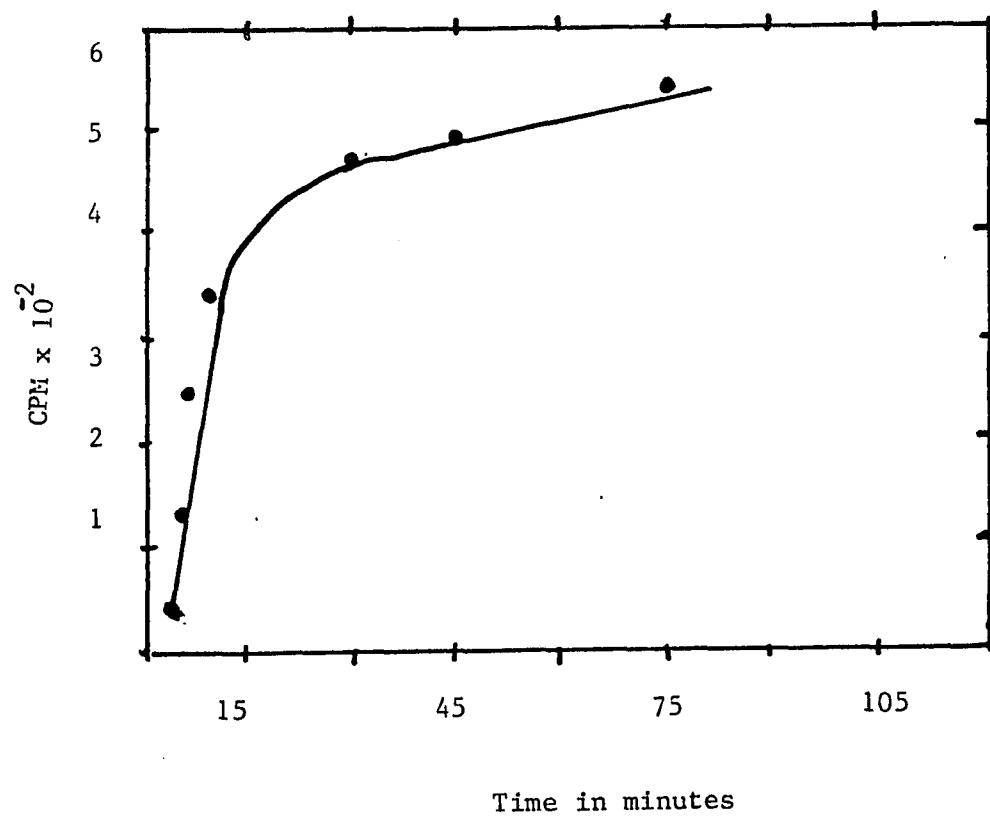


Figure 6.

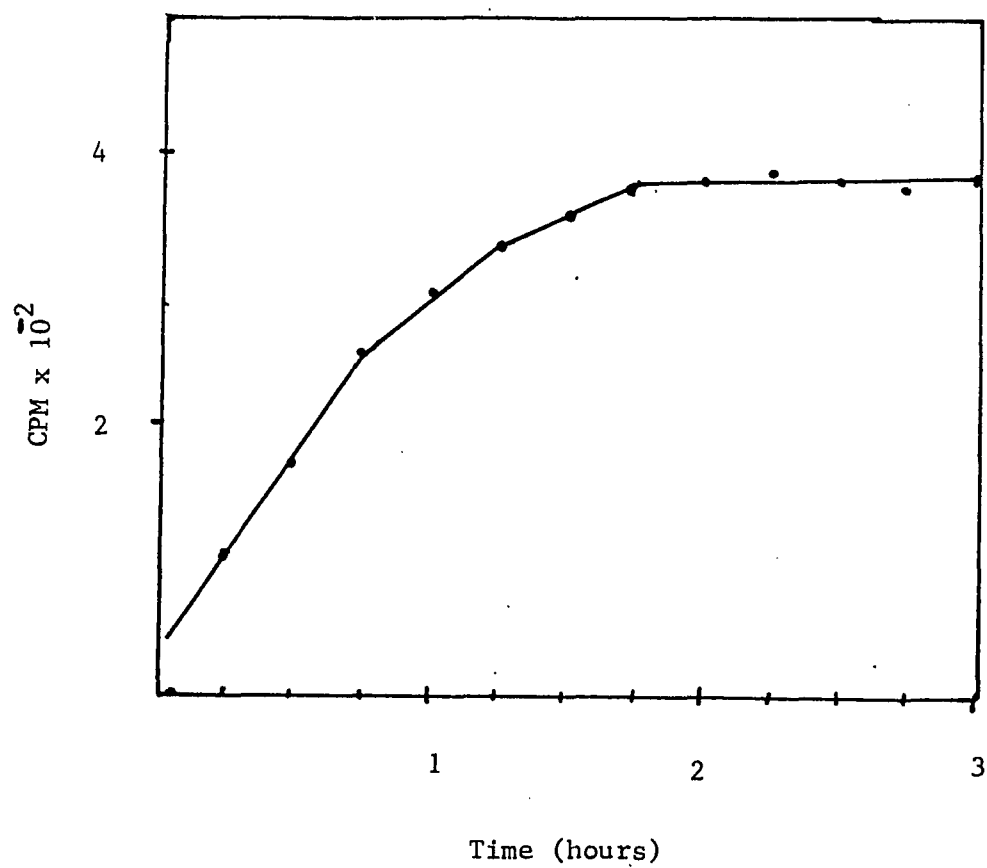
Tween extraction of extracellular matrix components from radioactive cultures of Volvox carteri f. nagariensis strain HK10.

A 10 ml heterogeneous culture of HK10 with spheroid density of $\sim 2 \times 10^3$ /10 ml culture was made radioactive with 0.3 ml $\text{NaH}^{14}\text{CO}_3$ stock (4×10^5 cpm/ml) for a total of 48 hours. Spheroids were washed with 3 volumes of non-radioactive medium and resuspended in 10 ml non-radioactive SVM. The medium was checked for radioactivity before and after washing spheroids to ensure no residual background radioactivity remained.

M_1 (before washing) = 1.571×10^3 cpm/20 μ l sample of medium.

M_2 (after washing) = 31.9 cpm/20 μ l sample of medium.

Spheroids were incubated with 1% Tween at room temperature with agitation for three hours. Aliquots of 0.5 ml were withdrawn at the indicated times and filtered through glass wool. Samples of 20 μ l were withdrawn in triplicate, transferred to 2.4 cm paper filter discs, dried, scintillant added, and the radioactivity measured in a Beckman scintillation counter.



radioactivity monitored.

Both HK10 and NB7 exhibited increased extraction of matrix with increased concentrations of detergent. There was a period of rapid extraction followed by a leveling off with both concentrations of Tween used (figures 7 and 8).

As seen in figure 7, the result of incubating HK10 spheroids with a 2% Tween mixture as compared to incubation with a 1% Tween mixture, was determined to be a 40% increase in extracted radioactive matrix. At the lower concentration of detergent, 67% of total extractible radioactivity was extracted after 30 minutes of detergent incubation, with a "plateau" effect observed, and no further radioactivity extracted after 55 minutes of incubation. At the higher concentration of detergent, 75% of the extractible radioactive matrix was extracted after 45 minutes, with no plateau observed until 1.25 hours of detergent incubation (figure 7).

It may be seen from the results of figure 8 that incubating NB7 spheroids with 2% Tween mixture as compared to 1% Tween mixture increased the extraction of radioactive matrix by nearly 100%. >85% of the total extractible radioactive matrix was extracted during the first two hours of incubation at the lower concentration of detergent, whereas no decrease in the rate of extraction was discernable at the higher detergent

Figure 7.

Extraction of extracellular matrix components of heterogeneous cultures of V. carteri f. nagariensis strain HK10 using two concentrations of a 1:1 mixture of the non ionic detergents Tween 40/ Tween 80.

A heterogeneous culture of HK10 was resuspended in 10 ml of fresh SVM, pH 8.0, at a spheroid density of 7,000 spheroids/10 ml. The culture was made radioactive by the addition of 0.15 ml $\text{NaH}^{14}\text{CO}_3$ stock to yield a final concentration of 2×10^5 cpm/ml. The spheroids were incubated with agitation for 24 hours at room temperature under constant illumination, washed exhaustively and resuspended in non radioactive SVM, pH 8.0.

The spheroids were divided into two aliquots of 5 ml each. Tween mixture was added to one tube to give a final concentration of 1%, to the second tube to give a final concentration of 2%. The cultures were agitated at room temperature for a period of three hours. Samples of 0.5 ml were removed at the indicated times and filtered through glass wool. Aliquots of 20 μl were removed, transferred to paper filter discs, dried, scintillant added, and the radioactivity measured with a Beckman liquid scintillation counter.

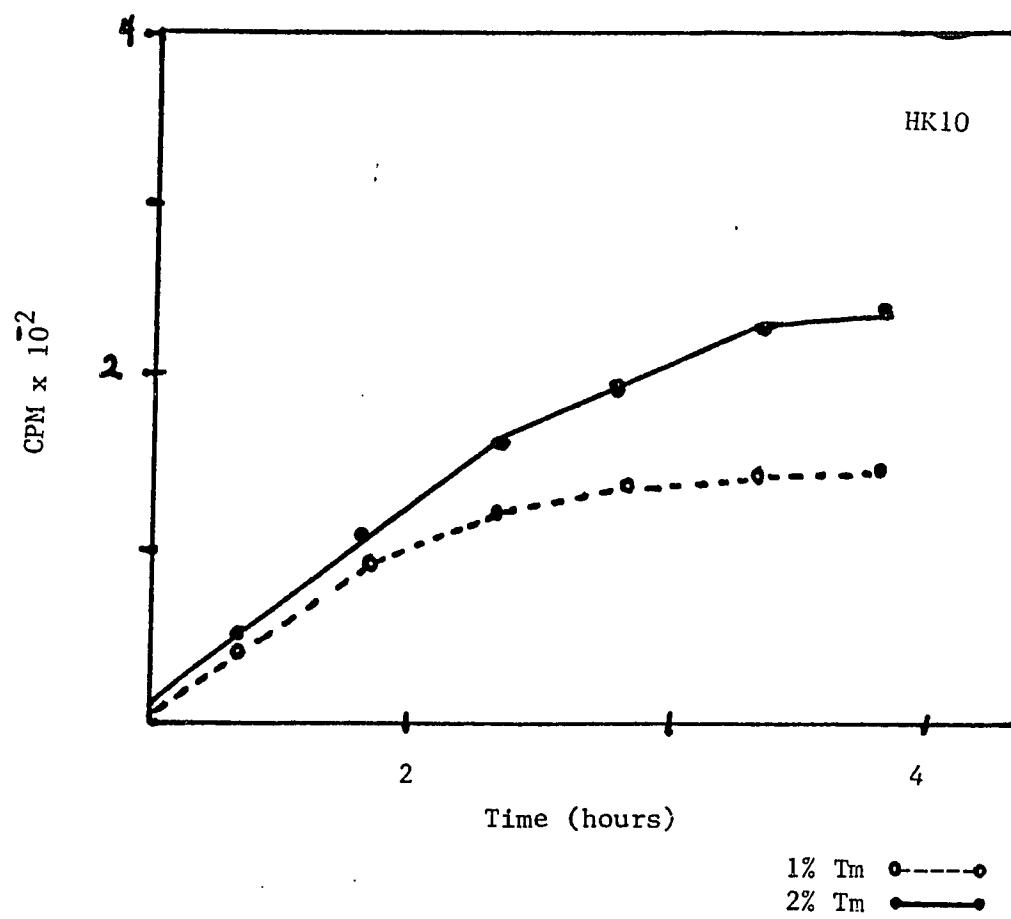
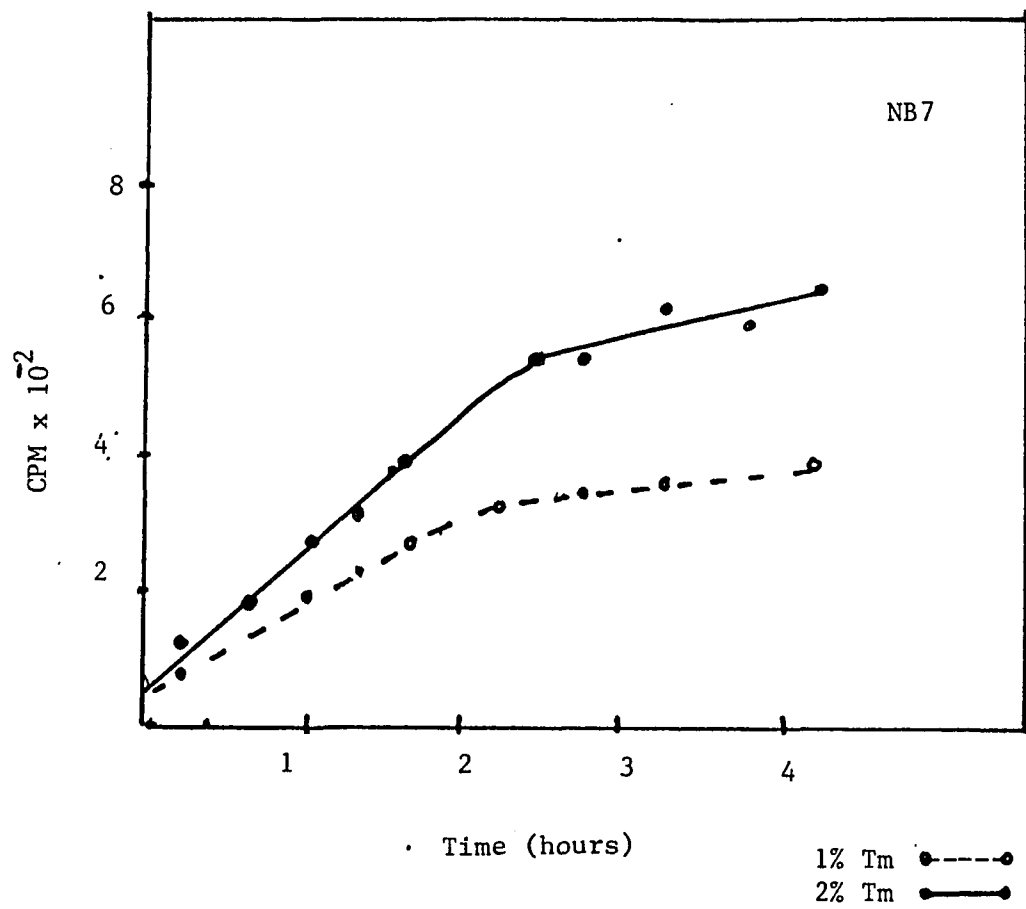


Figure 8.

Extraction of extracellular matrix components of V. carteri f. weismania strain NB7 with two concentrations of a 1:1 mixture of the non-ionic detergents Tween 40 and Tween 80.

Two 5 ml aliquots with a spheroid density of 600 spheroids/ ml were withdrawn from a heterogeneous culture of NB7. The cultures were made radioactive with 0.1 ml (1.34×10^5 cpm/ml) and 0.15 ml (2×10^5 cpm/ml) $\text{NaH}^{14}\text{CO}_3$ stock respectively, and incubated at room temperature with agitation for 18 hours. The cultures were combined, washed, and resuspended in non-radioactive SVM in two tubes each with a total volume of 3.0 ml (1,740 spheroids/ tube).

Tween mixture was added to each tube to final concentrations of 1% and 2% respectively. The tubes were incubated with agitation at room temperature for 4.25 hours. Aliquots of 0.3 ml were removed at the indicated times and filtered through glass wool. Twenty microliter samples of the filtrate were transferred to paper filter discs, dried, scintillant added, and radioactivity measured with a Beckman scintillation counter.



concentration until 0.5 hours more of incubation. After 2.5 hours of incubation of NB7 spheroids with a 2% Tween mixture, >80% of the total radioactive matrix extracted during the 4 hours of incubation was removed from the spheroids.

4) Detergent mediated extraction of Volvox matrix in the presence of a chelating agent.

Heterogeneous HK10 spheroids were made radioactive with $\text{NaH}^{14}\text{CO}_3$ overnight. The spheroids were resuspended in non-radioactive SVM, pH 8.0, with either no EDTA, 2mM EDTA, or 4mM EDTA added as a final concentration. Extracellular matrix components were extracted by the addition of a 1:1 mixture of the non-ionic detergents Tween 40/ Tween 80 to each culture, to yield a final concentration of 2% Tween. The cultures were incubated for 3 hours at room temperature. Samples were withdrawn from each culture at the indicated time intervals and the radioactivity of the extracted matrix measured.

The results of figure 9 show increased extraction of radioactive matrix from cultures incubated with EDTA with no decrease in the extraction rate. After 2.5 hours of incubation with 2% Tween and 2mM EDTA, 50% more radioactive matrix was extracted, as compared to the amount of radioactive matrix extracted using 2% Tween and no EDTA. Incubating cultures with 4mM EDTA resulted in a

Figure 9.

The effect of a chelating agent on detergent extraction of extracellular matrix components of V. carteri f. nagariensis strain HK10.

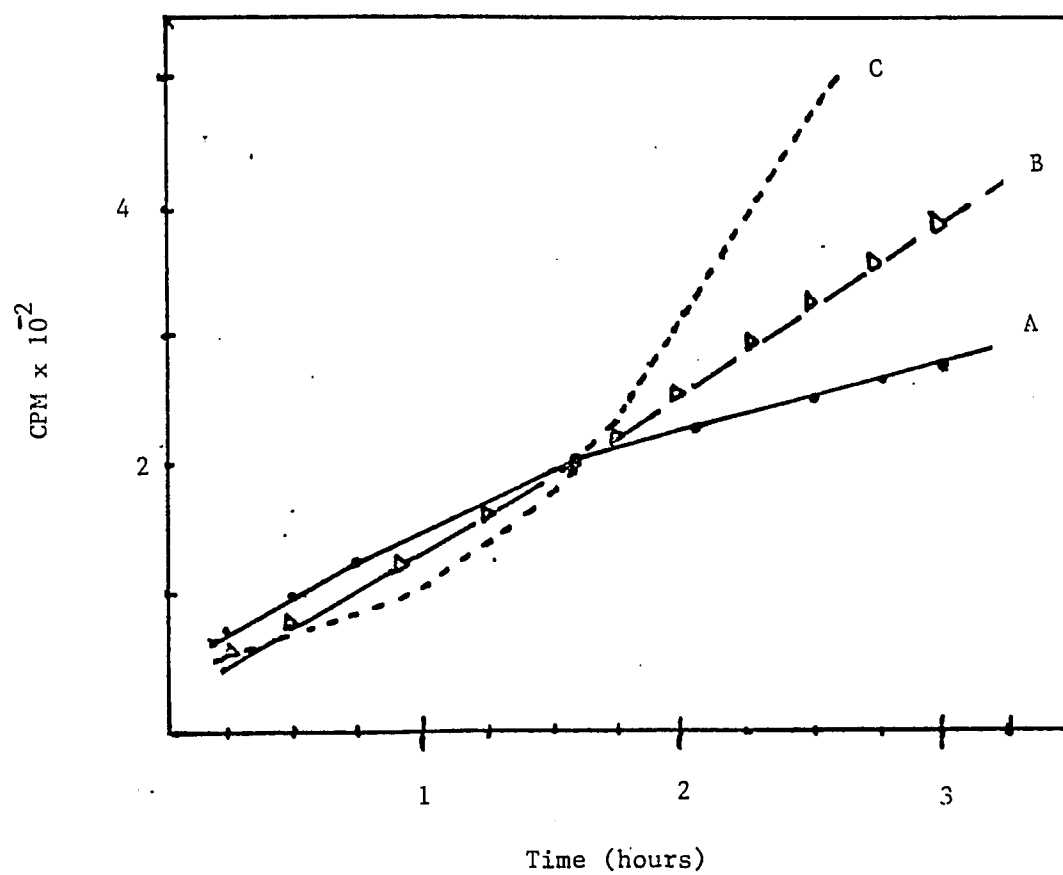
A 10 ml sample of a heterogeneous culture of HK10 with a spheroid density of ~200 spheroids/ml was made radioactive by the addition of $\text{NaH}^{14}\text{CO}_3$ stock (4×10^5 cpm/ml) and incubated for 18 hours at room temperature with constant illumination and agitation. The spheroids were washed with non-radioactive SVM, pH 8.0, and divided into three tubes with 3 ml each of non-radioactive SVM, pH 8.0.

Tube A = 0 mM EDTA

Tube B = 2 mM EDTA

Tube C = 4 mM EDTA

Tween mixture (1:1 Tween 40/ Tween 80) was added to all tubes to a final concentration of 2%. The tubes were incubated at room temperature with agitation for 3 hours. Aliquots of 0.3 ml were removed at the indicated times and filtered through glass wool. Samples containing 20 ul of the filtrate were transferred to paper filter discs, dried, scintillant added, and radioactivity measured with a Beckman liquid scintillation counter.



100% increase in extracted radioactive matrix as compared to cultures incubated with detergent minus EDTA. Increasing the concentration of EDTA in the medium from 2mM to 4 mM increased the extraction of radioactive matrix specifically over the latter 1.5 hours of the 3 hour incubation period (figure 9).

5) The effects of varying conditions of pH and the presence of a chelating agent on Tween mediated extraction of Volvox matrix.

Heterogeneous HK10 spheroids were made radioactive with $\text{NaH}^{14}\text{CO}_3$ for 18 hours. The spheroids were washed and resuspended in individual culture tubes under conditions of spheroid densities of either 350 or 700 spheroids/tube, at pH 5.0 or pH 9.0, and with or without the addition of 2 mM EDTA. A 1:1 mixture of Tween 40/ Tween 80 was added to each culture to yield a final concentration of 2% detergent/ tube. Samples were withdrawn from each culture at the indicated times and the radioactivity of the extracted matrix monitored.

The results of figure 10 show increased extraction of matrix material from all cultures incubated with EDTA as compared to the non-EDTA containing controls. After two hours of incubation, 400% more radioactive matrix was extracted from culture 2BE than from the corresponding control 2B.

Figure 10.

Tween extraction of extracellular matrix components from heterogeneous cultures of V. carteri f. nagariensis strain HK10 under conditions of varying pH and the presence of a chelating agent.

A 25 ml sample of a heterogeneous culture of HK10 with spheroid density ~150 spheroids/ml was made radioactive with $\text{NaH}^{14}\text{CO}_3$ (4×10^5 cpm/ml) overnight. Spheroids were washed and resuspended in tubes of non-radioactive SVM under the following conditions:

1 = 350 spheroids/ tube

2 = 700 spheroids/ tube

A = pH 5.0

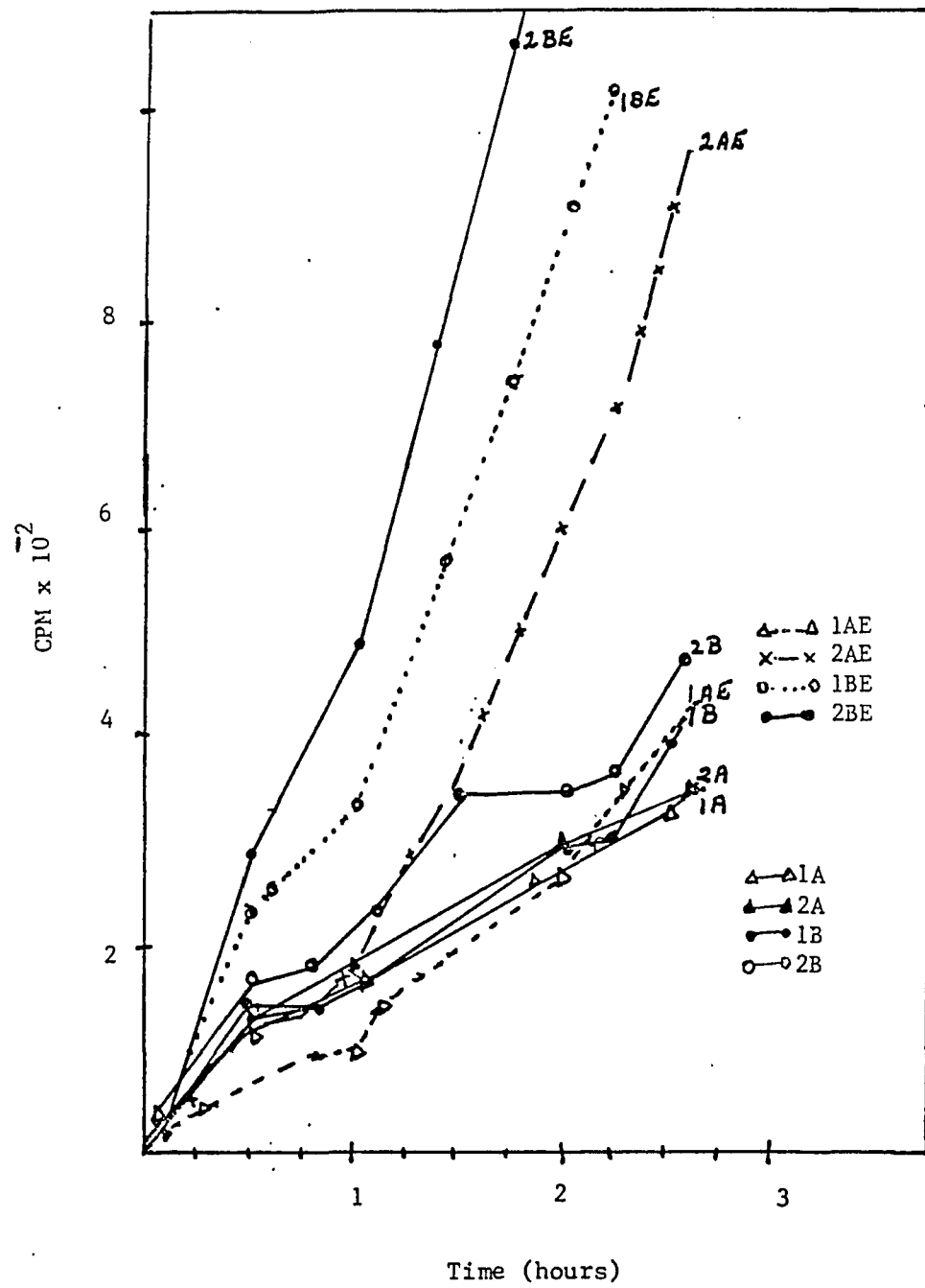
B = pH 9.0

E = +2mM EDTA

Tween mixture was added to each tube to give a final concentration of 2% and the cultures were incubated at room temperature with agitation for 2.75 hours. Non-radioactive control tubes were set up for each set of experimental conditions. Recovery was checked at 1 hour intervals by washing and resuspending non-radioactive control spheroids in SVM, pH 8.0, and observing viability.

Aliquots of 0.3 ml were removed from each tube at the indicated times and filtered through glass wool. Samples

containing 20 ul of the filtrate were removed in triplicate, transferred to paper filter discs, dried, scintillant added, and radioactivity measured in a Beckman scintillation counter.



Increasing the pH had the effect of enhancing the Tween mediated extraction of matrix components in those cultures incubated with EDTA. After two hours of incubation, culture 2BE, with twice the spheroid density as culture 1BE, exhibited a 40% increase in extracted matrix components. There appears to be increased Tween extraction of matrix components at the more basic pH, pH 9.0 as compared to pH 5.0. Comparing the results of cultures 2AE to 2BE, it was determined (figure 10) that after two hours of incubation >90% more radioactive matrix was extracted from culture 2BE, at pH 9.0.

6) Exclusion gel chromatography of Tween extracted Volvox matrix.

Large pore exclusion gels have been successfully used to remove detergents from solutions containing detergent extracted proteins (Holloway, 1973). Extracellular matrix components extracted from Volvox by a 1:1 mixture of the non-ionic detergents Tween 40/ Tween 80 were subjected to Sephadex G-100 column chromatography in an attempt to isolate the extracted proteins from the detergent.

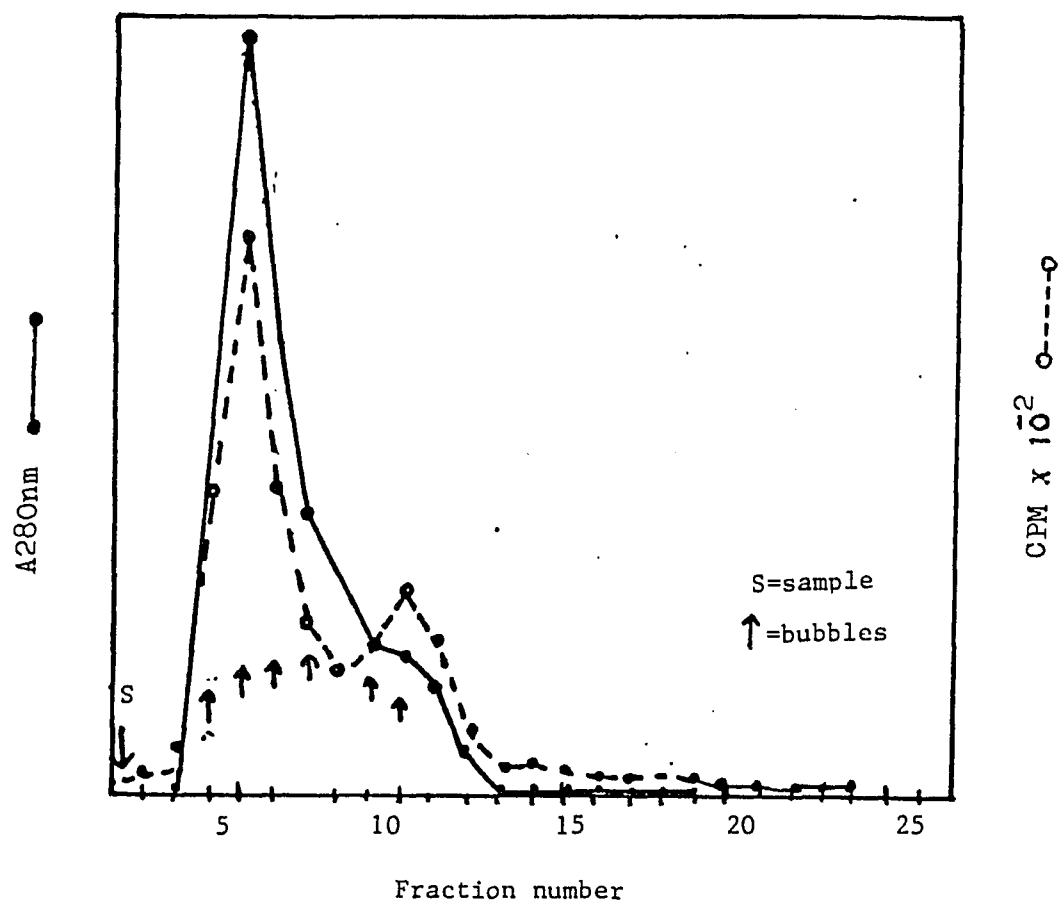
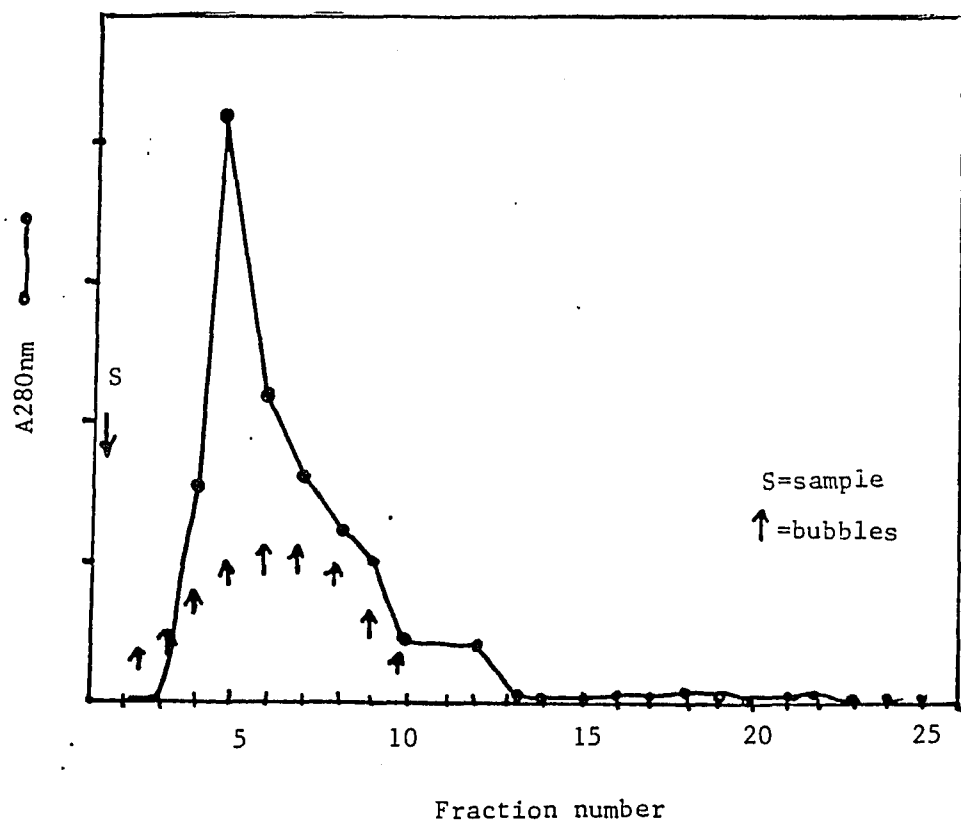
Heterogeneous HK10 spheroids were incubated with Tween at a final concentration of 2% for 3.5 hours. Detergent extracted components were isolated by filtration through glass wool, chromatographed on a column of Sephadex

Figure 11.

Tween "cleanup" of detergent extracted extracellular matrix components of V. carteri f. nagariensis strain HK10 on a column of Sephadex G-100.

A 5 ml sample of a heterogeneous culture of HK10 with a spheroid density of ~450 spheroids/ml was transferred to a culture tube to which Tween mixture was added to give a final concentration of 2%. The spheroids were incubated at room temperature with constant agitation for 3.5 hours. The culture was filtered through glass wool and the filtrate retained.

A 2 ml sample of the filtrate was layered onto a Sephadex G-100 column equilibrated with 0.02 M tris-Cl pH 8.1, 0.1 M NaCl, and 2 ml fractions collected. Fractions were monitored for the presence of protein by measuring absorbancy at 280 nm.



G-100, and the collected fractions monitored for protein. The Tween itself eluted just after the void volume, as evidenced (personal observation) by the appearance of bubbles in the eluant, and continued to elute together with the protein containing fractions. The results of figure 11 show the elution of one major protein containing component with a prominent shoulder, probably indicating insufficient separation of macromolecules by Sephadex G-100 (Curling, 1970). The attempt to isolate the extracted matrix from the detergent by this method was not successful, as the Tween appeared in all the protein containing fractions of eluant.

Heterogeneous HK10 and NB7 spheroids made radioactive with $\text{NaH}^{14}\text{CO}_3$ were incubated with Tween at a final concentration of 2% for 3.5 hours. Detergent extracted components were isolated from the spheroids by filtration and chromatographed on a column of Sephadex G-100. Fractions were monitored for protein and radioactivity.

The results of figure 12 show that the elution profiles of matrix extracted from both strains of Volvox are very similar, with the elution of radioactivity closely corresponding to the elution of protein; one major component with a significant shoulder (figure 12).

7) Extraction of extracellular matrix components from Volvox by the non-ionic detergent Triton X-100.

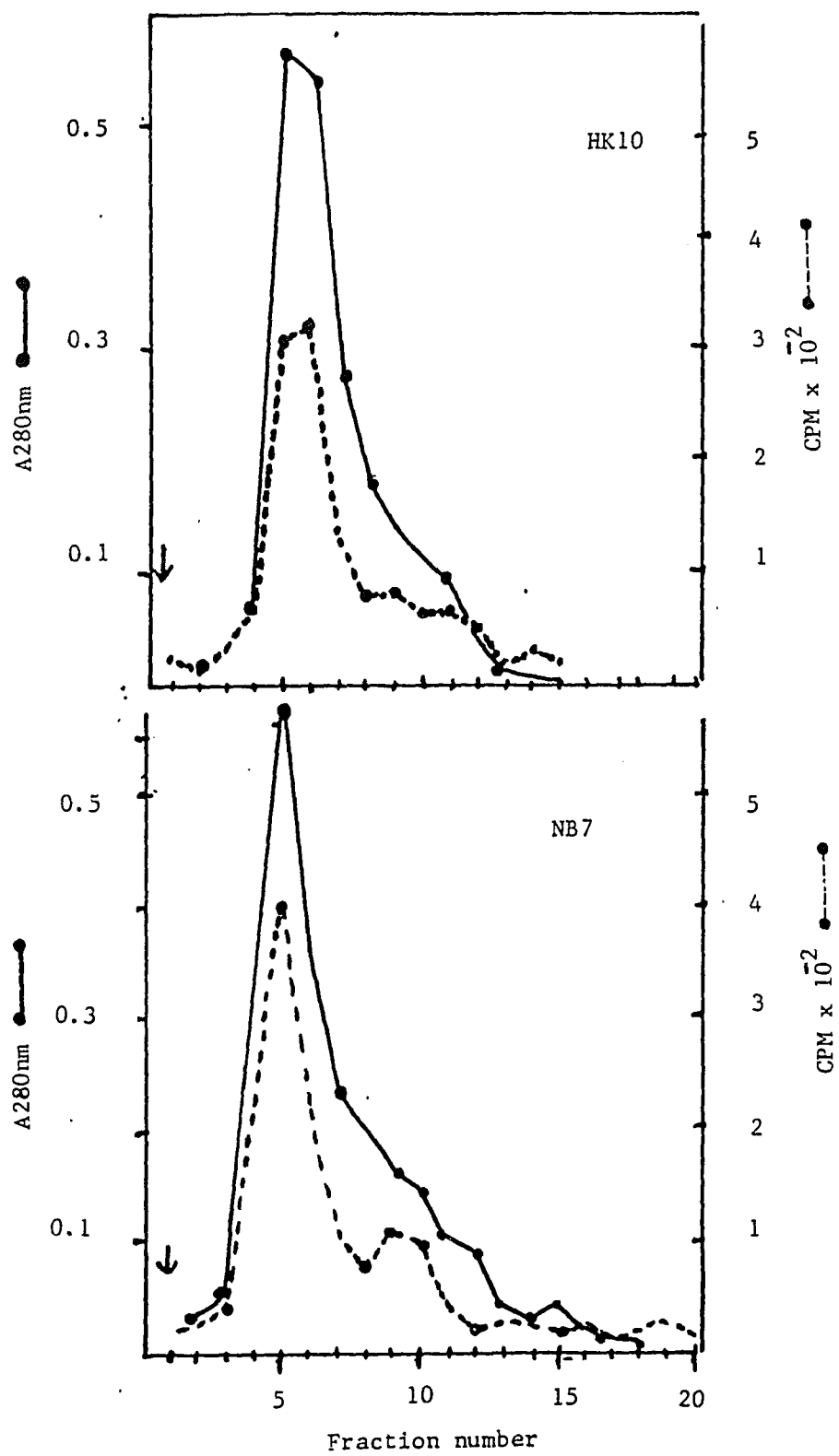
Figure 12.

A comparison of Tween extracted extracellular matrix components from Volvox carteri f. nagariensis strains HK10 and NB7 chromatographed on a column of Sephadex G-100.

Aliquots of 5 ml were withdrawn from heterogeneous cultures of HK10 and NB7. Spheroids were made radioactive with $\text{NaH}^{14}\text{CO}_3$ (4×10^5 cpm/ml). Cultures were incubated for 18 hours at room temperature with constant agitation. Spheroids were washed and resuspended in non-radioactive SVM for a total volume of 5 ml /culture.

A 1:1 mixture of the non-radioactive detergents Tween 40 and Tween 80 was added to each tube for a final concentration of 2%. The Tween treated cultures were incubated at room temperature with constant agitation for 3.5 hours. The cultures were filtered through glass wool and the filtrate retained.

A 2 ml sample of the filtrate was layered onto a column of Sephadex G-100 equilibrated with 0.02 M tris-Cl pH 8.1, 0.1 M NaCl. Fractions of 35 drops were collected and monitored for protein by measuring absorbancy at 280 nm. Aliquots of 20 μl were withdrawn from each fraction, transferred to paper filter discs, dried, scintillant added, and radioactivity measured with a Beckman liquid scintillation counter.



Heterogeneous HK10 spheroids made radioactive with $\text{NaH}^{14}\text{CO}_3$ for 18 hours were divided into two aliquots and incubated with two concentrations of the non-ionic detergent Triton X-100, 0.001% and 0.003%, for 4.75 hours. Samples were withdrawn at the indicated times and the extracted radioactivity monitored.

The results of figure 13 show increased extraction of matrix components at the higher concentration of detergent. After four hours of incubation, 33% more extracellular material was extracted from cultures incubated with 0.003% Tx-100, as compared to cultures incubated with 0.001% Tx-100. Unlike Tween mediated extraction, the rate of extraction did not level off (figure 13). It should be noted (personal observation) that incubation of Volvox spheroids with Triton X-100 at concentrations of 0.01% bleaches the spheroids and lethally affects the gonidia. At concentrations of 0.1% and greater, chlorophyll is leached out almost immediately, <5 minutes, indicating penetration of cellular membranes.

B. Physical extraction: Characterization of the crude matrix mechanically extracted from heterogeneous cultures of Volvox carteri f. nagariensis strain HK10.

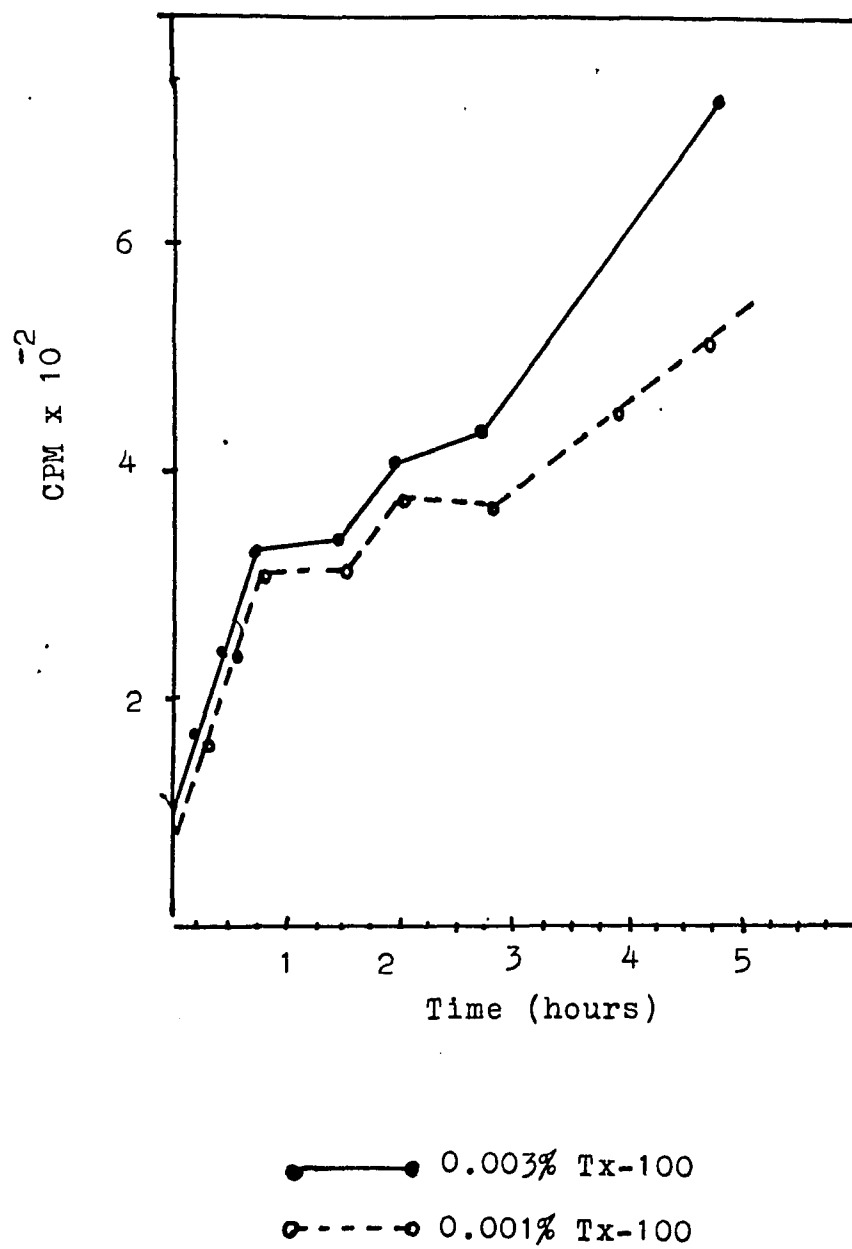
Many detergents used to extract the extracellular

Figure 13.

Extraction of extracellular matrix components from Volvox carteri f. nagariensis strain HK10 by varying concentrations of the non-ionic detergent Triton X-100.

A 10 ml sample of a heterogeneous culture of HK10 was made radioactive with 0.2 ml $\text{NaH}^{14}\text{CO}_3$ stock and incubated at room temperature with agitation for 18 hours. The spheroids were washed, resuspended in non-radioactive SVM, and divided into two tubes of 5 ml each.

Triton X-100 was added to tube 1 to yield a final concentration of 0.001%, and to tube 2 to yield a final concentration of 0.003%. The cultures were incubated with the detergent at room temperature with constant illumination and agitation for 4.75 hours. Aliquots of 0.25 ml were removed at the indicated times and filtered through glass wool. Samples containing 20 μl of the filtrate were transferred to paper filter discs, dried, scintillant added, and radioactivity measured with a Beckman liquid scintillation counter.



matrix of Volvox proved to be overly harsh and affected the viability of the organism. The adverse effect on viability caused by matrix extraction with high concentrations of Triton X-100 was attributed to the penetration of Volvox cell membranes (Helenius and Simons, 1975) and the extraction of essential membrane and/ or cellular components. Since it was observed that Tween treatment also proved damaging to the organism at high concentrations/ over long periods of time, it was decided to avoid all means of chemical extraction in this study. A successful (see Materials and Methods), purely mechanical means of extracting Volvox extracellular matrix was developed utilizing the Dounce tissue grinder.

The extracellular matrix of freshly harvested or frozen and thawed heterogeneous cultures of Volvox strain HK10 was extracted using the Dounce tissue grinder. Viscous cell free matrix preparations were recovered from the filtration series described in "Materials and Methods". The molecular composition of the crude extracellular matrix extracted from heterogeneous cultures of Volvox carteri f. nagariensis strain HK10 was characterized. Chemical analysis of the cell free matrix was accomplished using the Lowry assay for protein with BSA as a standard (Lowry et al., 1951) and the Dubois phenol-sulfuric acid assay for carbohydrate using mannose

and arabinose as standards (Dubois et al., 1956). Both carbohydrate and protein were found to be present in the mechanically extracted matrix with the ratios varying slightly, averaging ~2:1 carbohydrate : protein (table 5).

Multicomponent composition.

The crude matrix prep of HK10 was analyzed in order to determine whether the extracted matrix was homogeneous or multicomponent.

1) Exclusion gel chromatography.

Fractionation of the crude matrix extract on columns of Sepharose 4B proved most successful, as compared to prior attempts to fractionate the matrix on columns of Sephadex. Sephadex G-50, G-100, and G-125 did not have a large enough pore capacity to retard all of the matrix components and much protein and carbohydrate material eluted in the void volume. Sephadex G-200 was not resilient enough to withstand the pressure of the viscous matrix (personal observation).

Fractionation of the crude matrix prep on Sepharose 4B columns revealed the presence of two non-linked components. The first fraction was unretarded and eluted just after the void volume. Chemical analysis using the Dubois assay, showed it to be a complex carbohydrate

(polysaccharide) essentially free of protein. A lower molecular weight species eluted a distance behind and was composed of both protein and carbohydrate, as assayed by the Lowry and Dubois methods respectively (figure 14).

2) Ion exchange chromatography.

Resuspended matrix was chromatographed on columns of DEAE-cellulose. The results of figure 15 show that a non-adsorbing polysaccharide, devoid of protein, eluted at 0 M NaCl. It may be seen that a glycoprotein eluted at 0.07 M NaCl, indicating the presence of strongly acidic charged groups comprising the glycoprotein components of the matrix (McCracken and Barcellona, 1976).

3) Sephadex G-200 exclusion gel chromatography.

The purified glycoprotein fractions eluted from the Sepharose 4B columns were pooled, dialyzed exhaustively, concentrated by lyophilization, and subjected to further fractionation on a column of Sephadex G-200, in an attempt to separate any comigrating macromolecules without denaturation. It was of interest to determine whether the "glycoprotein peak" fractions eluted from the Sepharose 4B column were extracted from the arabinogalactan proteins found to be important components of extracellular matrices in algae and higher plants (Lamport, 1970, 1980; Lamport and Miller, 1971). The

Figure 14.

Sepharose 4B exclusion gel filtration patterns showing fractionation of extracellular matrix from physically disrupted spheroids of a heterogeneous culture of the green alga Volvox carteri f. nagariensis strain HK10.

A 10 liter culture of Volvox was harvested after five days growth and disrupted using the Dounce tissue grinder. The matrix was filtered through glass fiber filters and concentrated by lyophilization. The crude matrix preparation was resuspended in 0.1 M sodium phosphate buffer, pH 7.8, with 0.02% sodium azide added, to a total volume of 3 ml. A 2.5 ml aliquot of the resuspended matrix containing 3.74 mg protein/ml and 7.40 mg carbohydrate/ml, was layered onto a chromatography column packed with Sepharose 4B beads swollen and equilibrated with 0.1 M sodium phosphate buffer at room temperature. The column was eluted with two volumes of 0.1 M sodium phosphate buffer and 4.5 ml fractions were collected. Fractions were assayed for protein using the method of Lowry (with BSA as a standard) and measuring absorbancy at 750 nm, and for carbohydrate using the Dubois phenol-sulfuric acid method (with arabinose as a standard) and measuring absorbancy at 480 nm.

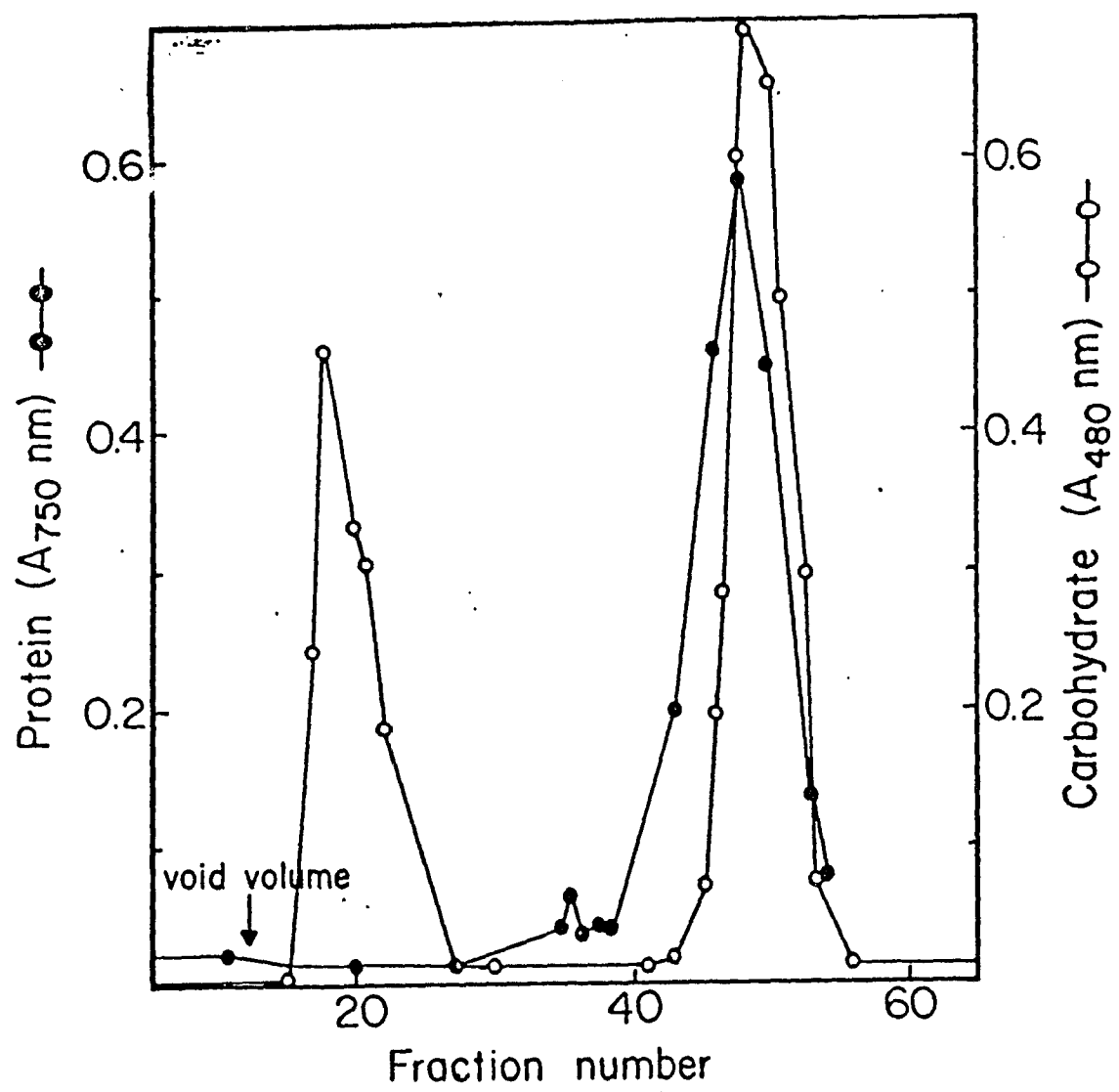


Figure 15.

Ion exchange chromatography of the extracellular matrix of Volvox carteri f. nagariensis strain HK10 on a column of DEAE cellulose.

DEAE cellulose was washed with 0.02 M tris-Cl pH 8.5, packed into a chromatography column and equilibrated with 0.02 M tris buffer. Lyophilized matrix extracted from a heterogeneous culture of Volvox was resuspended in 2 ml of 0.02 M tris buffer and layered onto the DEAE column. The column was eluted with a gradient of 0-0.5 M NaCl. 5 ml fractions were collected with the salt concentration increased after each ten fractions; 0 - 0.05 M - 0.07 M - 0.1 M - 0.2 M - 0.3 M - 0.5 M NaCl.

Two 0.5 ml samples were withdrawn from each fraction and assayed for the presence of protein and carbohydrate according to the methods of Lowry and Dubois respectively.

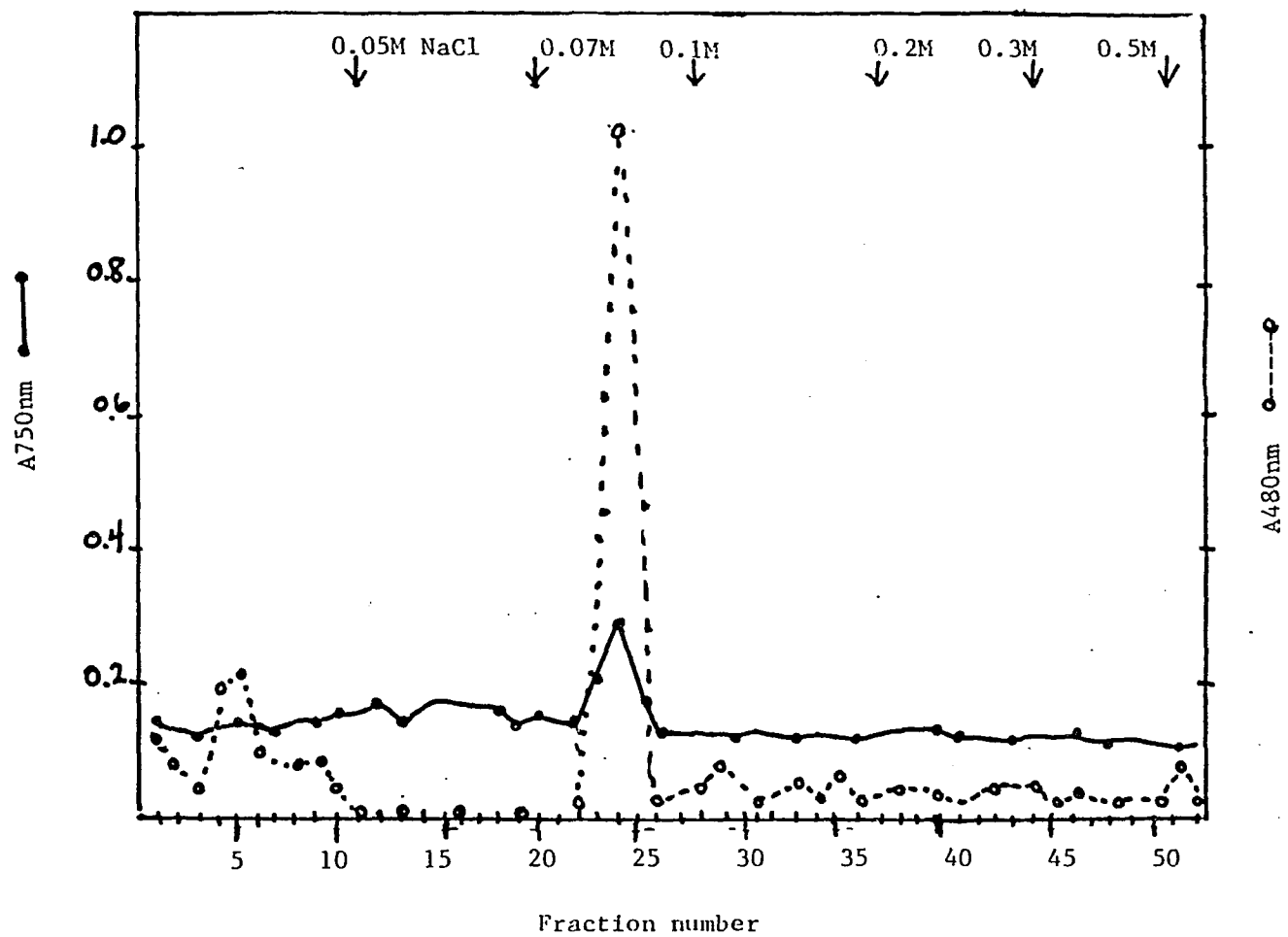


Figure 16.

Sephadex G-200 exclusion gel chromatography of purified glycoproteins extracted from the extracellular matrix of Volvox carteri f. nagariensis strain HK10.

Ten grams of Sephadex G-200 with a water regain of 20/1 were swollen in 400 ml buffer containing 0.02 M tris Cl pH 8.1, 0.1 M NaCl with 0.01% sodium azide. Fractions eluted from a column of Sepharose 4B containing glycoproteins extracted from the matrix of a heterogeneous culture of HK10 were dialyzed against four changes of distilled water over a period of 48 hours and concentrated by lyophilization. The concentrated glycoprotein peak was resuspended in 0.02 M tris buffer to a total volume of 2 ml containing 900 ug protein/ ml and 560 ug carbohydrate/ ml and layered onto a column of Sephadex G-200.

A void volume of 65 ml was determined by the elution of blue dextran. The column was eluted with two volumes of 0.02 M tris buffer and forty 4.5 ml fractions were collected. Fractions were assayed for the presence of protein and carbohydrate by the methods of Lowry and Dubois respectively.

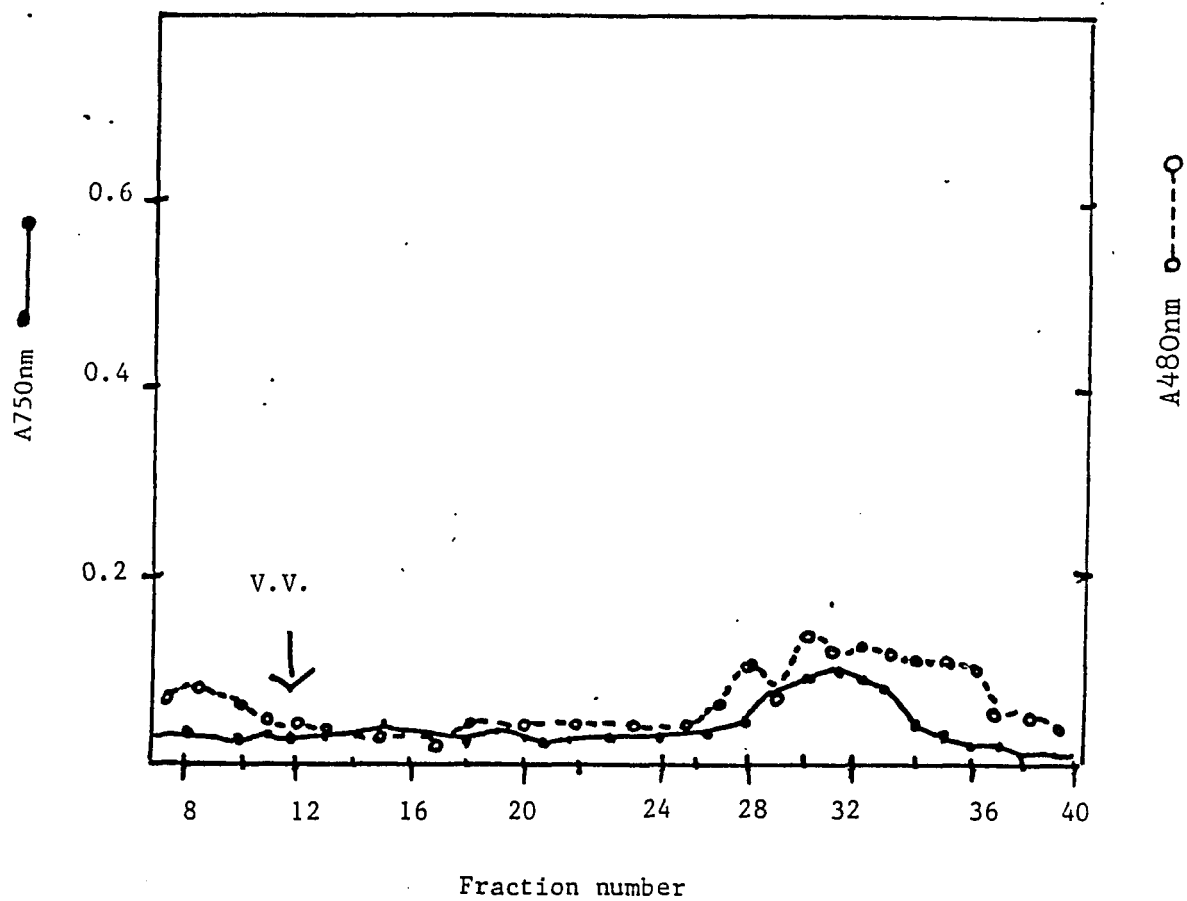


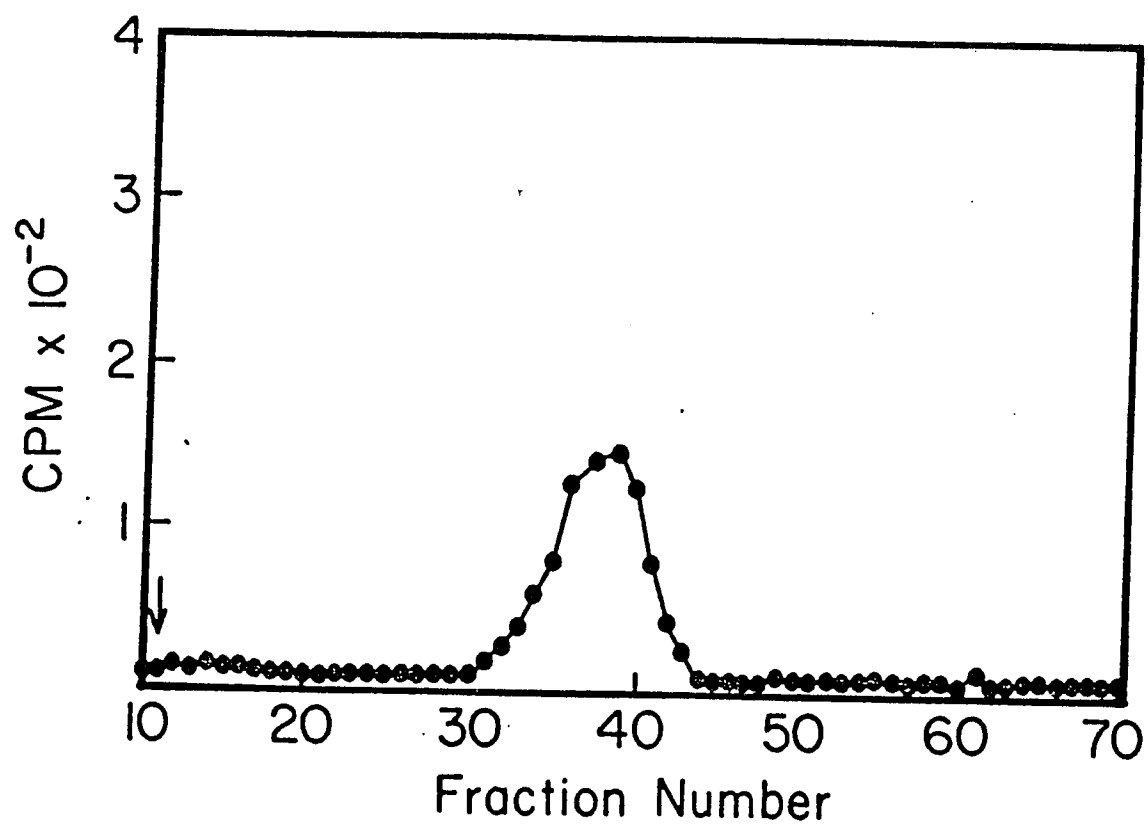
Figure 17.

Sephadex G-200 exclusion gel chromatography of radioactive matrix glycoproteins of Volvox carteri f. nagariensis strain HK10.

Sephadex G-200 beads were swollen and equilibrated in buffer containing 0.02 M tris Cl, pH 8.1, 0.1 M NaCl, and 0.01% sodium azide, and packed into a glass chromatography column.

^{14}C -matrix glycoproteins were extracted from heterogeneous cultures of HK10 made radioactive with $\text{NaH}^{14}\text{CO}_3$ by fractionation of total radioactive matrix lysate on columns of Sepharose 4B. Fractions containing glycoproteins were dialyzed against four changes of distilled water over a period of 48 hours at 4°C and concentrated by lyophilization. The concentrated "GP peak" was resuspended in 0.02 M tris buffer to a total volume of 0.5 ml (5×10^4 cpm/ sample) and layered onto a column of Sephadex G-200.

A void volume of 12 ml was determined by the elution of blue dextran. The column was eluted with two volumes of 0.02 M tris buffer and seventy 0.5 ml fractions were collected. Aliquots of 20 μl were removed from each fraction, transferred to GF/A filter discs, scintillant added, and radioactivity measured with a Beckman liquid scintillation counter.



eluted glycoprotein fractions contained both arabinose (assayed by the method of Dubois) and proline (incorporation of radioactive proline into the glycoprotein fractions). The results of elution profiles of the "glycoprotein peak" chromatographed on Sephadex G-200 (figure 16) show no further fractionation of this major matrix component.

Radioactive matrix was prepared as described in "Materials and Methods" and fractionated on a column of Sepharose 4B. The eluted radioactive glycoprotein fractions were chromatographed on a column of Sephadex G-200. It may be seen in figure 17 that the extracted radioactive glycoprotein components eluted as a single component.

4) Affinity column chromatography.

The extracted matrix components were chromatographed on a column of concanavalin A-agarose. Mechanically extracted matrix was resuspended in adsorbing buffer and applied to the affinity column. The column was washed with adsorbing buffer and fractions were collected. From measurements of the protein and carbohydrate content of the eluted fractions, it was determined that most of the matrix was not bound by the lectin concanavalin A (figure 18). It may be seen from the results of figure 18 that some matrix extract was adsorbed and could be eluted from

Figure 18.

Concanavalin A-agarose affinity chromatography:

Concanavalin A binding components of the extracellular matrix of Volvox carteri f. nagariensis strain HK10.

Concanavalin A agarose was washed in 0.1 M sodium acetate buffer pH 7.0 containing 0.5 M NaCl with 3 mM each $MnCl_2$, $MgCl_2$, and $CaCl_2$, and packed into a 1.5 cm x 20 cm chromatography column. A 1.0 ml sample of a lyophilized matrix preparation, extracted from a heterogeneous culture of HK10, containing 620 ug carbohydrate/ ml and 290 ug protein/ ml, was layered onto the column.

The column was eluted with 0.5 M NaCl buffer and 1 ml fractions were collected and monitored for protein content by measuring absorbancy at 280 nm. When optical density readings returned to background levels, the column was eluted with 0.02 M sodium borate buffer pH 6.0 and 15 1.0 ml fractions collected. When O.D. returned to 0 the column was eluted with saturated sodium borate buffer pH 8.0. Fifteen 1.0 ml fractions were collected and monitored for protein by measuring absorbancy at 280nm.

All fractions were assayed for protein by the method of Lowry and for carbohydrate by the method of Dubois.

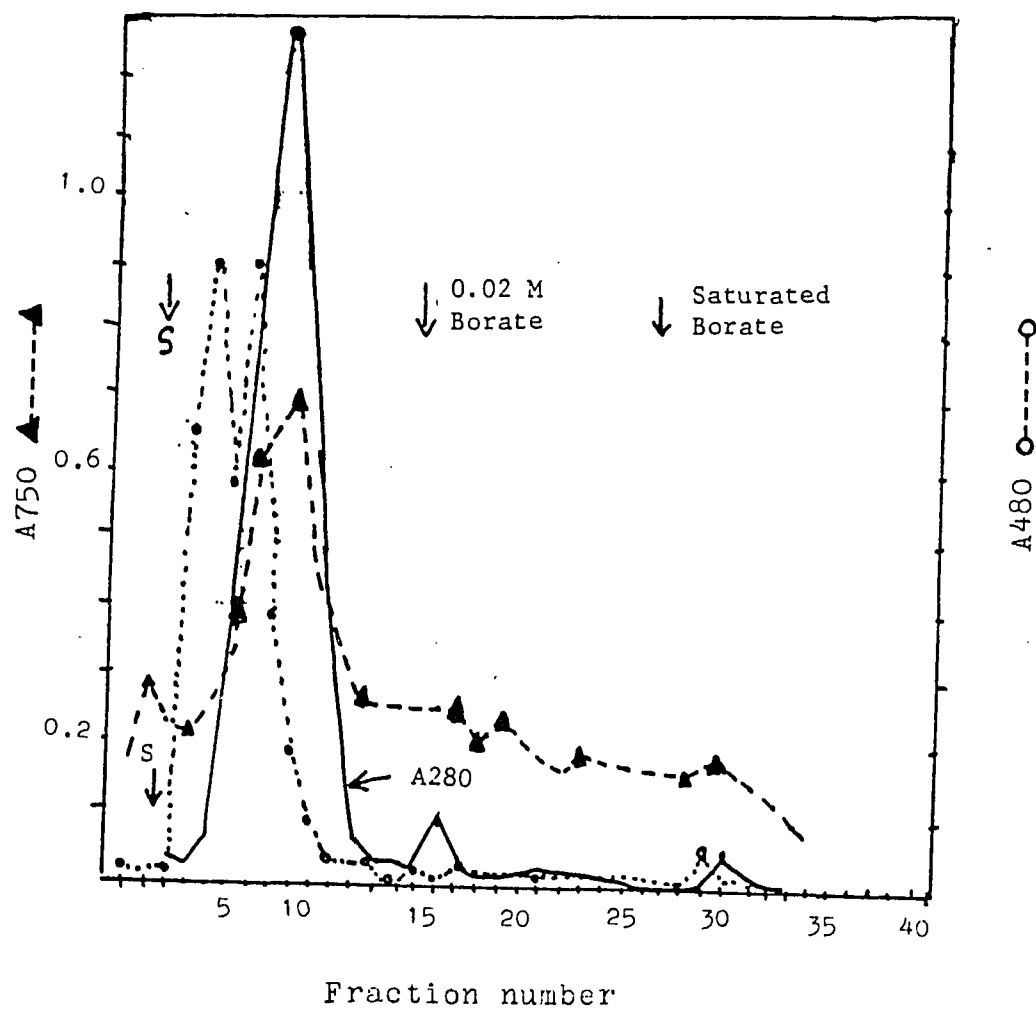
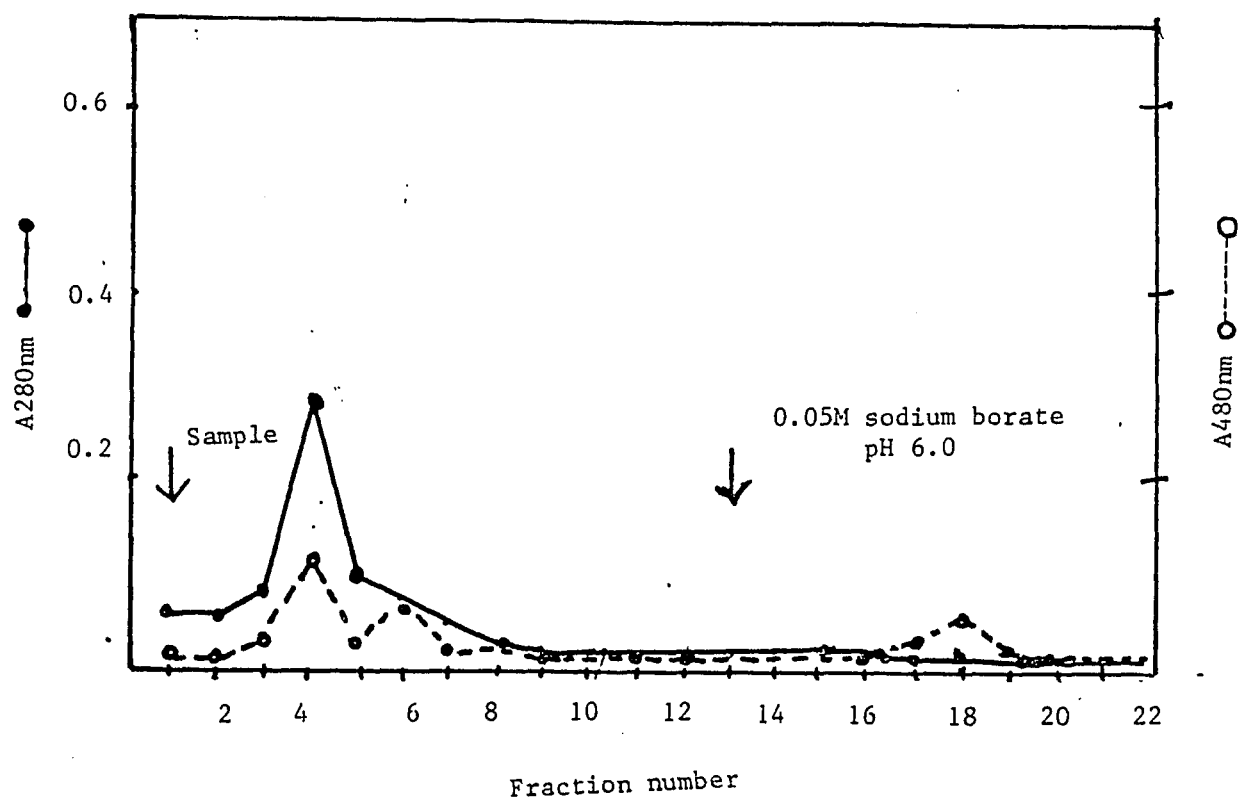


Figure 19.

Concanavalin A-agarose affinity chromatography of purified glycoprotein components of matrix extracted from heterogeneous cultures of V. carteri f. nagariensis strain HK10.

A 1.5 cm x 20 cm chromatography column was packed with concanavalin A agarose washed and equilibrated with adsorbing buffer containing 0.1 M sodium acetate pH 7.0, 0.5 M NaCl with 3mM each CaCl_2 , MnCl_2 , MgCl_2 and 0.01% sodium azide. Five fractions eluted from a column of Sepharose 4B containing glycoprotein components of the extracellular matrix, were dialyzed against four changes of distilled water for 48 hours at 4°C, concentrated by lyophilization, and resuspended in adsorbing buffer.

A 0.3 ml sample of resuspended glycoprotein concentrate ("GP peak"), containing 258 ug protein/ml and 102 ug carbohydrate/ ml, was layered onto the con A-agarose column equilibrated with adsorbing buffer. 1.5 ml fractions were collected and protein content monitored by absorbancy at A280. After 12 fractions were collected, when O.D. readings returned to 0, eluting buffer containing 0.05 M sodium borate, pH 6.0, was applied. Ten fractions were collected and absorbancy measured at 280nm. Fractions were assayed for the presence of carbohydrate by the method of Dubois.



the affinity column by 0.02 M sodium borate eluting buffer. An additional, minor, peak was eluted with the addition of saturated borate buffer. These results indicate that a limited degree of concanavalin A binding is exhibited by extracted matrix components.

The glycoprotein containing fractions of the extracellular matrix eluted from an exclusion gel of Sepharose 4B were dialyzed, concentrated by lyophilization, resuspended in adsorbing buffer and applied to the column of concanavalin A. From the results of figure 19 it may be seen that most of the glycoprotein was not adsorbed by the affinity column.

III. Changes in the extracellular matrix during the Volvox life cycle.

A. Biochemical analysis and exclusion gel chromatography of Volvox extracellular matrix during prominent stages of development.

Once the extracellular matrix of V. carteri strain HK10 had been characterized by identifying two distinguishable components, the large molecular weight polysaccharide and the lower molecular weight glycoprotein, it was necessary to determine what, if any, biochemical changes occur in

these two matrix components during the Volvox life cycle.

As seen in figure 1, the newly released, nascent Volvox spheroid undergoes morphological growth as it develops. Most of the growth is observed as deposition of extracellular matrix (Kochert, 1975). It was therefore of interest to compare the matrix of the newly released Volvox spheroid to the matrix of the fully expanded pre-release spheroids.

1) Newly released spheroids:

There is a reduced amount of carbohydrate, as assayed by the Dubois method, in nascent spheroids. Biochemical analysis showed a lower proportion of carbohydrate to protein in extracellular matrix extracted from the "juveniles" of strain HK10 when compared to matrix extracted from heterogeneous cultures (ratio of 1:1 as compared to 2:1 carbohydrate to protein).

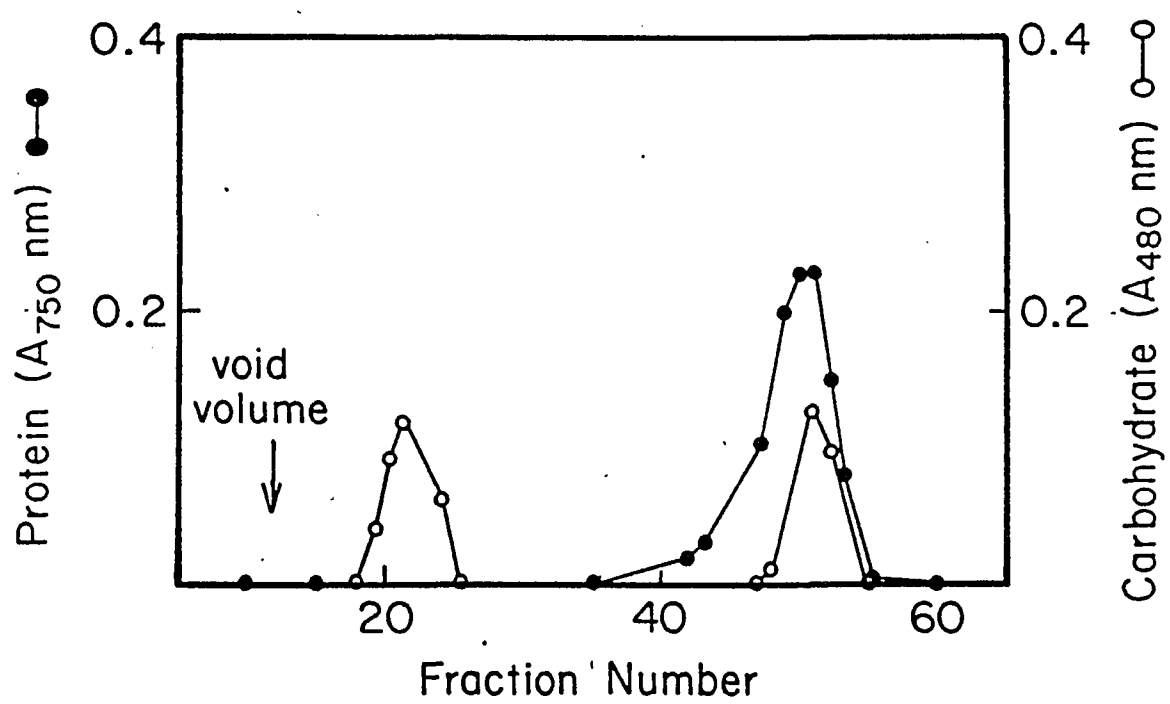
Matrix extracted from newly released HK10 spheroids was fractionated on a column of Sepharose 4B. It may be seen from the results in figure 20 that the large polysaccharide fraction is minimal at this stage of development as compared to the glycoprotein fraction. This is in contrast to the results of Sepharose 4B chromatography of matrix extracted from heterogeneous cultures (figure 14) where the polysaccharide fraction is

Figure 20.

Sepharose 4B gel filtration patterns showing fractionation of extracellular matrix extracted from newly released spheroids of V. carteri f. nagariensis strain HK10.

A 500 ml synchronous culture of HK10 was harvested after 96 hours (two life cycles under the illumination conditions described) as the maternal spheroids were completing the release process. The spheroids were filtered through 300 um - 125 um - 80 um Nitex. Newly released spheroids were retained by 80 um nitex and measured ~100 um in diameter.

The spheroids were disrupted using the Dounce tissue grinder, the homogenate filtered through glass fiber filters, and the matrix concentrated by lyophilization. The crude matrix preparation was resuspended in 0.1 M sodium phosphate buffer, pH 7.8, with 0.02% sodium azide added, to a yield a total volume of 3 ml. A 2.5 ml sample of the resuspended matrix preparation containing 800 ug protein/ml and 500 ug carbohydrate/ml was layered onto a Sepharose 4B chromatography column equilibrated with 0.1 M sodium phosphate buffer. The column was eluted with two volumes of 0.1 M sodium phosphate buffer and 4.5 ml fractions were collected. Two 0.5 ml aliquots were removed from each fraction and assayed for the presence of protein and carbohydrate (Lowry and Dubois methods).



prominant.

2) Pre-release spheroids: Fully expanded spheroids with the gonidia fully developed into juvenile spheroids.

Note: Care was taken to disrupt only the maternal spheroid, leaving the immature spheroids within intact.

Chemical analysis of matrix extracted from Volvox spheroids at the pre-release stage, showed carbohydrate to be the main biochemical component, with ratios of carbohydrate : protein ~3:1 (table 5). It is of interest to note there is a physical difference in the matrix at this stage. It is noticeably more viscous, and therefore more difficult to work with.

Matrix extracted from HK10 spheroids at the pre-release stage of development was fractionated on an exclusion column of Sepharose 4B. The results of figure 21 show that the polysaccharide fraction of the extracted matrix is more prominent at this stage of the life cycle than the glycoprotein fraction.

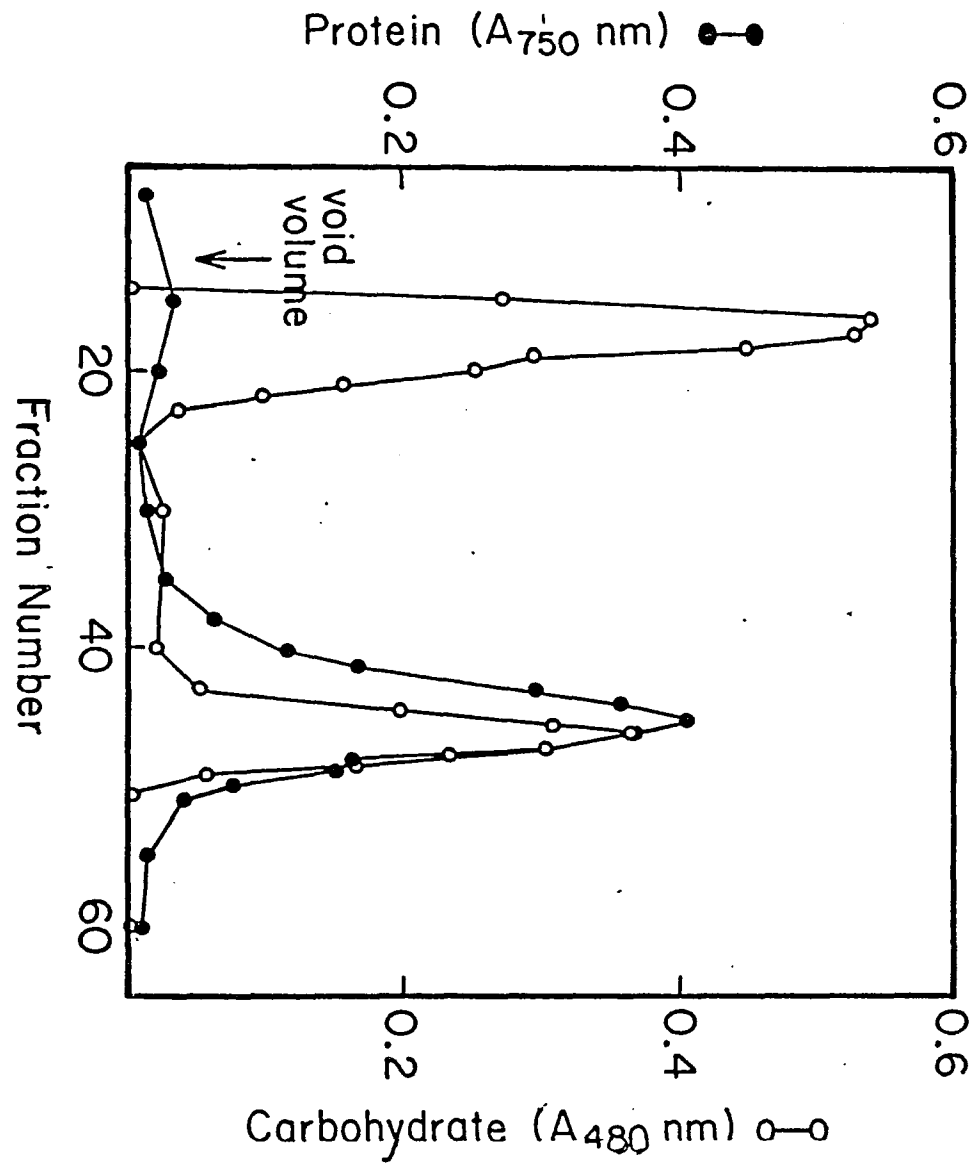
3) Embryogenesis.

A synchronous culture of HK10 was harvested as ~75% of the spheroids were undergoing cleavage. Chemical analysis of matrix extracted from these Volvox showed carbohydrate and protein to be present in nearly equal proportions, 1.4 mg carbohydrate/ml and 1.1 mg

Figure 21.

Sepharose 4B gel filtration patterns showing fractionation of extracellular matrix extracted from fully expanded, pre-release spheroids of V. carteri f. nagariensis strain HK10.

A 500 ml synchronous culture of HK10 spheroids at the fully expanded stage was harvested and retained on 300 um Nitex. The spheroids were resuspended in distilled water and disrupted with the Dounce tissue grinder until microscopic examination showed the maternal spheroids were disrupted while the juveniles were undamaged. The homogenate was filtered through glass fiber filters and the filtered maternal matrix concentrated by lyophilization. The lyophilized matrix preparation was resuspended in 0.1 M sodium phosphate buffer, pH 7.8, with 0.02% sodium azide added, to a final volume of 3 ml. A 2.5 ml sample of the resuspended matrix, containing 1.4 mg protein/ml and 2 mg carbohydrate/ml, was layered onto a Sepharose 4B chromatography column equilibrated with 0.1 M sodium phosphate buffer. The column was eluted with two volumes of buffer and 4.5 ml fractions were collected. Two 0.5 ml aliquots were withdrawn from each fraction and assayed for the presence of protein and carbohydrate according to the methods of Lowry and Dubois respectively.



protein/ml.

Matrix extracted from HK10 spheroids during embryogenesis (cleavage) were fractionated on an exclusion column of Sepharose 4B. The results of figure 22 show an increase in prominence of the carbohydrate fraction when compared to the results seen in figure 20 of matrix extracted from newly released spheroids.

4) The extracellular matrix extracted from a culture of HK10 during the release process.

A synchronous culture of HK10 was harvested at the onset of the release process. The matrix was extracted and fractionated on a column of Sepharose 4B. The results in figure 23 show that both the carbohydrate and glycoprotein are prominent components of the matrix at this stage of the life cycle. It may be seen that a small quantity of protein co-elutes with the polysaccharide component. This is not observed in fractionated matrix at the other stages of Volvox development reported in this study.

B. Radioactive isotope incorporation into the Volvox matrix during spheroid development.

The rationale for this study was to determine whether the matrix (or components thereof) was merely deposited

Figure 22.

Sepharose 4B gel filtration patterns showing fractionation of the extracellular matrix of Volvox carteri f. nagariensis strain HK10 during the cleavage (embryogenesis) stage of the life cycle.

A 500 ml synchronous culture of HK10 with 75% of the spheroids undergoing embryogenesis was harvested by filtration through 125 um Nitex, washed with distilled water, and frozen. The spheroids were thawed, disrupted using the Dounce tissue grinder, the homogenate filtered through glass fiber filters, and the matrix concentrated by lyophilization. The lyophilized matrix preparation was resuspended in 0.1 M sodium phosphate buffer, pH 7.8, with 0.02% sodium azide added, to a total volume of 3 ml. A 2.5 ml sample of the resuspended matrix containing 1.1 mg protein/ml and 1.4 mg carbohydrate/ml was layered onto a Sepharose 4B chromatography column equilibrated with 0.1 M phosphate buffer. The column was eluted with two volumes of 0.1 M sodium phosphate buffer and 4.5 ml fractions were collected. Two 0.5 ml aliquots were removed from each fraction and assayed for the presence of protein and carbohydrate according to the methods of Lowry and Dubois respectively.

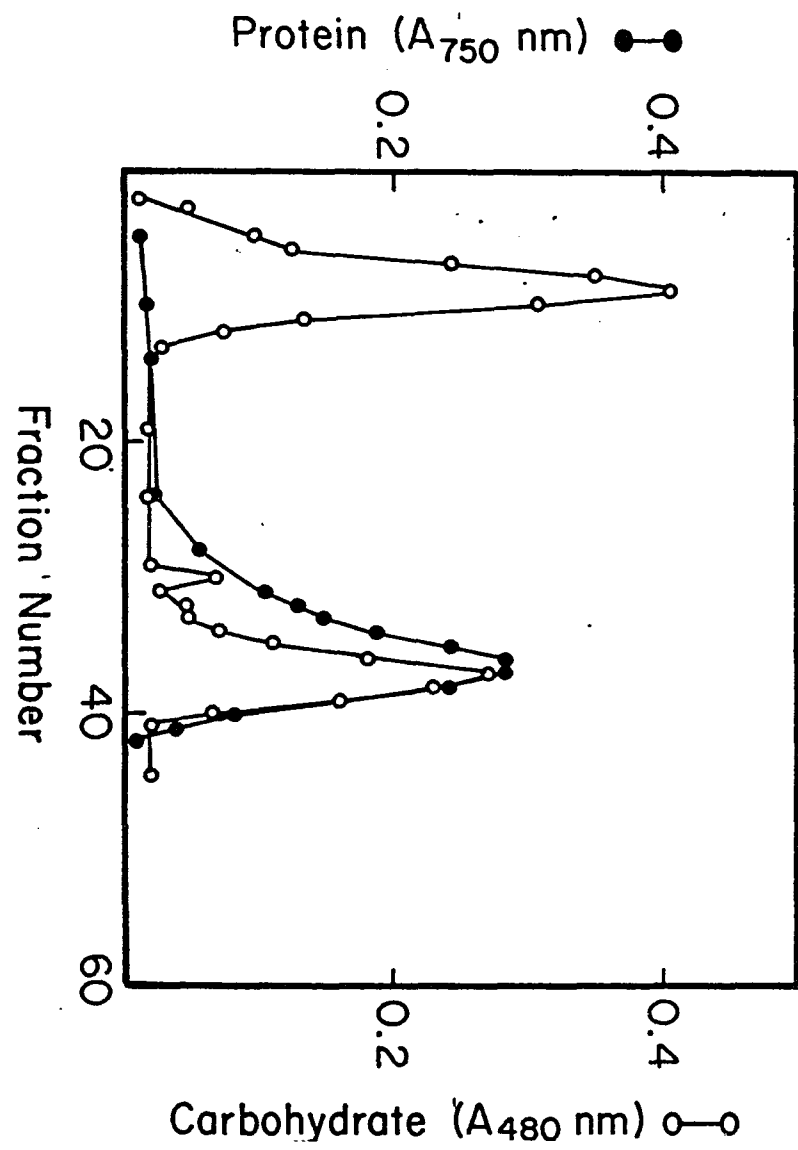
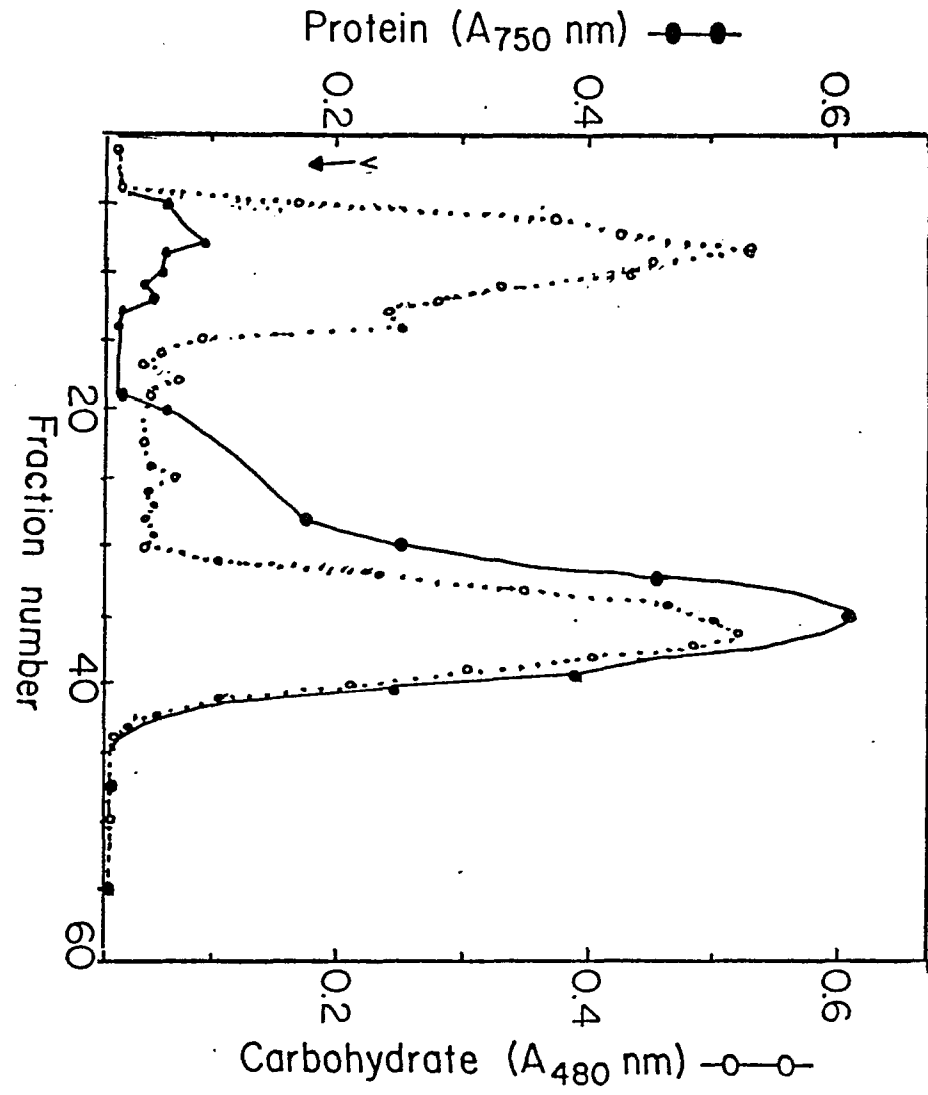


Figure 23.

Sepharose 4B gel filtration patterns showing fractionation of the extracellular matrix of a synchronous culture of Volvox carteri f. nagariensis during the release of the juvenile spheroids from the maternal organism.

A 500 ml synchronous culture of HK10 was harvested by filtration through 300 um Nitex as the first spheroids were starting to release. The retained spheroids were 300-400 um in diameter.

The spheroids were disrupted using the Dounce tissue grinder, the homogenate filtered through glass fiber filters, and the matrix concentrated by lyophilization. The crude matrix preparation was resuspended in 0.1 M sodium phosphate buffer, pH 7.8, with 0.02% sodium azide added to a total volume of 3 ml. A 2.5 ml sample of the resuspended matrix was layered onto a Sepharose 4B chromatography column equilibrated with 0.1 M sodium phosphate buffer. The column was eluted with two volumes of 0.1 M sodium phosphate buffer and 4.5 ml fractions were collected. Two 0.5 ml aliquots were removed from each fraction and assayed for the presence of protein and carbohydrate according to the methods of Lowry and Dubois respectively.



during spheroid expansion, or synthesized de novo during this period of growth.

1) Heterogeneous cultures:

Volvox HK10 spheroids grown heterogeneously were made radioactive for 18 hours with $\text{NaH}^{14}\text{CO}_3$. The matrix was extracted and fractionated on mini columns of Sepharose 4B and the fractions monitored for radioactivity. The elution profiles of the radioactive matrix closely resembled the elution profiles of non-radioactive matrix preparations. Two prominent matrix fractions were isolated and identified on the basis of biochemical assays; a large molecular weight polysaccharide and a glycoprotein. It may be seen from the results in figure 24 that the radioactive isotope was incorporated into both the polysaccharide and glycoprotein fractions of the extracted matrix.

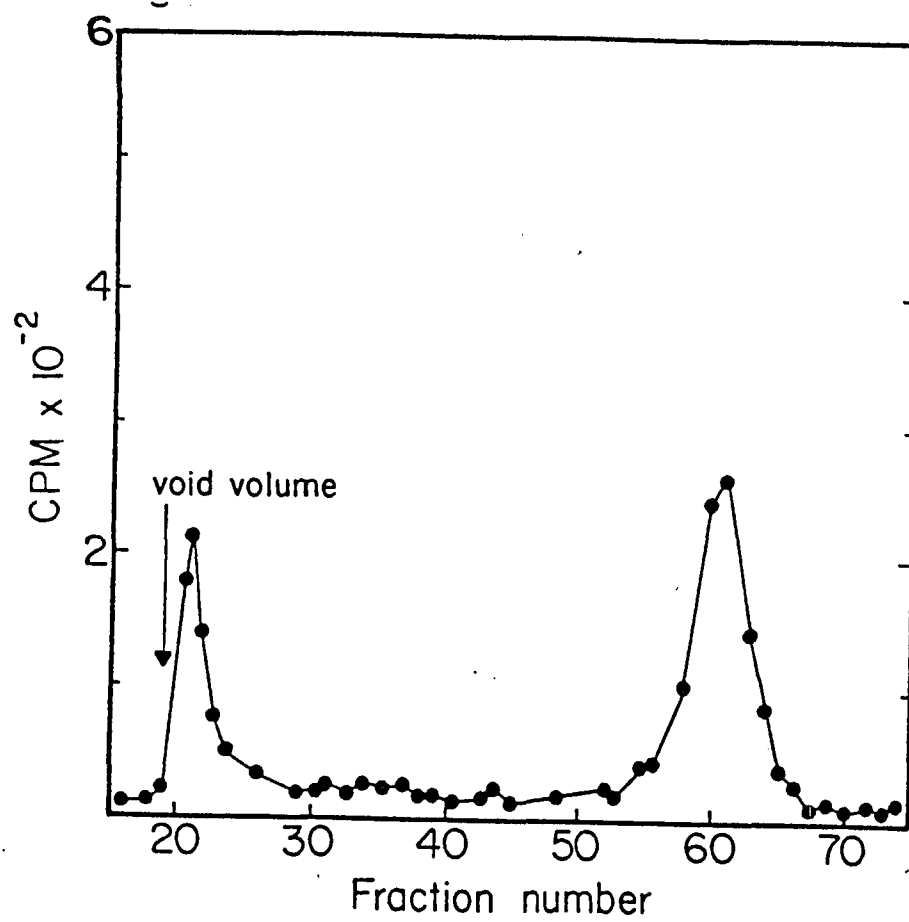
2) Synchronous cultures:

Volvox spheroids from synchronous cultures were pulse labeled at specific developmental stages to determine the stage during which the two identified matrix fractions, the large molecular weight polysaccharide and the glycoprotein, were synthesized.

Figure 24.

$\text{NaH}^{14}\text{CO}_3$ incorporation into the extracellular matrix of heterogeneous spheroids of V. carteri f. nagariensis strain HK10.

Two 5 ml aliquots of Volvox were withdrawn from a heterogeneous culture of five days growth, transferred into a culture tube containing 5 ml SVM, pH 8.0, and made radioactive with 0.3 ml ^{14}C -bicarbonate stock (4×10^5 cpm). Cultures were incubated at room temperature overnight with constant illumination and agitation. The spheroids were harvested, washed with distilled water, and the extracellular matrix extracted and concentrated by lyophilization. The matrix was resuspended in 0.02 tris-Cl, pH 8.0, 0.1 M NaCl, with 0.02% sodium azide added. A 0.5 ml sample of resuspended matrix (2.3×10^4 cpm/10 ul) was layered onto a Sepharose 4B mini column equilibrated with 0.02 M tris buffer. The column was eluted with two volumes of 0.02 M tris buffer and 75 0.5 ml fractions collected. Aliquots of 20 ul were removed from fractions, transferred to Whatman GF/A filter discs, dried, scintillant added, and radioactivity measured in a Beckman scintillation counter.



a. Newly released spheroids:

Spheroids from synchronous cultures were withdrawn immediately after release and labeled with $\text{NaH}^{14}\text{CO}_3$ for eight hours. The matrix was extracted and fractionated on a column of Sepharose 4B. The results seen in figure 25 show that newly released spheroids do not incorporate radioactive bicarbonate into the polysaccharide fraction of the extracted matrix. It may be seen (figure 25) that radioactive bicarbonate was incorporated into the glycoprotein fraction of the extracted matrix, indicating de novo synthesis of glycoprotein matrix components during the pre-cleavage stage of the life cycle.

b. Pre-release spheroids:

Volvox spheroids were withdrawn from a synchronous culture at the post inversion/ pre-release stage of the life cycle and pulse labeled with $\text{NaH}^{14}\text{CO}_3$. Radioactive matrix was extracted and fractionated on a column of Sepharose 4B. The results of figure 26 show incorporation of radioactive bicarbonate into both the polysaccharide and glycoprotein fractions of the extracted matrix, indicating synthesis of both components occurs during the pre-release stage of development.

The results of this study, seen in figures 24, 25, and 26, indicate that synthesis of the glycoprotein component of the extracellular matrix occurs during both of the two

Figure 25.

Sepharose 4B gel filtration patterns showing fractionation of radioactive matrix extracted from newly released spheroids of a synchronous culture of V. carteri f. nagariensis strain HK10.

A 5 ml aliquot of a synchronous culture of HK10 was transferred to a culture tube immediately after the juveniles were released from the maternal spheroids. The culture was made radioactive by the addition of 0.3 ml $\text{NaH}^{14}\text{CO}_3$ stock (4×10^5 cpm) and incubated at room temperature with constant illumination and agitation for 8 hours until the gonidia started to condense in preparation for cleavage. Spheroids were washed with 10 volumes of distilled water and frozen. The spheroids were thawed, disrupted with the Dounce tissue grinder, the homogenate filtered through glass fiber filters, and the radioactive matrix concentrated by lyophilization.

The lyophilized matrix preparation was resuspended in 0.02 M tris-Cl, pH 8.0, 0.1 M NaCl, with 0.02% sodium azide added. A 0.5 ml sample of resuspended matrix preparation (4.5×10^4 cpm) was layered onto a Sepharose 4B mini column equilibrated with 0.02 M tris buffer. The column was eluted with two volumes of tris buffer and seventy five 0.5 ml fractions were collected. Aliquots of 20 μl were withdrawn from each fraction,

transferred to GF/A filter discs, dried, scintillant added, and radioactivity measured in a Beckman liquid scintillation counter.

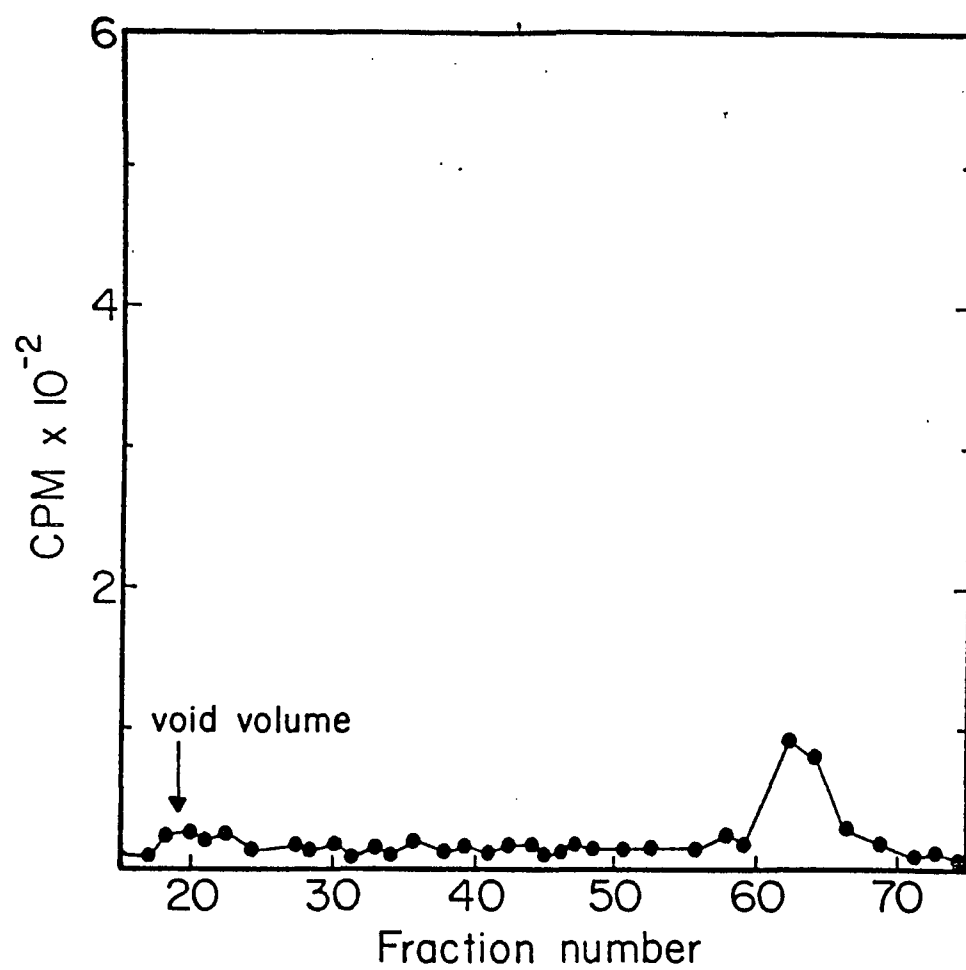


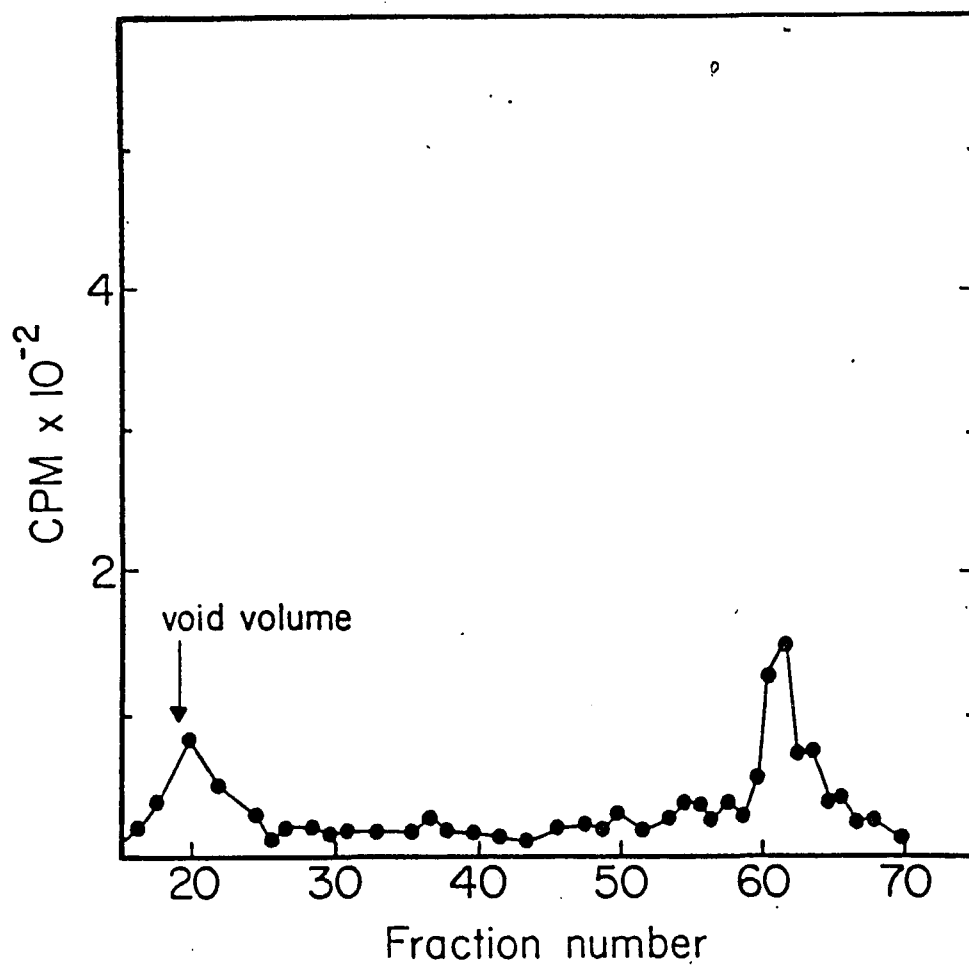
Figure 26.

Sepharose 4B gel filtration patterns showing fractionation of radioactive matrix extracted from fully expanded, pre-release spheroids of a synchronous culture of V. carteri f. nagariensis strain HK10.

A 5 ml sample of a synchronous culture of HK10 was transferred to a culture tube after the cleavage and inversion processes had been completed. The culture was made radioactive by the addition of 0.3 ml $\text{NaH}^{14}\text{CO}_3$ stock and incubated at room temperature with constant illumination and agitation for 8 hours through the period of spheroid expansion, until the first spheroids started to release. The spheroids were washed with 10 volumes of distilled water and frozen. The spheroids were thawed and homogenized with the Dounce tissue grinder until the maternal spheroids were disrupted, while the juveniles were left intact. The homogenate was filtered through glass fiber filters and the radioactive matrix concentrated by lyophilization.

The lyophilized matrix preparation was resuspended in 0.02 M tris-Cl buffer pH 8.0, 0.1 M NaCl, with 0.02% sodium azide added. A 0.5 ml sample of the resuspended matrix preparation (7.5×10^4 cpm) were layered onto a Sepharose 4B mini column equilibrated with 0.02 M tris buffer. The column was eluted with two volumes of tris

buffer and seventy five 0.5 ml fractions were collected. Aliquots of 20 ul were withdrawn from each fraction, transferred to GF/A filter discs, dried, scintillant added, and radioactivity measured in a Beckman liquid scintillation counter.



expansion stages of the Volvox life cycle. The large molecular weight polysaccharide component was synthesized primarily during the latter stage of expansion, the morphologically prominent pre-release expansion. This matrix component may be responsible for the tremendous increase in spheroid size and the moving apart of individual somatic cells observed at this stage of the Volvox life cycle.

IV. Volvox carteri f. weismania strain NB7

Strain NB7 differs from the HK10 strain used in the previous studies in that it contains fewer gonidia (8-10 as compared to 16 in "healthy" HK10) (Kochert, 1969, 1970; Starr, 1970). Recent ultrastructural studies reported by Kirk et al. (1986) show differences in the morphological arrangement of the matrix in the two strains, NB7 and HK10. Strain NB7 was also found to be more susceptible to dissolution by protease digestion (personal observation). This indicates a possible biochemical difference in matrix composition.

A. A comparison of extracellular matrix extracted from spheroids of strains HK10 and NB7:

Heterogeneous cultures of NB7 were grown under the same conditions as HK10. The extracellular matrix of NB7 spheroids was extracted, concentrated, and fractionated on a column of Sepharose 4B. The fractions were assayed by the methods of Lowry and Dubois for the presence of protein and carbohydrate respectively.

Two fractions of the extracted NB7 matrix were resolved by exclusion gel chromatography. The first, a large molecular weight polysaccharide, eluted just after the void volume. The second component was of lower molecular weight and was composed of both carbohydrate and protein (figure 27).

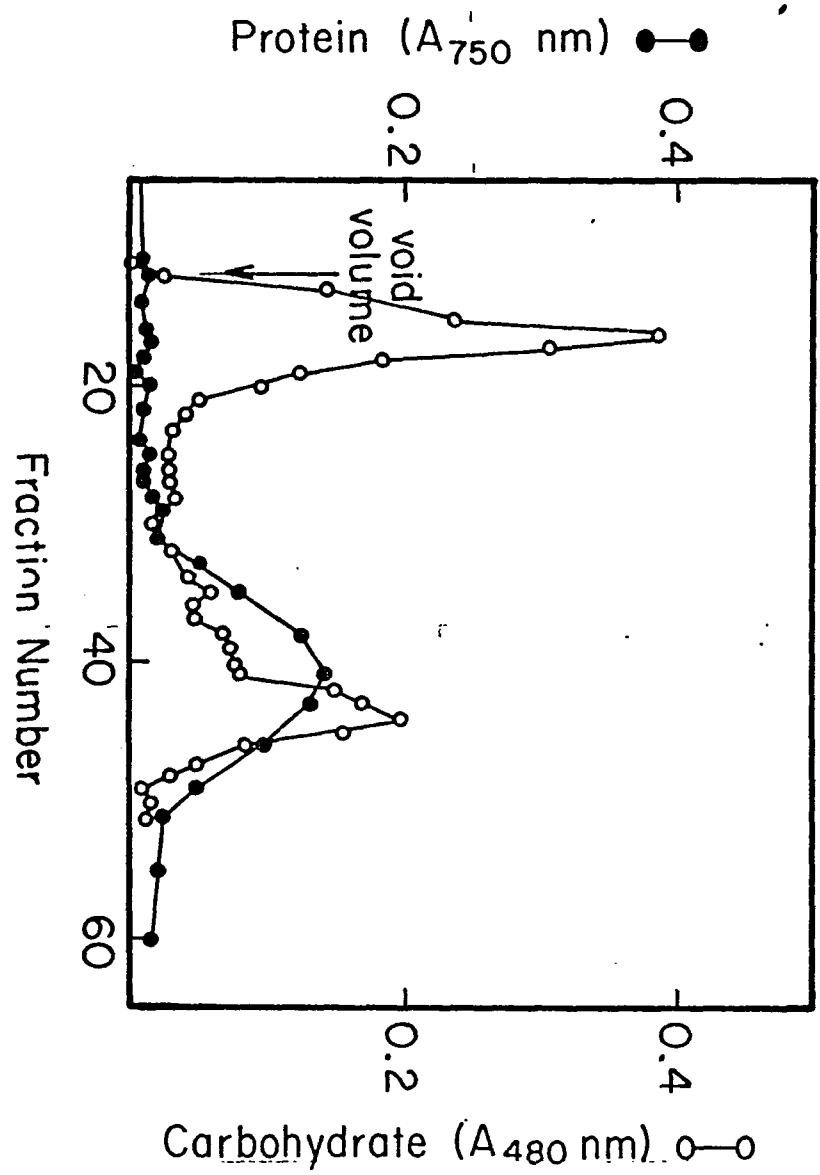
It may be seen, from a comparison of figure 14 (fractionation of HK10 matrix) and figure 27, that the Sepharose 4B elution profiles of the fractionated matrices extracted from the two strains of Volvox studied exhibit strong similarities. The individual matrix components are also observed to elute at similar positions during the fractionation of the matrices of these two strains of Volvox.

A marked difference is seen in the relative proportions of carbohydrate and protein comprising the glycoprotein components of the matrices of the two strains. In HK10 the glycoprotein component is a prominent molecular species, whereas in NB7 it is minor compared to the polysaccharide. This may be related to the increased

Figure 27.

Sepharose 4B exclusion gel filtration patterns showing the fractionation of extracellular matrix mechanically extracted from spheroids of a heterogeneous culture of the green alga Volvox carteri f. weismania strain NB7.

A 10 liter heterogeneous culture of NB7 was harvested after seven days of growth and the spheroids disrupted using the Dounce tissue grinder. The homogenate was filtered through glass fiber filters and the matrix concentrated by lyophilization. The crude matrix preparation was resuspended in 0.1 M sodium phosphate buffer, pH 7.8, with 0.02% sodium azide added, to yield a total volume of 3 ml. A 2.5 ml sample of the resuspended matrix, containing 400 ug carbohydrate and 250 ug protein, was layered onto a chromatography column of Sepharose 4B equilibrated with 0.1 M sodium phosphate buffer at room temperature. The column was eluted with two volumes of 0.1 M phosphate buffer and 4.5 ml fractions were collected. Two 0.5 ml aliquots were removed from each fraction and assayed for protein and carbohydrate content according to the methods of Lowry and Dubois respectively.



sensitivity of NB7 to spheroid dissolution by protease treatment.

B. Radioactive isotope incorporation into the extracellular matrix of NB7.

1) Fractionation of the extracellular matrix of NB7 spheroids on a column of Sepharose 4B.

A heterogeneous culture of NB7 spheroids was made radioactive with $\text{NaH}^{14}\text{CO}_3$ for 18 hours. Fractionation of radioactive matrix extracted from these spheroids on a mini column of Sepharose 4B showed the incorporation of radioactive isotope into both the polysaccharide and glycoprotein fractions. The elution profile of fractionated radioactive matrix seen in figure 28 closely resembles that of fractionated non-radioactive extracellular matrix extracted from NB7 spheroids seen in figure 27. The large molecular weight polysaccharide is observed to be a more prominent component of the extracted radioactive matrix than the glycoprotein (figure 28).

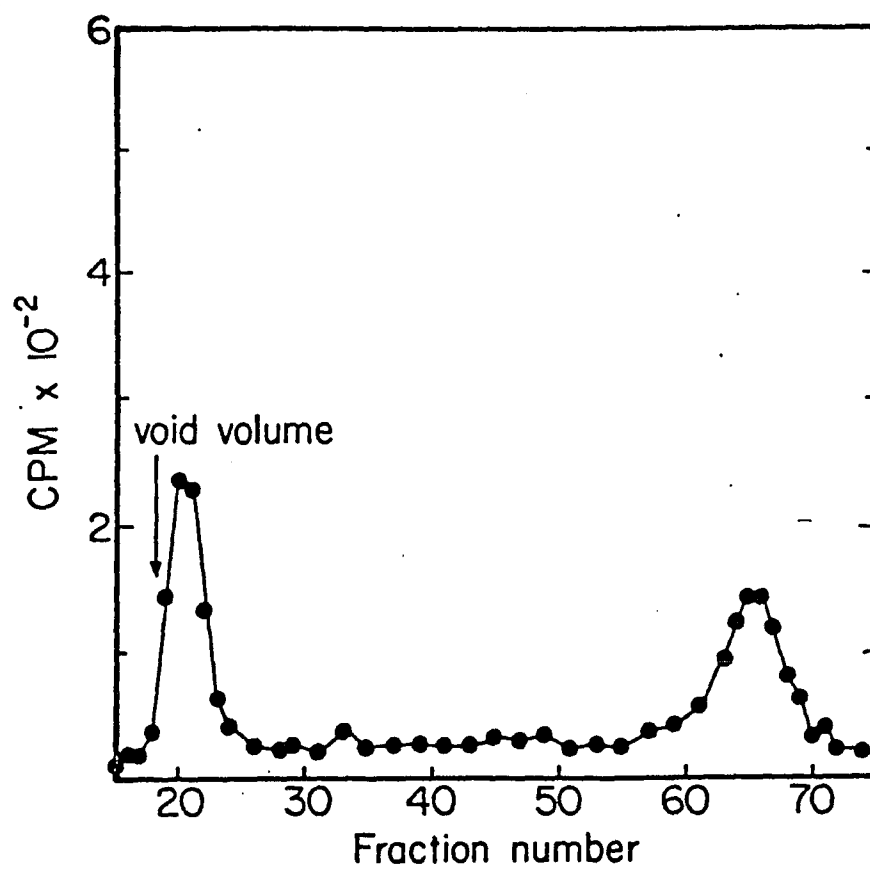
The results of the fractionation of radioactive NB7 matrix seen in figure 28 may be compared with the results of figure 24, showing the fractionation of radioactive matrix extracted from HK10 spheroids. It can be seen that the glycoprotein fraction of the extracted radioactive

Figure 28.

$\text{NaH}^{14}\text{CO}_3$ incorporation into the extracellular matrix of a heterogeneous culture of Volvox carteri f. weismania strain NB7.

A 10 ml aliquot of a heterogeneous culture of NB7 was transferred to a culture tube and made radioactive with 0.3 ml $\text{NaH}^{14}\text{CO}_3$ stock (4×10^5 cpm). The culture was incubated at room temperature for 18 hours with constant illumination and agitation. The spheroids were washed with 10 volumes of distilled water, resuspended in distilled water, and disrupted using the Dounce tissue grinder. The homogenate was filtered through glass fiber filters and the matrix concentrated by lyophilization.

The radioactive matrix preparation was resuspended in 0.02 M tris-Cl, pH 8.0, 0.1 M NaCl. A 0.5 ml sample of resuspended matrix (2.5×10^4 cpm/10 μl) was layered onto a 1.0 cm x 50 cm Sepharose 4B mini column equilibrated with 0.02 M tris buffer. The column was eluted with two volumes of 0.02 M tris buffer and 75 0.5 ml fractions collected. Aliquots of 20 μl were withdrawn from each fraction, transferred to GF/A filter discs, dried, scintillant added, and radioactivity measured in a Beckman liquid scintillation counter.



NB7 matrix is minor relative to the polysaccharide fraction, in contrast to the results of figure 24 that show the glycoprotein to be the more prominent component of the fractionated radioactive matrix of HK10 spheroids.

2) Fractionation of the extracellular matrix of NB7 spheroids on an exclusion gel of Sephadex G-200.

A heterogeneous culture of NB7 was made radioactive with $\text{NaH}^{14}\text{CO}_3$ for 18 hours. The radioactive matrix was mechanically extracted, fractionated on a column of Sephadex G-200, and the fractions monitored for radioactivity. The elution profile of the fractionated matrix showed the presence of two components, a more prominent high molecular weight species eluting just after the void volume, and a lower molecular weight fraction (figure 29).

V. Volvox carteri f. nagariensis strain HK10, mutant DIS 111.

The dissociator mutants of Volvox do not synthesize a functional extracellular matrix (Sessoms and Huskey, 1973). The gonidia and somatic cells of the DIS 111 mutant of the normal HK10 strain dissociate immediately after inversion. The gonidia do undergo cleavage to form

Figure 29.

Exclusion gel chromatography of mechanically extracted extracellular matrix components of V. carteri f. weismania strain NB7 on a column of Sephadex G-200.

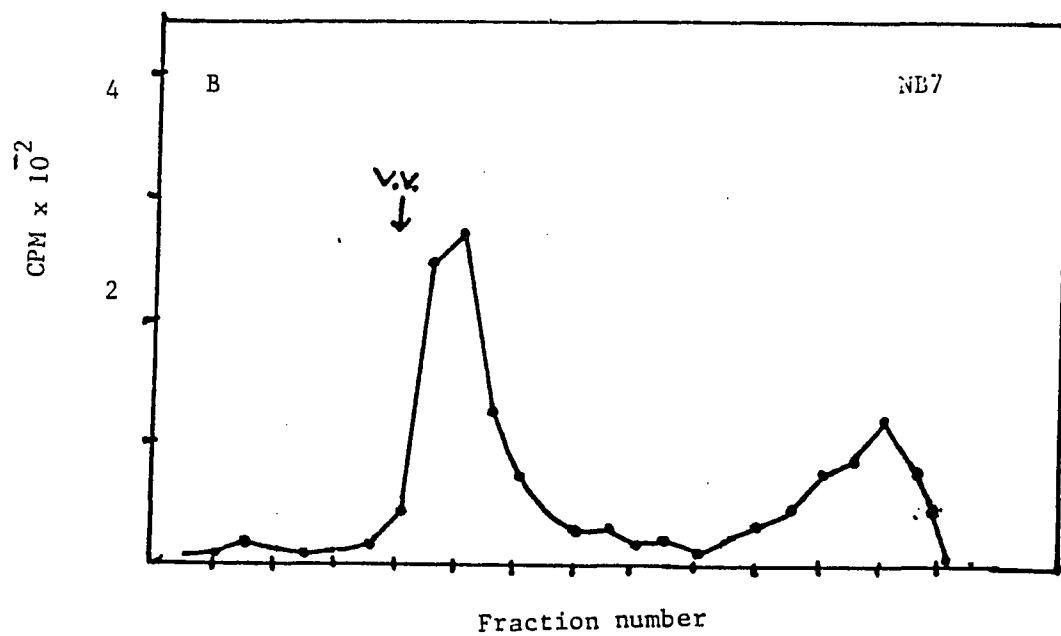
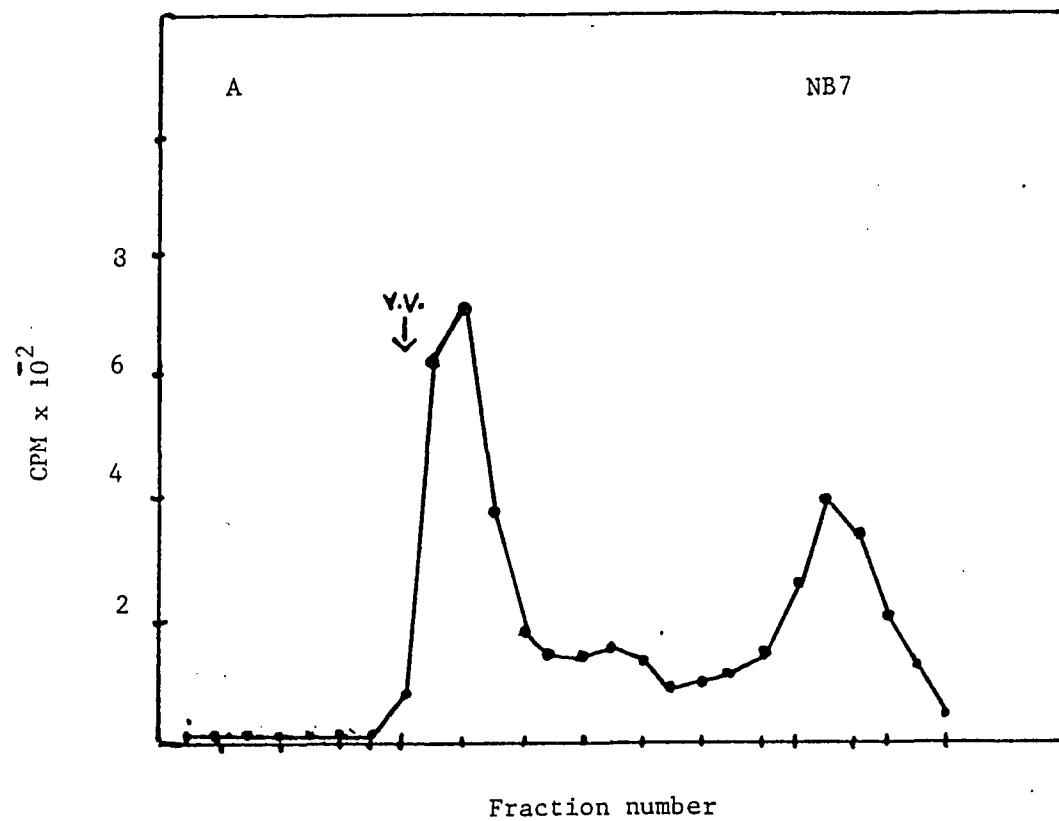
A 10 ml sample of a heterogeneous culture of NB7 was made radioactive for 18 hours with $\text{NaH}^{14}\text{CO}_3$. The spheroids were washed, resuspended in distilled water, and disrupted with the Dounce tissue grinder (seven strokes with the "A" ball). The homogenate was filtered through three successive plugs of glass wool and the filtrate retained. The specific activity of the filtrate was 1.03×10^6 cpm/ml.

A 1% solution of Blue Dextran (0.3 ml) in sucrose was layered onto a column of Sephadex G-200 equilibrated with 0.2 M tris Cl pH 8.1, 0.1 M NaCl, with 0.02% sodium azide added, and the void volume determined at 6 ml. The NB7 matrix filtrate was diluted 1:1 with 0.2 M tris buffer and layered onto the column of Sephadex G-200 equilibrated with the same buffer.

A = 0.3 ml matrix extract : 0.3 ml buffer

B = 0.1 ml matrix extract : 0.1 ml buffer

Fractions of 0.75 ml were collected and radioactivity monitored as follows. Aliquots of 20 ul were transferred from each fraction to paper filter discs, dried, scintillant added, and radioactivity measured with a Beckman liquid scintillation counter.



"juvenile spheroids" that immediately pass through a short lived pre-inversion stage, characterized by the positioning of the somatic cells on the interior of a ball of gonidia. Having no surrounding extracellular sheath or matrix, the ball of cells resembles a cluster of grapes, and the individual cells immediately separate upon inversion (personal observation). It was of interest to determine whether any extracellular matrix material was synthesized, and if so compare it to the normal matrix of V. carteri f. nagariensis strain HK10.

A. A Biochemical comparison of the extracellular macromolecules of DIS 111 cells and the extracellular matrix of normal HK10 spheroids.

Cultures of DIS 111 were grown for one week and the medium concentrated and dialyzed. Biochemical assays of the medium indicated the presence of both carbohydrate and protein. The presence of these macromolecules in the growth medium indicates that extracellular components are synthesized by the mutant, and released into the medium.

The dialyzed and concentrated medium was chromatographed on a column of Sepharose 4B, and the fractions assayed for the presence of protein and carbohydrate. It may be seen from the results of figure 30 that a polysaccharide fraction elutes immediately

after the void volume, indicating the presence of a large molecular weight macromolecule.

The results of figure 30 may be compared to the results seen in figure 14, showing the fractionation of matrix extracted from normal HK10 spheroids. The polysaccharide fraction recovered from the growth medium of DIS 111 is seen to be considerably less prominent than the polysaccharide fraction of normal Volvox, and appears as a split rather than single component. A second component was fractionated from the growth medium of DIS 111, as may be seen in figure 30, eluting at a similar position as the glycoprotein containing fractions of the normal Volvox matrix. The protein content of this component is diminished as compared to the extracted matrix glycoprotein of the normal spheroid.

B. Electron Microscopy: A comparative picture of the extracellular sheaths and matrix of Volvox carteri f. nagariensis strain HK10, and the matrix mutant DIS 111.

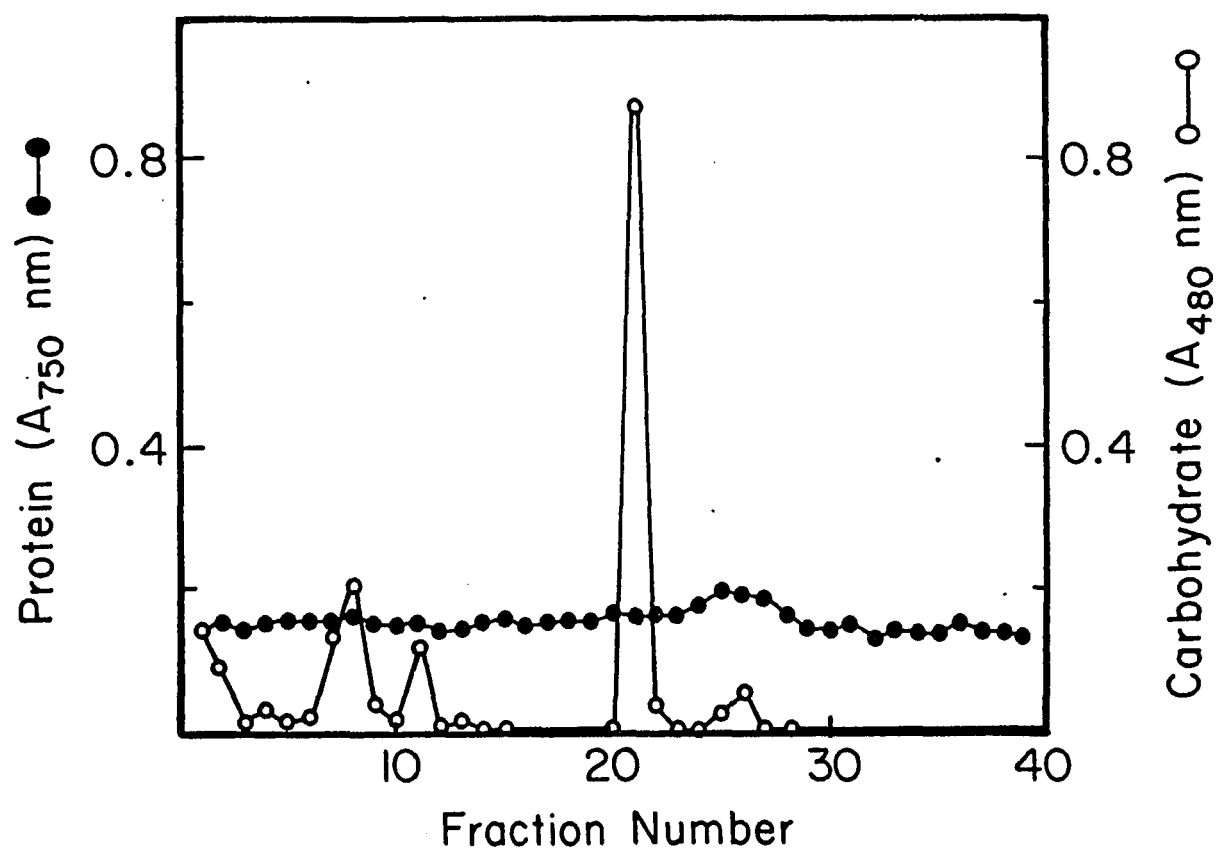
1) HK10: The normal spheroid:

The intact spheroids of strain HK10 were prepared for electron microscopy and stained with ruthenium red (Fulton, 1978). This enhances the contrast of the outer matrix sheaths surrounding the individual cells of HK10, and facilitates the visibility of the extracellular

Figure 30.

Extracellular macromolecules released into the growth medium by DIS 111, a mutant of V. carteri f. nagariensis strain HK10 that dissociates upon inversion and does not deposit and maintain a normal extracellular matrix.

DIS 111 was grown in 100 ml SVM pH 8.0 with no agitation. After one week's growth the flasks were gently swirled to release the cells adhering to the surface and the medium with the cells were filtered through GF/A filter discs. The filtrate was lyophilized, resuspended in distilled water, dialyzed against four changes of distilled water over 48 hours, and refiltered. The dialyzed filtrate was lyophilized, resuspended in 2 ml 0.1 M sodium phosphate buffer pH 7.8, and layered onto a chromatography column of Sepharose 4B. Fractions of 4.5 ml were collected and the fractions assayed for the presence of protein and carbohydrate according to the methods of Lowry and Dubois respectively.



matrix.

The matrix of the normal spheroid is seen in figure 31 as an electron dense region surrounding the gonidia and, to a lesser degree, the somatic cells. Figure 32 shows an electron dense layer "lining" the inner surface of the normal HK10 spheroid and continuous with the flagellar canal of the somatic cells. Electron dense fibrillar material is visible throughout the extracellular regions of the spheroid in both figures 31 and 32.

2) DIS 111: The matrix mutant.

A suspension of the cells of the matrix "dissociator" mutant, DIS 111, was prepared for electron microscopy and stained with ruthenium red to enhance the visibility of any extracellular material deposited by the organism.

Figures 33 and 34 show that the surfaces of the gonidia and somatic cells are devoid of the prominent electron dense layer that surrounds the individual cells of the normal HK10 spheroid. The cells themselves appear to be irregularly shaped, as though devoid of cell wall material to maintain structure. The electron micrographs (figures 33 and 34) show electron dense fibrillar material to be present in the areas immediately proximal to the cells of DIS 111, indicating the synthesis or release of extracellular components.

Figure 31.

Electron micrograph showing the extracellular matrix surrounding gonidia and somatic cells of Volvox carteri f. nagariensis strain HK10.

A 10 ml aliquot of a heterogeneous culture of strain HK10 was prefixed in 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 2 hours, followed by fixation in fresh 1.5% glutaraldehyde for 0.5 hours at room temperature, and 0.5 hours on ice. After washing spheroids in 0.1 M sodium cacodylate buffer for 10 minutes, spheroids were post-fixed in 1% osmium tetroxide, pH7.0 with 0.08% ruthenium red in 0.1 M sodium cacodylate added, for 3 hours on ice. The spheroids were washed in 0.1M sodium cacodylate buffer for 10 minutes and dehydrated in a series of ethanol changes; 50% ethanol - 10 minutes, 70% ethanol - overnight, 95% ethanol - 10 minutes, 100% ethanol - three changes of 10 minutes each, propylene oxide - two changes of 5 minutes each. Specimens were infiltrated with a 1:1 mixture of Spurr/propylene oxide for three hours, and then in pure Spurr overnight. The specimens were transferred to beam capsules, embedded in pure Spurr, and polymerized in a 70°C oven for 12 hours.

The specimens were sectioned with a glass knife,

transferred to carbon coated copper grids and stained with Reynold's (lead citrate) stain. The sections were viewed and filmed using a Zeiss electron microscope 9S-2 at a magnification of 4,900 X.

Note: Two adjacent spheroids are visible, with extracellular matrix and outer matrix sheaths discernable in each spheroid.

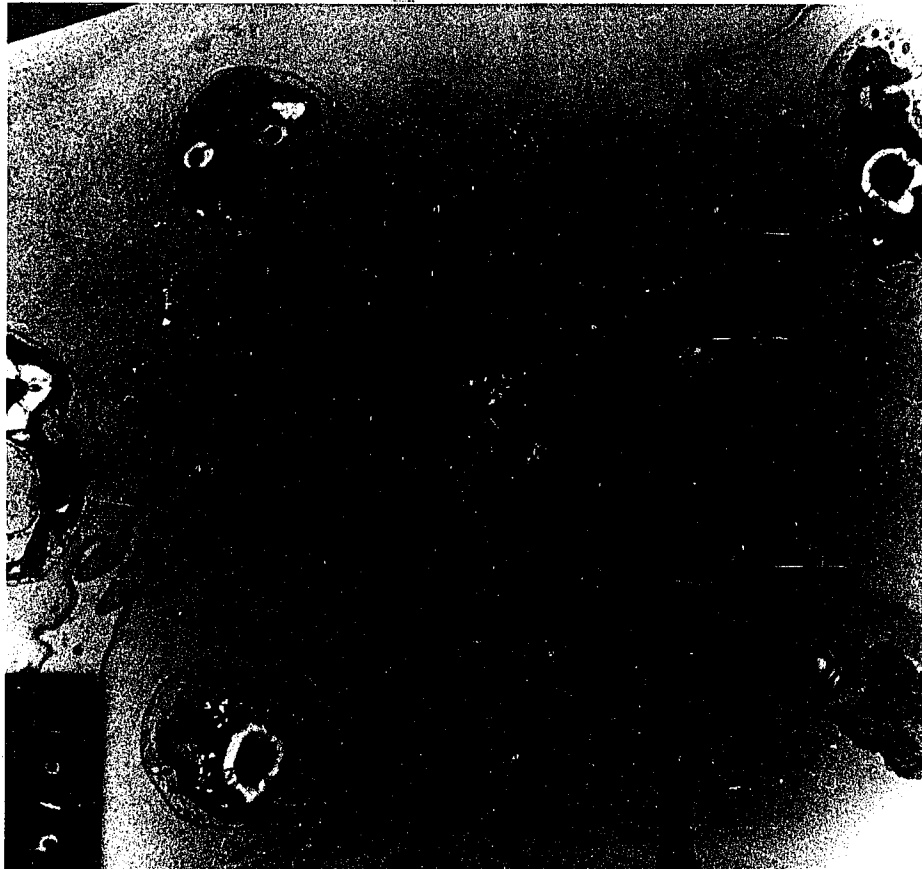


Figure 32.

Electron micrograph showing the extracellular material surrounding a somatic cell of V. carteri f. nagariensis strain HK10 continuous with the flagellar canal and the external matrix sheath.

A 10 ml aliquot of a heterogeneous culture of HK10 was prepared for electron microscopy by fixation in 1.5% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.2, for 5 minutes followed by fixation in 1.5% glutaraldehyde for 0.5 hours chilled on ice. The spheroids were washed in 0.1 M sodium cacodylate for 10 minutes and post-fixed and stained in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, with ruthenium red added to a final concentration of 0.08%, for 3 hours on ice. The spheroids were washed with sodium cacodylate buffer for 10 minutes, dehydrated through a series of ethanol changes; 50% ethanol - 10 minutes, 70% ethanol - overnight, 95% ethanol - 10 minutes, 100% ethanol - three changes, 10 minutes each, propylene oxide - two changes, 5 minutes each. The specimens were infiltrated with a 1:1 mixture of Spurr/ propylene oxide for three hours, and in pure Spurr overnight. The specimens were transferred to beam capsules, embedded in pure Spurr, and polymerized in a 70°C oven for 12 hours.

The specimens were sectioned using a glass knife, transferred to carbon coated grids, and stained with Reynold's (lead citrate) stain. The sections were viewed and photographed using a Zeiss 9S-2 electron microscope at a magnification of 9,500 X.

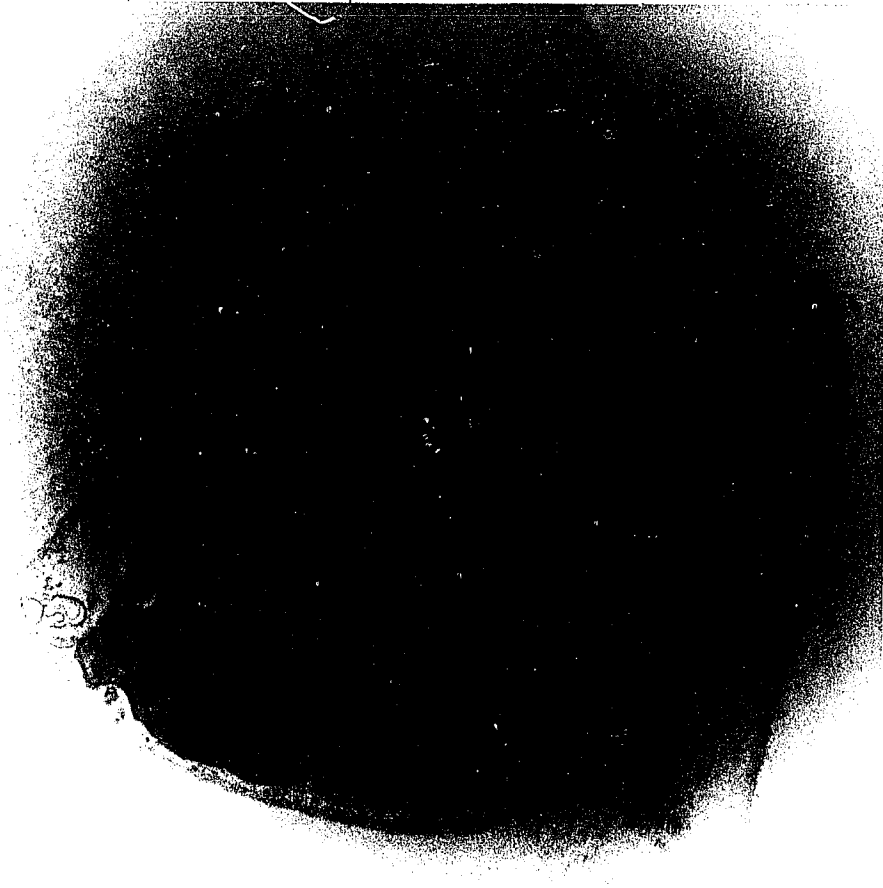


Figure 33.

Electron micrograph of Volvox carteri f. nagariensis strain HK10 matrix mutant DIS 111.

DIS 111 cells were prepared for electron microscopy using the fixation, embedding, and staining procedures described for the electron microscopy of the normal HK10 spheroids (figures 31 and 32). The specimens were sectioned using a glass knife, stained with Reynold's (lead citrate) stain, and viewed and photographed using a Zeiss 9S-2 electron microscope at 9,500 X magnification.

DIS 111 fails to synthesize normal extracellular matrix and dissociates immediately after undergoing the inversion process of embryogenesis. There is a large gonidium and smaller somatic cell visible in the photograph.

Note: The extracellular matrix surrounding normal gonidia visible in electron micrographs of normal HK10 spheroids is noticeably absent. The irregularity of the surface of the somatic cell is contrasted with the outer border of the normal somatic cell, and the outer matrix layer is absent.



Figure 34.

Electron micrograph of the outer surface of a gonidium of the matrix mutant, DIS 111, of V. carteri f. nagariensis strain HK10.

DIS 111 cells were suspended in 10 ml SVM pH 7.0 and prepared for electron microscopy as described for normal spheroids of V. carteri f. nagariensis strain HK10 (figures 31 and 32). The cells were fixed, stained, and embedded as described. The specimens were sectioned with a glass knife, transferred to carbon coated copper grids, stained with Reynold's (lead citrate) stain, and viewed and photographed with a Zeiss 9S-2 electron microscope at 9,500 x magnification.

Note the absence of the outer surface layer as visible on the surface of gonidia of the normal spheroid seen in figures 31 and 32.



VI. Palmelloid Chlamydomonas.

The addition of the chelating agent EDTA to the normal growth medium simulates stressful growth conditions and induces normal cells of Chlamydomonas (+ mating type) to form immobile clusters of eight cells embedded in a matrix like material (Iwasa and Murakami, 1968, 1969). Palmelloid Chlamydomonas was studied in order to biochemically compare the matrix that holds the more primitive unicellular cells of Chlamydomonas together under stressful conditions to the extracellular matrix of "colonial" Volvox

A. Extraction and fractionation of extracellular matrix from palmelloid Chlamydomonas.

Palmelloid clusters of Chlamydomonas were disrupted and the extracellular material extracted, concentrated, and fractionated on an exclusion gel of Sepharose 4B. Fractions eluted from the column were assayed for the presence of carbohydrate and protein.

The elution profiles of fractionated extracellular components extracted from palmelloid Chlamydomonas seen in figure 35 demonstrate the presence of at least two major macromolecular fractions; one a prominent carbohydrate, the other a closely migrating protein/

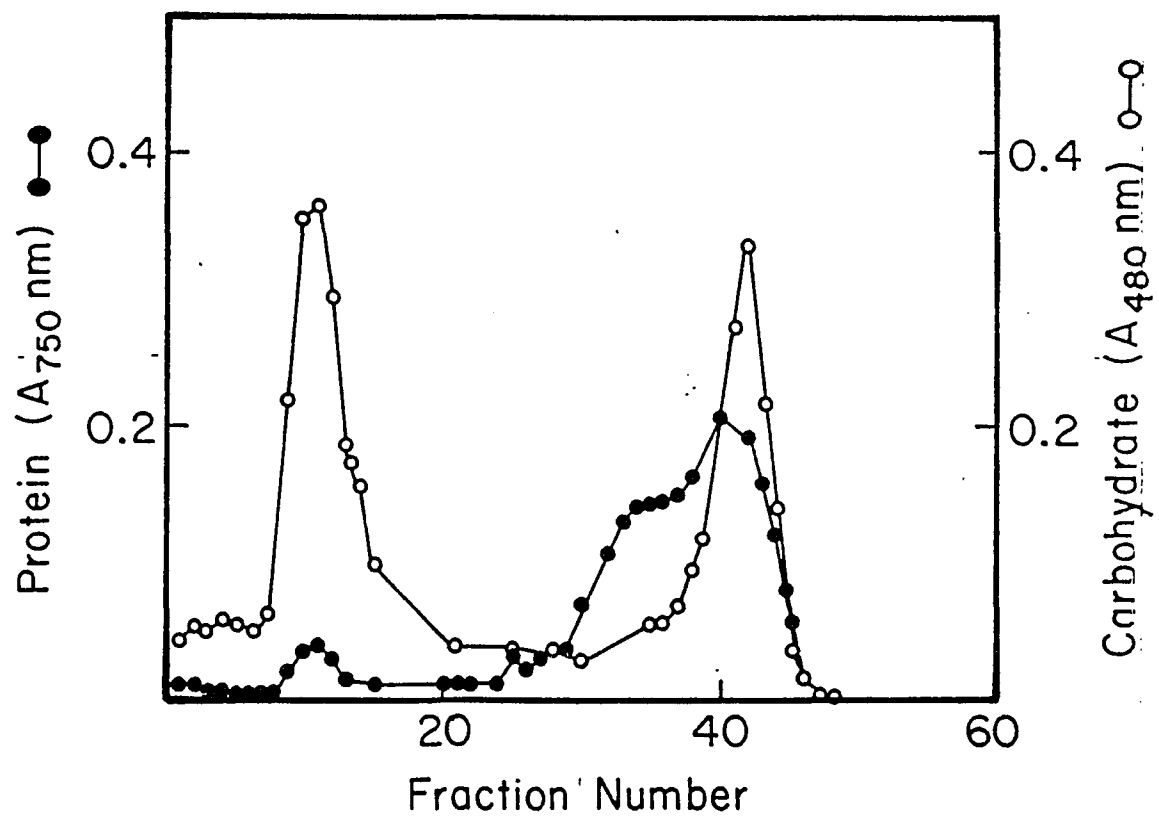
Figure 35.

Palmelloid Chlamydomonas: Fractionation of extracellular "matrix" extracted from palmelloid clusters by exclusion gel chromatography.

Cells of wild type Chlamydomonas, (+) mating type, were induced to undergo palmella formation by the addition of sodium EDTA ($12.5 \times 10^{-5} \text{M}$) to the normal growth medium. The palmelloid clusters were centrifuged at $12,000 \times g$ for 10 minutes and the pellet washed and resuspended in distilled water. The pelleted clusters were disrupted using the Dounce tissue grinder and cellular material was removed intact by filtering through GF/A. The filtrate containing the extracted material was concentrated by lyophilization.

The concentrated extract was resuspended in 0.1 M phosphate buffer pH 7.8 to a total volume of 2.5 ml containing 0.90 mg carbohydrate/ ml (as assayed by the Dubois method using arabinose as a standard) and 1.4 mg protein/ ml as assayed by the Lowry method using BSA as a standard). The extract was layered onto a column of Sepharose 4B equilibrated with 0.1 M phosphate chromatography buffer. A void volume of 55 ml was determined by the elution of blue dextran from the column. The column was eluted with two volumes of buffer and fifty 4.5 ml fractions were collected. Two 0.5 ml

samples were removed from each fraction and assayed for protein and carbohydrate using the methods of Lowry and Dubois respectively.



carbohydrate of lower molecular weight. These results may be compared to the results of figures 14 and 27, showing the presence of two macromolecular components, a large molecular weight polysaccharide and a glycoprotein, in extracellular matrix mechanically extracted from heterogeneous spheroids of Volvox, strains HK10 and NB7.

B. Electrophoresis of palmelloid Chlamydomonas glycoprotein components on SDS-polyacrylamide gradient gels.

(see figures 41, 42, and 43).

Extracellular glycoprotein containing fractions isolated from palmelloid Chlamydomonas were dialyzed, concentrated, resuspended in sample solvent, and electrophoresed on 5%-15% SDS-polyacrylamide gradient slab gels. The gels were silver stained, photographed, and dried.

The results seen in figures 41-43 show the glycoprotein fraction of the palmella "matrix" surrounding the unflagellated Chlamydomonas cells during stressful conditions, is resolved by electrophoresis into 16-20 distinct polypeptide bands with additional minor bands visible. It may be seen (figure 41) that some of the extracellular Chlamydomonas polypeptide bands appear to be common to Volvox extracellular matrix

glycoproteins, resolved by electrophoresis and silver staining. Many of the numerous polypeptide bands resolved by silver staining are likely to originate from the Chlamydomonas cell wall, known to be composed of much hydroxyproline rich glycoprotein (Catt et al., 1976; Goodenough and Heuser, 1985).

VII. SDS-polyacrylamide gel electrophoresis of extracellular matrix extracted from Volvox.

A. SDS-polyacrylamide gel electrophoresis of Tween extracted Volvox matrix components.

Heterogeneous HK10 spheroids were made radioactive with either ^3H -arginine or with $\text{NaH}^{14}\text{CO}_3$. Volvox matrix was extracted with 1.5% Tween mixture and the proteins precipitated with 5% TCA at 4°C . The pellets were neutralized as described in Materials and Methods, redissolved in sample solvent, and electrophoresed on a 10% SDSpolyacrylamide gel (Laemmeli, 1970; Studier, 1973).

It may be seen from the results of figure 36 that Tween treatment extracted protein compounds of high molecular weight which were resolvable by electrophoresis on 10% slab gels into at least seven major polypeptide bands.

Figure 36.

SDS-polyacrylamide gel electrophoresis of extracellular matrix components extracted from heterogeneous spheroids of Volvox carteri f. nagariensis by a 1:1 mixture of Tween 40 and Tween 80.

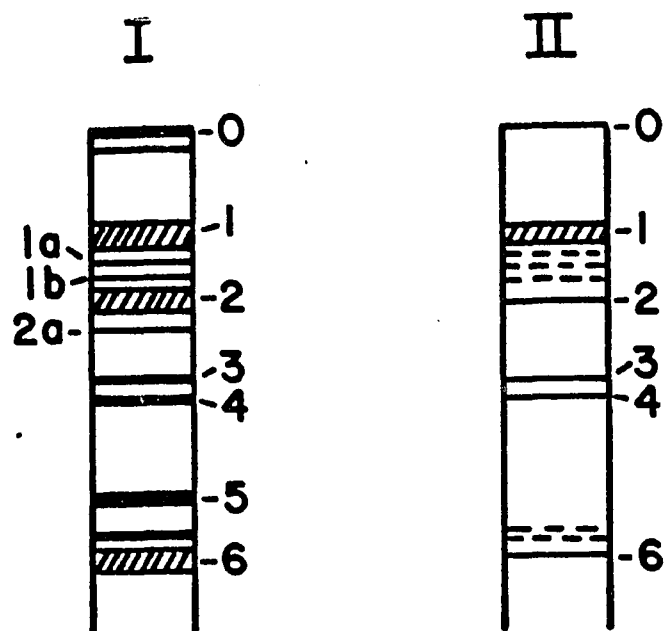
SDS-polyacrylamide slab gels (10%) were prepared as described and pre-electrophoresed at 100 V for two hours. HK10 spheroids were made radioactive with ^{14}C -bicarbonate or ^3H -arginine. Matrix proteins were extracted by incubating spheroids with 1.5% Tween mixture (50% Tween 40/ 50% Tween 80) at room temperature for three hours with constant agitation. The extracted matrix was precipitated with 5% TCA at 4°C . The pellets were neutralized by the addition of 1N NaOH with the use of the indicator dye bromocresol purple, redissolved in sample solvent, and 25 μl layered into the wells of a 5% stacking gel. Gels were electrophoresed at 150 V for 3.5 hours or until the tracking dye reached 1 mm from the bottom of the gel. Gels were developed by scintillation autoradiography (fluorography).

Diagrammatic representation of 10% slab gel developed by fluorography.

I = matrix proteins made radioactive with $\text{NaH}^{14}\text{CO}_3$

II= matrix proteins made radioactive with ^3H -arginine.

0 = origin of separating gel. 1 mm represents 2 mm migration into the gel.



B. SDS-polyacrylamide gel electrophoresis of detergent extracted Volvox matrix proteins of strain HK10 eluted from a chromatography column of Sephadex G-100.

The extracellular matrix was extracted from cultures of heterogenous Volvox strain HK10 made radioactive for 18 hours with $\text{NaH}^{14}\text{CO}_3$ by a 1:1 mixture of the non-ionic detergents Tween 40: Tween 80, or by the non-ionic detergent Triton X-100. Tween extracted matrix was chromatographed on a column of Sephadex G-100. Matrix proteins eluted from the column were precipitated with TCA, neutralized, and electrophoresed on 10% SDS-polyacrylamide slab gels. Triton X-100 extracted matrix was precipitated with TCA, neutralized, and electrophoresed alongside the Tween extracted matrix components.

It may be seen in figure 37 that four polypeptide bands were resolvable by 10% SDS-polyacrylamide slab gel electrophoresis of Tween extracted matrix components eluted from a column of Sephadex G-100. The polypeptides resolved were of high molecular weight, and the largest was composed of at least two closely migrating polypeptides. No qualitative differences were discernable between the gel patterns of pooled fractions 4-7 or fractions 8-11.

The results of figure 37 show that many more extracellular components are extracted from Volvox

Figure 37.

SDS-polyacrylamide gel electrophoresis of detergent extracted components of the extracellular matrix of V. carteri f. nagariensis strain HK10.

Two 10 ml aliquots of a heterogeneous culture of HK10 with 200 spheroids/ml were made radioactive with the addition of 0.4 ml of $\text{NaH}^{14}\text{CO}_3$ stock (2×10^6 cpm/10 ml culture). The cultures were incubated at room temperature under constant illumination, with agitation, for 24 hours. The spheroids were harvested by filtration through 7 μm Nitex, washed and resuspended in distilled water at a total volume of 5 ml.

Tube A: A 1:1 mixture of Tween 40 : Tween 80 was added to yield a final concentration of 2% Tween mixture. The tube was incubated at room temperature with constant agitation for 3 hours and filtered through glass wool.

Extract A₁: 2 ml of the filtrate was layered onto a chromatography column of Sephadex G-100 equilibrated with 0.02 M Tris buffer. Fractions 4-7 eluted from the column were collected, precipitated with 10% TCA for 0.5 hours at 4°C.

Extract A₂: Fractions 8-11 eluted from the above Sephadex G-100 column, precipitated with 10% TCA.

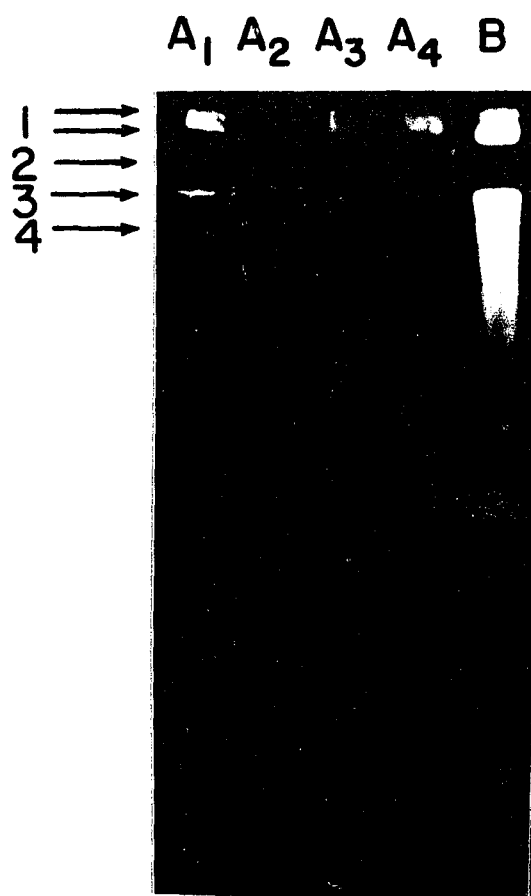
Extract A₃: 1.8 ml of the filtrate were layered onto a chromatography column of Sephadex G-200 equilibrated

with Tris buffer. Fractions 4-9 eluted from the column were collected, and precipitated with 10% TCA at 4°C for 0.5 hours.

Extract A₄: 1 ml of the filtered matrix extract was diluted with 1 ml Tris buffer and precipitated with 10% TCA at 4°C for 0.5 hours.

Tube B: Triton X-100 was added to the culture to yield a final concentration of 0.003%. The culture was incubated at room temperature for three hours with constant agitation, and the spheroids filtered through glass wool. The extracted matrix was precipitated by the addition of 10% TCA at 4°C for 0.5 hours.

The TCA precipitated proteins were centrifuged at top speed in a microfuge and the pellets neutralized by the addition of NaOH to a neutral endpoint indicated by the addition of bromocresol purple. Sample solvent was added to the neutralized proteins in a ratio of 1:1, and the samples placed in a boiling water bath for 2 minutes. The samples were layered into the wells of a 5% spacer gel overlaid on a 10% SDS acrylamide separating gel pre-electrophoresed for 1 hour at 100 V. The gel was electrophoresed at room temperature for 3 hours at 150 V, prepared for fluorography according to the method of Bonner and Lasky, and filmed using Kodak Royal X-Omat 5 x 7 sheets.



spheroids by Triton X-100. The two largest, closely migrating polypeptide bands resolved, as well as band #3, appeared to be similar to those resolved by Tween treatment, however additional material was present that was not resolvable by the method used. The increased extraction of matrix components by Triton X-100 seen in figure 37 may be correlated with the results of figure 13, showing that unlike the plateau effect produced by Tween extraction, Tx-100 extraction of Volvox matrix components does not level off.

C. SDS-polyacrylamide gel electrophoresis of extracellular matrix mechanically extracted from HK10 spheroids, and extracellular material extracted from a suspension of DIS 111 cells (a mutant of HK10 defective in the synthesis of extracellular matrix).

Heterogeneous spheroids of strain HK10 and a cell suspension of DIS 111 were made radioactive with $\text{NaH}^{14}\text{CO}_3$ for 18 hours. HK10 spheroids were disrupted using the Dounce tissue grinder. The extracellular matrix was extracted, concentrated by lyophilization, resuspended in sample solvent, and electrophoresed on 10% SDS-polyacrylamide gel slabs (figure 38).

The polypeptide banding pattern of mechanically extracted Volvox extracellular matrix seen in figure 38

closely resembles the polypeptide banding pattern seen in autoradiograms of detergent extracted matrix of Volvox (figure 37). There are four distinct high molecular weight polypeptide bands resolved. Band #1 appears to be the result of two closely migrating polypeptides. Band #3 appears as a prominent component of the extracted matrix.

Extracellular material concentrated from a suspension of DIS 111 cells was resuspended in sample solvent and electrophoresed alongside extracellular matrix components mechanically extracted from HK10 spheroids. The results of figure 38 show the extracellular material synthesized by DIS 111 was resolved into at least three high molecular weight polypeptide bands in common with the extracellular matrix of the normal spheroid. One band present in the extracellular matrix extracted from the normal spheroid (band #4) was noticeably absent from the extracellular material synthesized by the matrix mutant.

D. SDS-polyacrylamide gel electrophoresis of radioactive extracellular matrix mechanically extracted from a heterogeneous culture of Volvox and fractionated on an exclusion gel of Sepharose 4B.

Extracellular matrix mechanically extracted from a

Figure 38.

SDS-polyacrylamide gel electrophoresis of mechanically extracted extracellular matrix of a heterogeneous culture of Volvox carteri f. nagariensis strain HK10, and extracellular proteins of the matrix mutant DIS 111.

Two 10 ml samples of a heterogeneous culture of HK10 and two 10 ml samples of a cell suspension of DIS 111 were each made radioactive with the addition of 0.2 ml $\text{NaH}^{14}\text{CO}_3$ stock (1×10^6 cpm/ 10 ml culture) and incubated for 24 hours at room temperature with constant illumination.

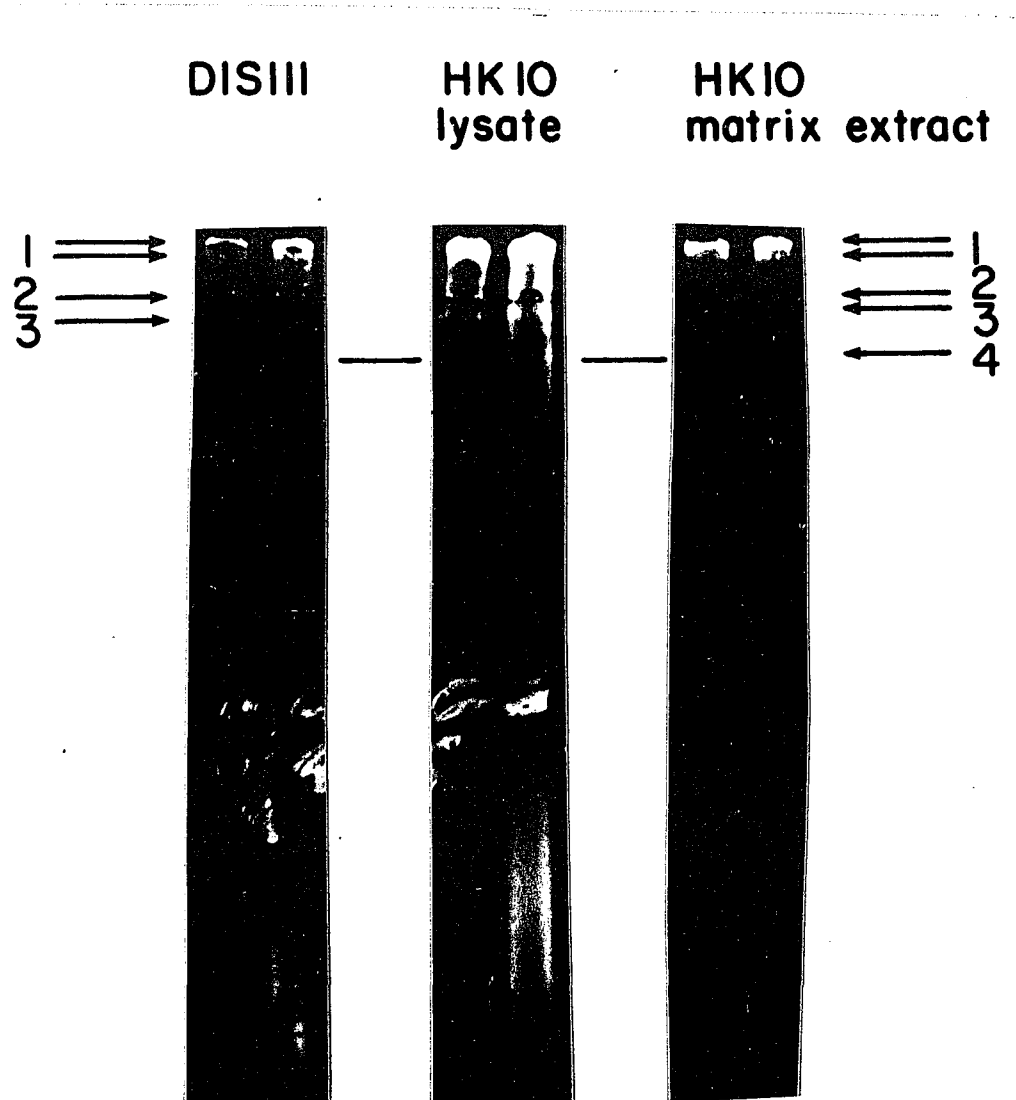
The HK10 cultures were filtered through 7 um Nitex, washed and resuspended in distilled water. One culture of HK10 was disrupted with 7 strokes of the Dounce tissue grinder, the matrix filtered through glass wool, and concentrated by lyophilization. The second culture of HK10 was extracted with 3 volumes of acetone at 4°C , and the cells lysed in sample solvent with glass beads in a mini homogenizer.

One culture of DIS 111 was gently disrupted with two strokes of the "A" ball of the Dounce tissue grinder, the suspension filtered through glass wool, the filtrate dialyzed against 4 changes of distilled water and concentrated by lyophilization. The second culture of DIS 111 was dialyzed against distilled water, extracted with

3 volumes of acetone at 4°C, and the cells lysed in sample solvent in a mini homogenizer.

All samples in sample solvent were placed in a boiling water bath for 2 minutes. Aliquots of 5 ul were removed from all samples, transferred to paper filter discs, dried, scintillant added, and radioactivity measured in a Beckman liquid scintillation counter. Radioactivity/ 5 ul sample: HK10 matrix - 1185 cpm, HK10 lysate - 4293 cpm. DIS 111 extracellular - 350 cpm, DIS 111 lysate - 1018 cpm.

Samples of 20 ul and 40 ul were layered into the wells of a 5% spacer gel overlaid on a 7.5% SDS acrylamide slab gel pre-electrophoresed for 1 hour at 100 V. The gel was electrophoresed for 3 hours at 150V, prepared for fluorography according to the method of Bonner and Lasky, and filmed with Kodak Royal X-Omat film.



heterogeneous culture of HK10 spheroids made radioactive with $\text{NaH}^{14}\text{CO}_3$ was fractionated on a chromatography column of Sepharose 4B. The glycoprotein containing fractions were combined and electrophoresed on a 7.5% SDS-polyacrylamide slab gel alongside the unfractionated radioactive matrix.

The results of figure 39 show that the polypeptide banding pattern of the fractionated matrix was more clearly resolved by SDS-gel electrophoresis than the unfractionated matrix. The large amount of unresolved matrix material seen in figure 39 is attributable to the polysaccharide components of the extracted matrix which are not fractionated by polyacrylamide gel electrophoresis. In contrast, exclusion gel chromatography isolated the proteinaceous components of the matrix which were then more effectively separated and resolved by SDS-polyacrylamide gel electrophoresis.

The banding patterns produced by electrophoresis on the 7.5% SDS-polyacrylamide gel seen in figure 39 may be further compared to the banding pattern resolved by electrophoresis on a 10% SDS-polyacrylamide gel seen in figure 37. The four major polypeptide bands seen in figure 37 are seen to be resolved (figure 39) into additional components when electrophoresed on an SDS-polyacrylamide gel with greater porosity.

Figure 39.

SDS-polyacrylamide gel electrophoresis of mechanically extracted components of the extracellular matrix of V. carteri f. nagariensis strain HK10.

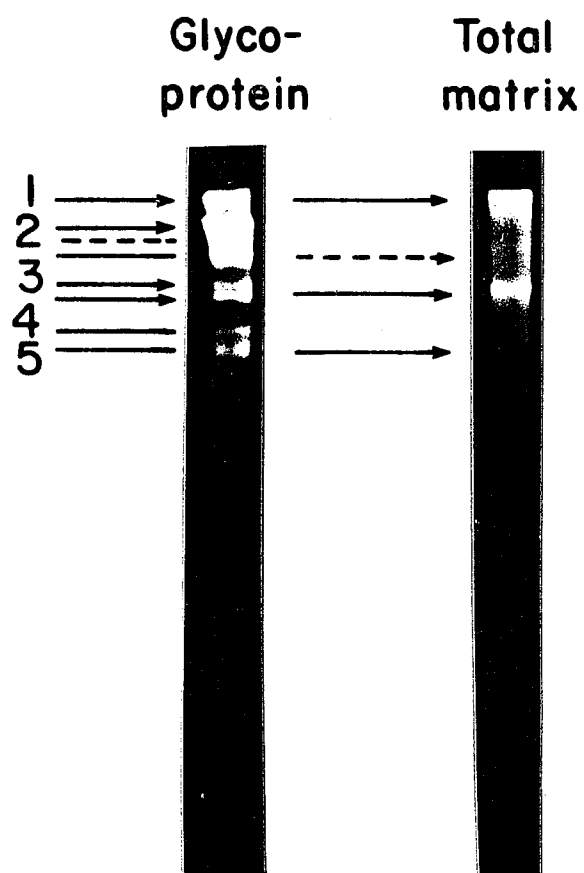
Two 10 ml samples were withdrawn from a heterogeneous culture of HK10, transferred to culture tubes, made radioactive with the addition of 0.2 ml $\text{NaH}^{14}\text{CO}_3$ stock in SVM pH 8.0 (1×10^6 cpm/10 ml culture), and incubated for 24 hours at room temperature with constant illumination and agitation. The extracellular matrix was extracted using the Dounce tissue grinder, filtered through glass fiber filters, and concentrated by lyophilization.

The crude matrix preparation was resuspended in 0.1 M sodium phosphate buffer, pH 7.8, with 0.02% sodium azide added, and 0.5 ml layered onto a mini chromatography column of Sepharose 4B equilibrated with 0.1 M sodium phosphate buffer. The column was eluted with two volumes of 0.1 M sodium phosphate buffer and 70 fractions collected. The glycoprotein containing fractions were dialyzed against four changes of distilled water over 48 hours, and concentrated by lyophilization.

The concentrated glycoprotein "GP peak" was resuspended in sample solvent for electrophoresis, and placed in a boiling water bath for 2 minutes. For

electrophoresis of total matrix extract, crude matrix preparation was resuspended in sample solvent and placed in a boiling water bath for 2 minutes. Aliquots of 10 ul were removed from each sample, transferred to GF/A filter discs, dried, scintillant added, and radioactivity measured with a Beckman liquid scintillation counter. GP peak 1= 9,705 cpm/25ul; GP peak 2= 2,170 cpm/25ul; total matrix 1= 14,070 cpm/ 25 ul;total matrix 2= 6,990 cpm/25 ul.

Aliquots of 25 ul or 50 ul (as indicated) of each sample were layered into the wells of a 5% stacking gel overlaid on a 7.5% SDS polyacrylamide gel, pre-electrophoresed for 1 hour at 100 V. The gel was electrophoresed at 150 V for 3 hours, prepared for fluorography according to the method of Bonner and Lasky, and filmed with Kodak Royal X-Omat 5 x 7 sheets.



VIII. Electrophoretic analysis of fractionated matrix.

The glycoprotein containing fractions isolated by exclusion gel chromatography on a column of Sepharose 4B were pooled, dialyzed, and concentrated by lyophilization. The matrix glycoproteins were electrophoresed on SDS - polyacrylamide slab gels in order to;

- 1) determine the internal polypeptide heterogeneity of the glycoprotein migrating as a single component on exclusion gels of Sepharose 4B and Sephadex G-200.
- 2) follow changes therein during specific developmental stages of the normal HK10 life cycle.
- 3) compare the matrix glycoproteins of the two strains of Volvox, HK10 and NB7.
- 4) determine any resemblance existing between matrix glycoprotein polypeptides of Volvox and those of palmelloid Chlamydomonas.

A. 7.5% and 10% SDS-polyacrylamide slab gel electrophoresis; Coomassie Blue staining.

Lyophilized Volvox matrix glycoproteins were isolated from heterogeneous spheroids of strains HK10 and NB7, resuspended in sample solvent, and electrophoresed on 7.5% and 10% SDS-polyacrylamide gel slabs overlaid with

5% stacking gels. The discontinuous buffer system of Laemmli (1970) was used, and the gel was stained with 0.1% Coomassie Brilliant Blue (Weber and Osborn, 1975).

The results, seen by diagrammatic representation in figure 40, indicate the presence of a major high molecular weight polypeptide band present in matrix glycoproteins extracted from HK10 spheroids that was absent from matrix glycoproteins extracted from NB7 spheroids. A number of slightly faster migrating bands were not clearly resolved but appeared in the same molecular weight regions as BSA and G6PD respectively. These three polypeptide bands corresponded to bands #2,3, and 4 as seen in figures 37 and 39. It may be seen in figure 40 that a prominent intermediate weight polypeptide band is resolvable in matrix glycoproteins of strain NB7 that is absent in matrix glycoproteins extracted from spheroids of strain HK10. In glycoprotein samples of both strains, a number of small bands migrated just behind the dye front with lysozyme, only two of which were resolvable with the method used.

Although some polypeptide bands were resolvable, since much of the gel is unclear when stained with Coomassie Blue, other techniques of staining were used.

Figure 40.

Diagrammatic representation of major protein bands comprising the glycoproteins of Volvox extracellular matrix.

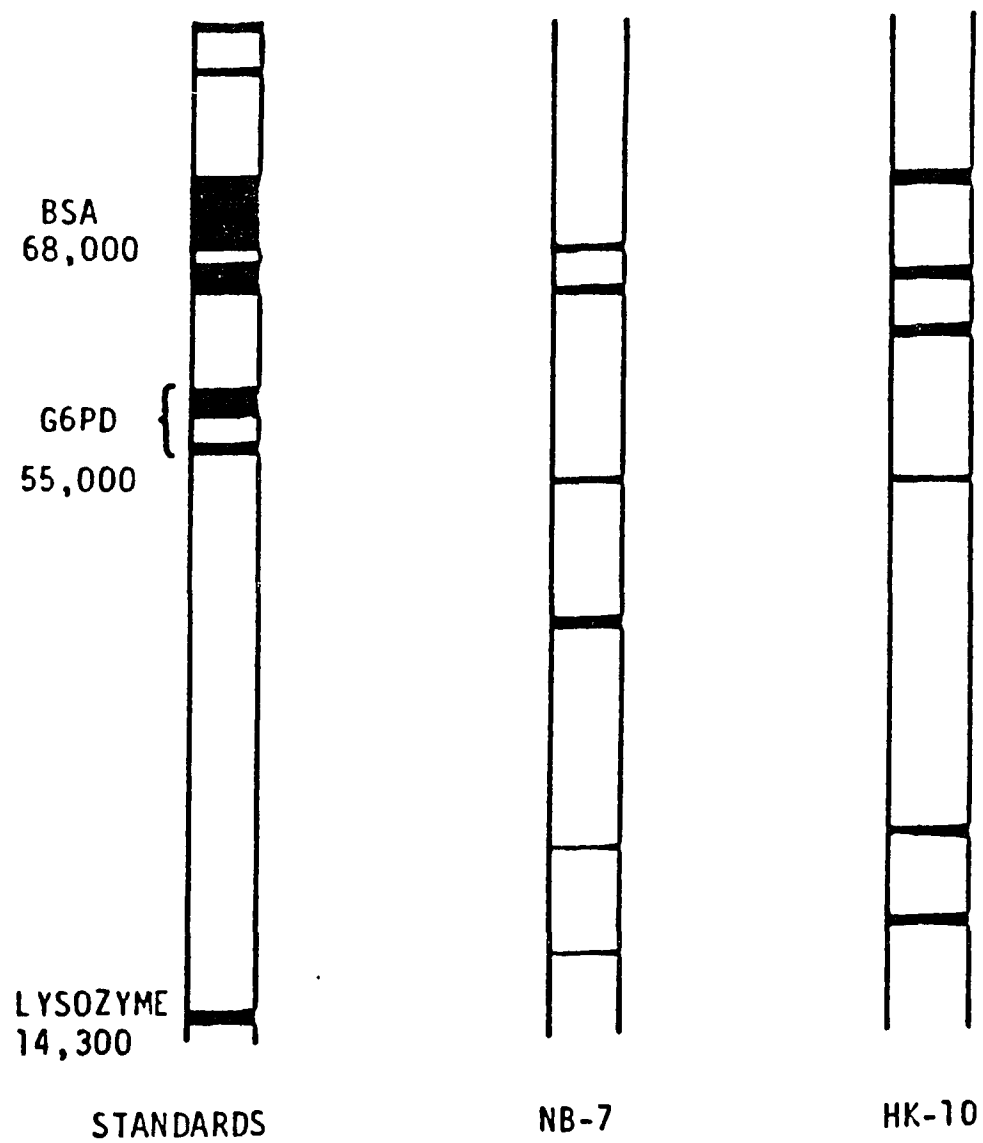
Ten liter heterogeneous cultures of Volvox carteri f. nagariensis strain HK10 and Volvox carteri f. weismania strain NB7 were grown in SVM pH 8.0 and harvested by filtration through 80 um Nitex. The spheroids were disrupted using the Dounce tissue grinder. The extracellular matrix was extracted by filtration through glass fiber filters and concentrated by lyophilization. The crude matrix preparation was fractionated by exclusion gel chromatography on a column of Sepharose 4B, and the glycoprotein containing fractions dialyzed and lyophilized.

The concentrated GP peaks were resuspended in sample solvent and placed in a boiling water bath for 2 minutes. Samples of 25 ul and 50 ul were layered into the wells of a 5% stacking gel overlaid on a 10% SDS acrylamide separating gel. Volumes of 10 ul and 25 ul of a mixture of molecular weight standards in sample solvent (containing bromophenol blue as a tracking dye) were layered into wells 1 and 2. The gel was electrophoresed at 150 V for 3 hours, until the bromophenol blue tracking

dye reached the bottom of the gel.

The gel was fixed in Coomassie Blue fixative, stained with Coomassie Brilliant Blue, and destained in 7.5% acetic acid with at least four changes overnight. The gel was dried onto Whatman 3MM chromatography paper and photographed.

DIAGRAMMATIC REPRESENTATION OF MAJOR PROTEIN BANDS
COMPRISING GLYCOPROTEINS OF VOLVOX EXTRACELLULAR MATRIX



B. 7.5%-15% SDS-polyacrylamide gradient gels; silver staining.

Matrix glycoproteins were isolated from heterogeneous spheroids of strains HK10 and NB7, and HK10 spheroids at specific developmental stages; newly released, condensed (pre-cleavage), and pre-release. The glycoproteins were electrophoresed on SDS-polyacrylamide gradient gels and silver stained to enhance resolution.

The results seen in figures 41-43 reveal differences in the protein subunit composition of the matrix glycoproteins extracted from two strains of Volvox, HK10 and NB7. The highest molecular weight polypeptide band resolved in glycoproteins extracted from heterogeneous spheroids of strain HK10, was absent in glycoproteins isolated from NB7. A prominent polypeptide band appears in all stages of NB7 that is never found in glycoproteins isolated from the matrix of HK10 spheroids at any stage of the life cycle. The lowest molecular weight polypeptides appear to be common to both strains of Volvox studied.

The glycoprotein components of extracellular matrix extracted from Volvox carteri f. nagariensis strain HK10 cultures at specific developmental stages of the life cycle were compared to matrix glycoproteins extracted

from heterogeneous cultures. Specific polypeptide bands are present in newly released, condensed (just prior to cleavage), and pre-release (fully expanded) Volvox. Differences are noted, specifically in a high molecular weight polypeptide band prominent in condensed Volvox, which appears to shift and is noted as a faster migrating band in pre-release cultures. Both these bands appear in matrix extracted from heterogeneous cultures, whereas neither one appears in the matrix of newly released spheroids (photos and dried gels). A large molecular weight polypeptide resolved in matrix glycoproteins extracted from heterogeneous, newly released, and condensed spheroids of strain HK10 is noticeably absent from glycoproteins extracted from spheroids about to release. One possible explanation may be the onset of release enzyme activity at this stage, with this specific polypeptide being an early target of enzymatic digestion.

Figures 41, 42, and 43.

SDS-polyacrylamide gel electrophoresis showing banding patterns of glycoproteins extracted from the matrix of heterogeneous cultures of V. carteri f. nagariensis strain HK10, V. carteri f. weismania strain NB7, synchronous cultures of newly released and pre-release HK10 spheroids, and the "matrix" of palmelloid clusters of Chlamydomonas reinhardi.

Matrix preparations were fractionated on exclusion gels of Sepharose 4B equilibrated with 0.1 M sodium phosphate buffer, pH 7.8. Glycoprotein containing fractions were dialyzed against four changes of distilled water over 48 hours and concentrated by lyophilization. The lyophilized glycoproteins, "GP peak", were resuspended in distilled water to give a suspension of 100 ug/ ul. Samples of 10 ul were withdrawn and 90 ul electrophoresis sample solvent added to give a solution of 10 ug glycoprotein/ ul. A 20 ul aliquot of the sample was further diluted to give a final concentration of 2.5 ug glycoprotein / ul.

The samples were placed in a boiling water bath for two minutes, cooled to room temperature, and 2 ul-25 ul of each sample layered into the wells of a 7.5%-15% SDS-polyacrylamide gradient gel. A mixture of protein standards in sample solvent, containing bromophenol blue as a tracking dye, was placed in a boiling water bath,

cooled to room temperature, and 5 ul layered into one of the two outer sample wells. The gel was electrophoresed at 150 V for 3.75 hours, or until 0.75 hours after the tracking dye reached the bottom of the gel.

The gel was fixed in a solution of formaldehyde and methanol overnight, stained with silver nitrate, dried onto Whatman 3 MM filter paper, and photographed.

Key to polypeptide bands:

- HK - HK10 high molecular weight polypeptide band.
- NB - NB7 polypeptide band never seen in HK10 extracts.
- PR - high molecular weight band(s) not seen in pre-release matrix.
- S - appears to be a shift in mobility of band "PR".
- C - low molecular weight band appearing in matrix extracted from condensed spheroids (prior to embryogenesis).

Molecular weight standards:

1. BSA - 66,000 D.
2. egg albumin - 45,000 D.
3. pepsin - 34,700 D.
4. trypsinogen - 24,000 D.
5. β -lactoglobulin - 18,400.
6. lysozyme - 14,300.

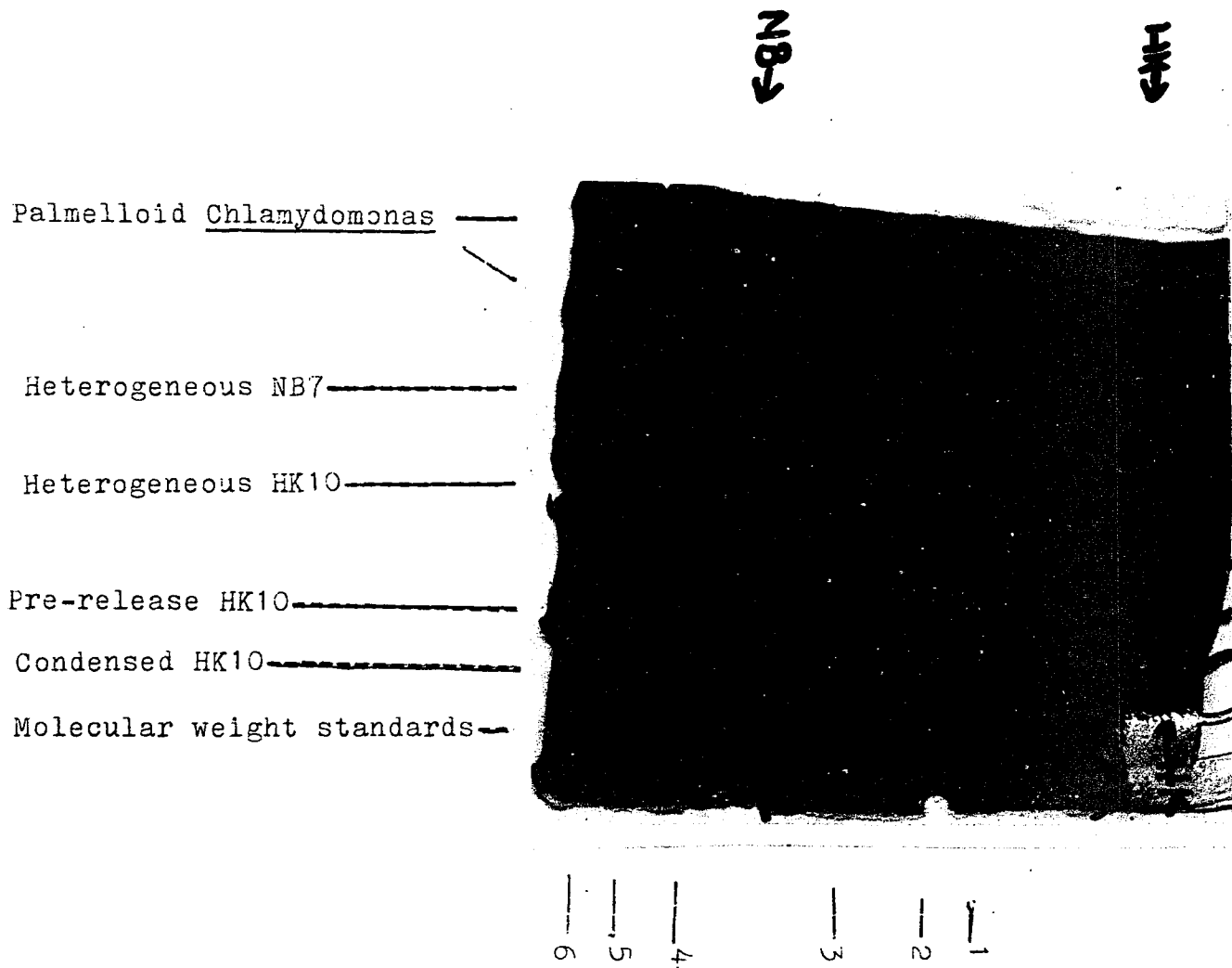


Figure 41.

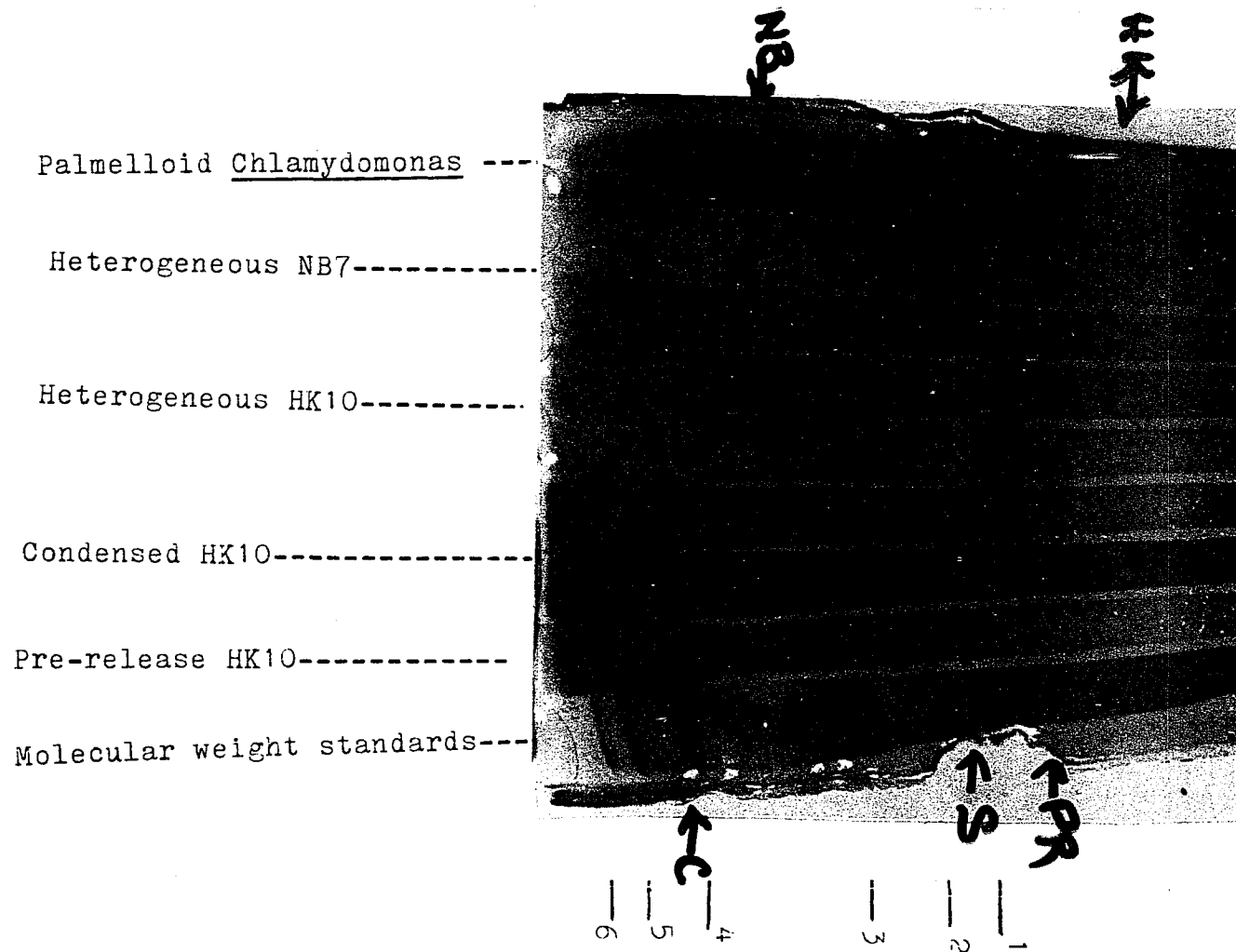


Figure 42.

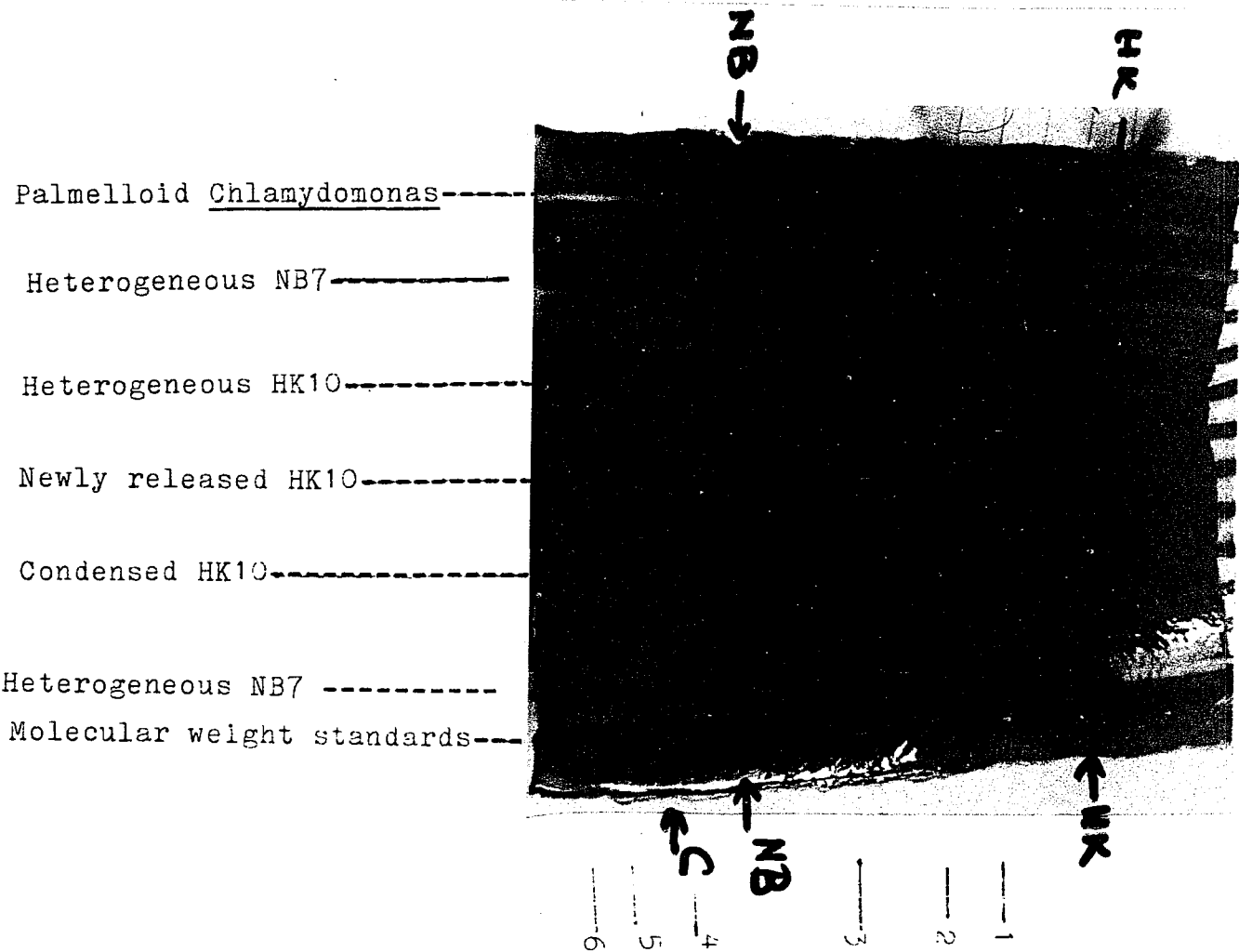


Figure 43.

DISCUSSION

The Volvocales exemplify the transition from unicellular simplicity to multicellular complexity. Volvox, the most complex and highly evolved member, is both a colony of Chlamydomonad unicells, and a multicellular organism consisting of functionally specialized cells. The extracellular matrix is an elaboration of the primitive algal cell wall; a link between the unicellular and multicellular life forms and an adaptation that buffers the organism against adverse environmental conditions.

This study was undertaken with the goal of investigating Volvox development with primary emphasis on characterization of the extracellular matrix. The results of this investigation may be organized into four general areas for the purpose of summary and discussion.

I. Development of optimal growth conditions for study of the matrix, including; synchronization, isolation of spheroids at desired stages of the life cycle, identification of matrix components using biochemical techniques, and the incorporation of radioisotopes into matrix components.

II. Detergent mediated extraction of the extracellular

matrix including; extraction of matrix components of two strains of Volvox using solubilizing agents under varying conditions of detergent concentration, spheroid density, pH, and the presence of a chelating agent. The detergent extracted components were separated using exclusion gel chromatography and SDS-polyacrylamide gel electrophoresis.

III. Development of a technique for mechanical extraction of the extracellular matrix; characterization of the molecular composition of the mechanically extracted matrix material and separation of the individual matrix components utilizing the techniques of exclusion gel, ion exchange, and affinity column chromatography, and SDS-polyacrylamide gel electrophoresis.

IV. A comparative study of changes in matrix components extracted from; Volvox carteri f. nagariensis strain HK 10 at distinct developmental stages in the asexual life cycle, two morphologically similar yet reproductively isolated strains of Volvox (HK10 and NB7), the normal HK10 spheroid and a mutant defective in matrix synthesis (DIS 111). Volvox matrix was also compared to the extracellular material secreted by Chlamydomonas during the stress induced palmelloid stage, whereby vegetative cells become non-flagellate and proliferate while embedded within a gelatinous matrix.

I. The development of optimal growth conditions.

A. Synchronization:

Standard Volvox medium (Provasoli and Pintner, 1959) at a pH range of 7.0-8.0 under conditions of either continuous light, a light/ dark regime of 16 hours light/ 8 hours dark, or 31 hours light/ 17 hours dark, was satisfactory for the growth of both HK10 and NB7 in quantities up to 10 l, and for the growth of the dissociator mutant, as well as for the maintenance of stock cultures under continuous illumination. Synchronized cultures were more easily grown and consistently maintained at pH 8.0, with a synchrony inducing (Mitchison, 1971; Kirk and Kirk, 1976) light/ dark cycle of 31 hours light/ 17 hours dark. Optimal synchrony was maintained in culture volumes up to 1 liter.

B. Isolation of stage specific spheroids:

Synchronous cultures were routinely filtered through Nitex monofilament cloth. This was found to ensure the isolation of spheroids at specific stages of the life cycle and to eliminate error attributable to the inclusion of low numbers of asynchronous individuals. For

the purpose of obtaining pre-release spheroids cultures were filtered through 300 um Nitex. The pre-release spheroids were efficiently trapped while the early releasing individuals present were eliminated. 125 um Nitex was routinely used to isolate pre-cleavage stage spheroids, while the newly released juveniles passed through all but 60-80 um filters (see Appendix, section X).

C. Identification of matrix components:

Non-cellular components of Volvox were found to comprise much of the spheroid mass in a heterogeneous population (table 1). However, the amount of extracellular material present at any given time in a spheroid is a function of the developmental stage (figure 1). Spheroid expansion results in a 300% increase in the diameter of the organism from its nascent state (stage 1) through maturity (stage 7). It was determined that the proportion of spheroids in the various stages of development in a heterogeneous culture varies as a function of spheroid density and age of the population (table 2). Any statement regarding the ratio of extracellular to cellular components of Volvox should therefore be based on data obtained from synchronous cultures with spheroid size/ stage clearly defined.

It was determined (table 4), that the protein content of the extracellular matrix increases as a function of expansion, as seen from the analysis of matrix extracted from spheroids at specific developmental stages. The matrix extracted by the techniques used (see Materials and Methods) does not, however, reflect the total extracellular matrix in the spheroid. Ultrastructural studies clearly indicate the existence of multiple layers of matrix material, found to differ morphologically and histochemically (Kirk et al., 1986), hence more than likely to differ in susceptibility to various extraction techniques.

The ratio of carbohydrate to protein in extracellular matrix mechanically extracted from two morphologically similar strains of Volvox, HK10 and NB7, varies with the specific stage (and size) of spheroid development. The change in carbohydrate: protein ratio reflects an increase in carbohydrate containing components in the extractible matrix as a function of spheroid growth (table 5). Based on the morphological changes occurring during the asexual life cycle, depicted in figure 1, the results of tables 4 and 5 indicate that as spheroid growth and development progress, there is a concomitant deposition of extracellular matrix composed of both protein and carbohydrate macromolecules. The increase in protein/ spheroid is evident from the results of table 4.

The increasing proportion of carbohydrate to protein as a function of spheroid expansion is shown by the data in table 5. The results of recent investigations by Kopan, Kurn, and Ovadia (1985) showing an increase in carbohydrate containing macromolecules in the post-embryonic matrix of HK10 corroborate the findings of this study.

D. Incorporation of radioisotopes:

Volvox and Chlamydomonas have been found to contain a carrier mediated uptake system for the facilitation of arginine incorporation (Kirk and Kirk, 1978). However, in this study, although arginine was taken up readily by the spheroids it was not incorporated into the Volvox matrix with much success (personal observation). Arginine appears to be synthesized into cellular proteins (Kirk and Kirk, 1976).

Lamport, in a comparative amino acid analysis of the cell wall of Chlamydomonas and the extracellular matrix of Volvox, determined that hydroxyproline comprised 21% of the amino acid residues of the matrix (Kochert, 1975; Miller and Lamport, 1972). Radioactive proline was therefore the isotope of choice for the production of radioactive matrix proteins. Volvox, a photosynthetic autotroph, was thought to be a likely candidate for

incorporating radioactive carbon into polysaccharide. The results (figure 4) clearly show the incorporation of $\text{NaH}^{14}\text{CO}_3$ and radioactive proline into the extracellular matrix mechanically extracted from two strains of Volvox, HK10 and NB7. Since ^{14}C -proline was empirically found to be incorporated into matrix macromolecules more efficiently than ^3H -proline (figure 4), it was the radioactive amino acid of choice.

II. Detergent extraction of matrix components.

A. Solubilization of macromolecular components:

Densely populated cultures of Volvox were observed to have a cloudy appearance which is the result of the release of proteinaceous material into the medium (table 6). Voigt (1985) reported similar release of macromolecules into the culture medium of Chlamydomonas during the cell enlargement phase of the vegetative cell cycle. These macromolecules were shown to be proteinaceous components of the cell wall. Pulse-chase experiments utilizing radioactive proline resulted in the appearance of radioactive macromolecules in the culture medium concomitant with a decrease in the radioactivity of the cell wall.

The results of these investigations support the observation that Volvox extracellular macromolecules are released into the culture medium during development. It was therefore important for the study of the extracellular matrix to; 1) devise a procedure to extract extracellular macromolecules from Volvox without interfering with the viability of the spheroids, 2) characterize the extracellular macromolecules released from asexual spheroids of Volvox.

Margolis-Kazan and Blamire (1978 and personal communication) found that the non-ionic detergent Tween (50% Tween 40/ 50% Tween 80) facilitated the isolation of Volvox protoplasts, apparently by solubilizing components of the matrix. Tween treated cultures of Volvox have a cloudy appearance. TCA precipitation of the extracted material resulted in a pellet that yielded positive assays for both protein and carbohydrate, indicating that the detergent mediated the release of extracellular carbohydrate, protein, and/or glycoprotein into the medium.

The use of non-ionic detergents has been documented as a procedure for the extraction of extracellular components, specifically extracellular and membrane proteins (Helenius and Simons, 1975; Stohlman et al., 1976). Tween 40, Tween 80, and Triton X-100 have been used to extract cytochrome oxidase from mitochondrial

membranes because they are non denaturing detergents. The non-ionic detergents act by binding and displacing part of the phospholipid associated with the enzyme after isolation (Robinson and Capaldi, 1977). Triton X-100 has been used to solubilize viral envelope glycoproteins (Hearing et al., 1976) and is a preferred method of protein extraction because it preserves the 3D structure of proteins. Many membrane proteins can in fact be solubilized only in the form of protein-detergent complexes. Non-ionic detergents are used for this purpose with increasing frequency because they are less likely than ionic detergents to cause alterations in the state of aggregation or conformational properties of the proteins. Triton X-100 has also been used as a successful means of extracting plant cell wall material (Cathala et al., 1978).

The results reported in this study demonstrate that the detergent mediated extraction of extracellular components using Tween follows biphasic kinetics, with a period of rapid extraction of extracellular components followed by extraction at a reduced rate (figures 2, 5, and 6). This may be explained by differential adherence of soluble matrix components to insoluble, fibrillar portions of the matrix network (Crayton, 1982; Kirk et al., 1986), so that the more loosely bound material is extracted more rapidly. For both HK10 and NB7, the extraction of matrix

components was found to be proportional to the concentration of detergent used over the range, 0.8%-2.0% (figures 7 and 8).

B. The effects of EDTA and pH on matrix extraction.

The presence of a chelating agent and the pH of the extracting environment were found to be important factors in detergent mediated matrix extraction. These results are relevant in light of recent findings on the study of proteoglycans (Toole, 1981; Dorfman, 1983; Hook, 1984). The chelating agent EDTA was found to increase the extraction of extracellular components under all conditions investigated (figures 9 and 10). Hook (1984) cites the removal by EDTA of extracellular material secreted by fibroblasts. The importance of divalent cations for spheroid integrity and the normal functioning of Volvox and other organisms is well documented (Hearing et al., 1976; Cheung, 1982; Jaenicke, 1982; Gilles et al., 1985, see Introduction). The effect of EDTA in binding the divalent cations necessary for the normal interaction and adherence of the matrix macromolecules appears to facilitate the extraction of these matrix components.

Increased extraction of extracellular material occurs at basic pH (figure 10). This finding may be correlated with

the negatively charged nature of the polysaccharides associated with extracellular matrix proteoglycans, the GAG (glycosaminoglycans) in general, and the acid mucopolysaccharides present in the matrix of Volvox in particular (Crayton, 1980). A basic environment would neutralize the negatively charged side chains involved in the adherence of the macromolecules in situ, thus facilitating the extraction of these matrix components.

C. Exclusion gel chromatography of Tween extracted matrix components.

It may be seen that extracellular proteins were extracted from both strains HK10 and NB7 by Tween treatment. Chromatography of both radioactive and non-radioactive matrix, extracted from both strains of Volvox, on a column of Sephadex G-200 revealed a major proteinaceous component of the matrix as well as some minor components.

Tween adheres to the extracted matrix glycoproteins (Helenius and Simons, 1975 and personal observation). Tween extracted matrix material was therefore chromatographed on columns of Sephadex G-100 and G-200 in order to remove the detergent from the extracellular components (Holloway, 1973). There was apparently some binding existing between the detergent and the

proteinaceous extract which resulted in the co-elution of the detergent (as evidenced by bubbles appearing in the eluant) and the matrix components (monitored by the absorbancy of the eluted fractions at 280 nm) (fig.11). The usefulness of non-ionic detergents for the extraction of proteins has been limited by difficulties experienced in the removal of detergents from extracted proteins. The detergent behaves as a large molecular weight aggregate and, although useful for the extraction of extracellular macromolecules, would not be practical for the purification of high molecular weight matrix glycoproteins.

D. SDS-polyacrylamide gel electrophoresis of detergent extracted matrix.

Low concentrations of non-ionic detergents were used in this study (see figure 13) to successfully extract matrix components. Comparing matrix components extracted by the two non-ionic detergents, Tween and Triton X-100 using SDS acrylamide gel electrophoresis, reveals (figure 37) that both non-ionic detergents extract certain matrix components in common, Triton X-100 extracts much more material, and extracts components of the matrix not extracted by Tween treatment. This is consistent with the general reaction of the organism to Tx-100 treatment.

Volvox is lethally affected by prolonged incubation with the detergent (>3 hours), and by short incubation periods at concentrations of 0.01% or greater (personal observation). At these concentrations of Tx-100, chlorophyll is extracted almost immediately.

Triton X-100 is routinely used for the extraction of membrane proteins (Robinson and Capaldi, 1977; Willadsen and Sumper, 1982). It appears that after prolonged exposure to the detergent, or even after shorter periods of exposure at increased concentrations, cellular membranes are penetrated with the accompanying bleaching and lethality of the cell. The ionic detergent SDS has a similar but immediate effect on cell viability (personal observation).

III. Mechanical extraction of matrix components.

The use of the Dounce tissue grinder was routinely employed for extraction of extracellular matrix components to avoid the use of chemicals that adversely affect or alter the normal development of the spheroids. Freeze thawing was found to facilitate the mechanical disruption of Volvox spheroids without altering the composition of the extracted extracellular matrix. Therefore, spheroids were always frozen after harvesting

and thawed immediately prior to homogenization.

A. Characterization of physically extracted matrix components.

The protein and carbohydrate composition of the mechanically extracted matrix components (table 5), corresponded to accounts of the molecular composition of the matrix as determined by other investigators (Kochert, 1975; Crayton, 1980). The existence of multiple areas of matrix in the intercellular regions of the mature Volvox spheroid has been reported (Kirk et al., 1986). The extracellular matrix, consisting of fibrillar and fluid regions is undoubtedly composed of more than one species of macromolecule. The mechanically extracted matrix was analyzed using various fractionation techniques to see whether the physically extracted material was homogeneous or multicomponent.

B. Fractionation of mechanically extracted Volvox matrix components.

1) Exclusion gel chromatography:

The results of exclusion gel chromatography indicate (figure 14) the mechanically extracted matrix is multicomponent. The elution profile obtained after

fractionation on exclusion gels yields both a polysaccharide (without any detectable protein) and a glycoprotein (or proteoglycan) component. These would seem to correspond to histochemical analysis of the Volvox matrix (personal observation from in situ PAS staining; Burr and McCracken, 1973; Darden, 1973).

The glycoprotein component of the matrix isolated by exclusion gel chromatography was not fractionated any further when subjected to chromatography on a column of Sephadex G-200 (figures 16 and 17). This indicated the isolation of closely linked polysaccharide and protein components.

2) Ion exchange chromatography:

The matrix has been described as being composed of highly acidic mucopolysaccharides (Lamport and Catt, 1984). The results of ion exchange chromatography on an anion exchange column (figure 15) indicate adsorption of a carbohydrate containing component at 0.07 M NaCl, as would be expected for a highly acidic macromolecule.

3) Affinity column chromatography:

Lectins have been widely employed as probes in the study of extracellular matrix components (Sharon and Lis, 1972). These agglutinins have been used in situ in the study of Volvox surface macromolecules. Concanavalin A

was specifically shown to alter normal patterns of Volvox development when bound to the spheroid surface (Kochert and Jaworski, 1972; Kurn and Sela, 1979).

Extracted matrix components were found not to bind to any appreciable extent to affinity columns of concanavalin A-agarose (figure 18). Most of the mechanically extracted matrix applied to con A affinity columns is non adsorbed. However, a minor proteinaceous component is adsorbed and can be eluted by borate buffer. These results indicate that the extracellular matrix components extracted by the method used in this study do not include all the matrix components seen ultrastructurally and cytochemically, or the components of the matrix as probed in situ using the lectin concanavalin A.

IV. Comparative studies of the extracellular matrix.

A. Changes occurring in the extracellular matrix of Volvox carteri f. nagariensis strain HK10 during normal development.

1) Matrix composition:

In this study correlations were found to exist between biochemical changes occurring in the matrix and

morphological growth of Volvox during the normal life cycle (figure 1). Increased deposition of proteinaceous material occurs in the matrix as the spheroid expands (table 4). There is a reduced amount of carbohydrate in matrix extracted from nascent spheroids compared to heterogeneous spheroids (table 5), (~1:1 vs. ~2:1, carbohydrate to protein). An increase in matrix carbohydrate is observed as the spheroid matures and expands. Carbohydrate containing materials are the major components of the maternal matrix extracted from fully expanded spheroids with a ratio of carbohydrate to protein of ~3:1.

It is interesting to note a physical difference in the matrix at this stage. It is noticeably more viscous and slimy and therefore more difficult to extract (personal observation). This may be related to the presence of developing Volvox "embryos" within the maternal spheroid at this stage of the life cycle, when the matrix functions as a buffer to protect the juveniles during maturation. A matrix, or mucilage, of extremely high viscosity is characteristic of other members of the Volvocales. Tautvydas (1978), in studying the colony wall and mucilage of Eudorina, found that the chief cause of the viscosity of the mucilage was the presence of a mannose-rich polysaccharide. This concurs with the increase in both carbohydrate content and viscosity of

the matrix during spheroid expansion. The solubilizing effect of the proteolytic lysin, the "release enzyme" identified and characterized by Jaenicke and Waffenschmidt (1979), does not appear to affect the viscosity of the matrix at this time. This may be explained by the viscosity resulting from increased synthesis and deposition of polysaccharide components of the matrix at this stage of development, rather than proteinaceous components.

2) Fractionation of matrix components:

As described in Discussion, section III, matrix mechanically extracted from heterogeneous cultures of Volvox is composed of two prominent fractions, a polysaccharide and a glycoprotein (figure 14). Fractionation of crude matrix extracted from nascent spheroids on columns of Sepharose 4B revealed the large polysaccharide fraction to be minor relative to the glycoprotein fraction (figure 20). This may be related to the lesser amount of assayable matrix carbohydrate at this stage of development (1:1 carbohydrate/ protein as compared to 2:1 carbohydrate/ protein in matrix extracted from heterogeneous spheroids). In contrast, polysaccharide is the more prominent component in matrix extracted from fully expanded, pre-release spheroids (figure 21). At this stage of development there is a

corresponding increase in the carbohydrate content of extracted matrix, with a resulting 3:1 ratio of carbohydrate/ protein.

There appears to be an increase in the relative quantity of protein comprising the glycoprotein containing fractions of Volvox matrix extracted during the release process (figure 23). This is very likely directly related to the enzymes regulating the release process itself. Jaenicke and Waffenschmidt have characterized the release enzyme of Volvox as a glycoprotein (Jaenicke and Waffenschmidt, 1979, 1981; Jaenicke, 1982). Glycoproteins have been implicated in autolysis (both in vegetative and sexual stages) of other Volvocales as well (Schlosser et al., 1976; Schlosser, 1981).

3) Fractionation of radioactive matrix components:

Fractionation of matrix extracted from heterogeneous spheroids made radioactive with $\text{NaH}^{14}\text{CO}_3$ shows the incorporation of radioactivity into two major components (figure 24). Pulse labeling spheroids from synchronous cultures for specific developmental time periods shows that the glycoprotein component of the extracted matrix was synthesized during both expansion stages of the Volvox life cycle studied (figures 25 and 26). The large polysaccharide component was synthesized primarily during

the latter stage of expansion (figure 26), the morphologically prominent pre-release expansion.

Vegetative growth in the related Volvocale Eudorina occurs with the colony enlarging from 50 um in diameter after cleavage to 400 um in diameter at the next cleavage period. This growth is marked by enlargement of polysaccharide rich extracellular matrix accompanying growth of asexual reproductive cells (Tautvydas, 1978). It appears likely that the polysaccharide components of the Volvox extracellular matrix may in fact be responsible for the tremendous increase in spheroid size (with an increase in spheroid diameter of as much as from ~100um-~400 um) and the moving apart of somatic cells seen during spheroid growth and expansion. (figure 1).

4) Electrophoretic analysis of fractionated matrix glycoproteins:

Matrix glycoproteins extracted from heterogeneous spheroids of strain HK10, isolated from matrix polysaccharides by exclusion column chromatography, were compared to matrix glycoproteins of spheroids at specific developmental stages of the life cycle using the technique of SDS-Polyacrylamide gel electrophoresis. The results indicate that specific polypeptide bands are common to extracellular matrix glycoproteins physically extracted from HK10 spheroids at all stages, whereas

other polypeptide bands are present only at certain stages of development. (see figures 41-43).

The shift in electrophoretic mobility observed is possibly the result of chemical modification of matrix components during development. Sulphation of specific matrix glycoproteins has been demonstrated during (and implicated in the control of) two distinct developmental processes, inversion and sexual induction (Sumper and Wenzl, 1980; Wenzl and Sumper, 1981; Willadsen and Sumper, 1982; see Introduction). Protein glycosylation has been shown to alter the electrophoretic mobility of proteins on SDS-polyacrylamide gels. A well developed glycosyl transferase system has been demonstrated in Volvox as well as other algae (Chrispeels, 1976), and localized in the Golgi apparatus (of the somatic cells of Volvox) (Dauwalder et al., 1980). It is likely that shifts in electrophoretic mobility are the results of stage specific glycosylation or other modifications of Volvox matrix proteins.

It would be of interest to determine whether the different polypeptide bands observed in newly released spheroids, in condensed spheroids, and in spheroids just prior to the release process, are the result of de novo synthesis or a shift in electrophoretic mobility due to protein modification. It should be possible to expose the spheroids to radioactive proline for short periods of

time and follow any observable shift in electrophoretic mobility. Alternately, the de novo appearance of "unlabeled" polypeptide bands as a result of a "cold chase" would be noted.

B. Volvox carteri f. weismania strain NB7.

Spheroids of strain NB7 are morphologically similar to and are generally considered to be closely related to spheroids of strain HK10. Certain morphological, ultrastructural, and biophysical distinctions exist, however, and the two strains are reproductively isolated (Starr, 1969). Normal spheroids of strain NB7 contain 8-10 gonidia as compared to 16 in "healthy" spheroids of strain HK10 (Starr, 1969 and personal observation). The portions of the matrix that serve as regions of flagellar insertion are ultrastructurally different in the two strains (Kirk et al., 1986). Spheroids of strain NB7 are more susceptible to dissolution by protease treatment than spheroids of strain HK10 (Kurn and Sela, 1979 and personal observation). Inducers of one strain will not act upon the other (Kochert and Yates, 1974) nor will lysins of strain HK10 effect the dissolution of the matrix of strain NB7 (Kurn and Sela, 1979). This indicates a possible biochemical difference in the

composition of the extracellular matrices of the two strains.

Mechanical extraction of the matrix of spheroids of strain NB7 yields a viscous solution/ suspension (mucilage) of $\sim 2/3$ carbohydrate and $\sim 1/3$ protein (table 5). Fractionation of the matrix by column chromatography resolved two major non-linked components. The first, a high molecular weight polysaccharide, eluted just after the void volume and the second component, of lower molecular weight, was a glycoprotein (figures 27 and 28). A marked difference is seen, however, in the relative proportion of carbohydrate and protein in the glycoprotein peak when comparing the elution profiles of matrix extracted from the two strains. In HK10 the glycoprotein peak is a prominent molecular species, whereas in NB7 it is minor compared to the polysaccharide fraction. This may be related to the increased sensitivity of NB7 to spheroid dissolution by protease treatment.

Comparison of the of the extracellular matrix glycoproteins of the two strains of Volvox using slab gel electrophoresis reveals differences in the protein subunit composition (figures 40-43). The highest molecular weight polypeptide band was absent from glycoproteins isolated from the matrix of NB7, whereas a prominent polypeptide appears in all stages of NB7 that

is never found in any stage of HK10. There are many molecular weight polypeptides common to both strains.

The close biochemical and morphological resemblance of these two strains of Volvox has been noted as well by other investigators. The increased spheroid susceptibility to pronase treatment of NB7 spheroids may be related to the absence of a large molecular weight polypeptide band present in matrix extracted from HK10 spheroids. The presence of an intermediate molecular weight polypeptide band not found in the matrix of HK10 spheroids, is characteristic of NB7 spheroids. Enhanced glycosylation of matrix proteins, which would result in the presence of glycopeptides of increased molecular weight, has been known to increase steric hinderance to proteases. This effect has been well documented for enzymes such as RNase (Lamport, 1980) and may be related to the increased resistance of HK10 spheroids to dissolution by proteases.

Despite the morphological similarity of NB7 and HK10, recent ultrastructural studies (Kirk et al., 1986) have shown that specific differences exist between these two strains. Likewise, despite the strong biochemical similarities between the extracellular matrices of the two strains observed in this study, distinct biochemical differences exist. The sexual inducers of the two strains do not cross react, nor does the autolysin of one strain

affect the other, indicating a probable difference in matrix (i.e. autolysin substrate) composition. The presence of major polypeptide bands specific for each strain, NB7 and HK10, appears to be yet another manifestation of the biochemical uniqueness of these two strains of Volvox.

C. DIS 111.

This matrix mutant of the normal HK10 strain dissociates immediately after inversion (Sessoms and Huskey, 1973). Biochemical analysis of the growth medium revealed the presence of protein and carbohydrate, indicating the release of matrix like components. Exclusion gel chromatography (Sephacrose 4B) reveals that the polysaccharide component is greatly diminished in this mutant (figure 30). If the polysaccharide components of the matrix are responsible for maintaining spheroid integrity by nature of the glycosaminoglycan side chains, then the absence of polysaccharide in the mutant may be correlated with the absence of matrix and lack of spheroid integrity.

Electron micrographs of the normal HK10 spheroid (figures 31 and 32) clearly show matrix material surrounding the individual gonidia and somatic cells. The sheath material surrounds the somatic cells and is

contiguous with the lining of the outer surface of the spheroid. Electron microscopy comparing the matrix and outer cell surfaces of the normal and mutant organisms (figures 31-34) supports the finding that some matrix like components are present on the outer cell surfaces of the mutant. However, the normal configuration of the matrix as observed in electron micrographs of normal HK10 spheroids is absent in DIS 111, and the outer surfaces of the individual cells are irregularly shaped.

D. Palmelloid Chlamydomonas.

The unicellular Volvocale Chlamydomonas is induced to elaborate extracellular matrix material in response to biochemically stressful conditions. Extraction and fractionation of this primitive matrix reveals many similarities to the matrix of the more highly evolved member of the order, Volvox. The elution profile of palmelloid extract chromatographed on a column of Sepharose 4B, consists of a predominantly polysaccharide containing component and a glycoprotein (figure 35). This is not surprising since ultrastructural and histochemical studies of the matrix of more primitive Volvocales (Lang, 1965; Fulton, 1978; Crayton, 1980, 1982; Kirk et al., 1986) indicate distinct regions of carbohydrate and

proteinaceous material within the colony walls and matrices of these related organisms.

Electrophoretic analysis of the glycoprotein component of the fractionated palmelloid "matrix" indicates some similarities to the polypeptide composition of the matrix glycoproteins of Volvox. What is surprising is the apparant complexity of the banding pattern of palmelloid Chlamydomonas matrix glycoproteins, with many more bands resolved than the matrix glycoproteins of either strain of Volvox studied (figures 40 and 41). This could be a result of the presence of Chlamydomonas cell wall proteins in the palmelloid extract.

Electrophoretic analysis of cell wall glycoproteins extracted from Chlamydomonas reveals much complexity (Lamport and Miller, 1971; Miller and Lamport, 1972; Roberts, 1974; Voigt, 1985). Davies and Lyall (1973) compared the glycoprotein components of the cell walls of normal Chlamydomonas to those extracted from cell wall mutants. Voigt (1985) recently analyzed Chlamydomonas cell wall macromolecules released into the culture medium. Both these studies indicate that the glycoprotein cell wall components of Chlamydomonas exhibit a high degree of complexity.

What is intriguing is the possibility that the palmelloid "matrix" is an elaboration of specific Chlamydomonas cell wall macromolecules, similar to a

description of the extracellular matrix of Volvox as an elaboration and extension of "cell wall" material surrounding the individual cells of a Volvox spheroid. These are not unreasonable possibilities in light of recent findings. Fulton (1978) showed chemical and ultrastructural data which suggest that the matrix of Pandorina is similar to the Chlamydomonas cell wall. Crayton (1982) demonstrated the presence of similar extracellular acidic polysaccharides in the cell walls of Chlamydomonas and the matrix of Eudorina. Goodenough and Heuser (1985), in collaboration with Kirk, used freeze etching to examine the matrix surrounding the cells of V. carteri. They found a close resemblance between this region of the Volvox matrix and the expandable region of the Chlamydomonas cell wall.

For further study:

Comparative investigations of Volvocalean extracellular matrices:

The intriguing similarities exhibited by extracellular matrix components of the two strains of Volvox studied, and the common features shared with palmelloid Chlamydomonas, should be studied in greater detail using

the techniques developed during this investigation. The biochemical characterization of the extracellular matrix of Volvox should be extended and elaborated by a comparison with the matrices of; 1) more primitive, purely colonial members of the Volvocales where no functional differentiation exists (such as in Gonium and Pandorina), 2) the more highly evolved forms (such as Platydorina and Eudorina), where functional, although no morphological, differentiation occurs.

It would further be of interest to see whether an even closer resemblance exists between the palmelloid matrix of Chlamydomonas and the extracellular components of the more primitive Volvocales. Lamport (1980) cites many stabilizing and protective effects of protein glycosylation including increased resistance to protease degradation and increased thermal stability. The glycosylated matrix proteins may therefore have enabled thermophilic adaptation to evolve. Pickett-Heaps (1975) cites the naturally occurring phenomenon of palmella formation in the Chlamydomonales and Tetrasporales as a possible mechanism to protect the organism from dessication, as the individual cells rapidly revert to the flagellated unicellular form following suitable stimulation such as flooding the colonies with water.

Unicellular organisms may therefore adapt a colonial mode of existence, thus becoming temporarily

"multicellular" for the purpose of survival, possibly protected by the buffering capacity of the intercellular matrix. An in depth comparative investigation of the extracellular matrices of the Volvocales, inclusive of biochemistry and ultrastructure, would aid in determining the survival and evolutionary advantage provided by the colonial matrix. Such a study would provide greater understanding of the phylogenetic increase in complexity associated with the Volvocine line of development.

Correlation of biochemistry and morphology:

With the elucidation of the ultrastructural complexity and compartmentalization of the Volvox extracellular matrix it would be of interest to attempt to localize the proteoglycan components of the matrix by means of radioisotope or flourescene tagged antibodies and electron microscopy. In view of the method of isolation employed, it appears likely that the mechanically extracted macromolecules characterized in this study were extracted from the more fluid deep zone of the matrix. No doubt other surface glycoproteins described by various investigators are isolated from other intercellular locations.

The role of matrix components in the control of Volvox

development:

The change in patterns of synthesis of extracellular matrix components during the Volvox life cycle indicated in this study suggests a role of these matrix components in regulating Volvox development. A recent investigation by Kopan, Kurn, and Ovadia (1985) reports that the incubation of spheroids with various matrix proteoglycans influences spheroid expansion, causing it to cease, and enhances mobility. Embryonic proteoglycans were found to cause aggregation whereas proteoglycans extracted from mature spheroids did not exhibit this effect. Possibly the presence of these high molecular weight "mature" macromolecules may serve as a trigger to the spheroid to signal the termination of embryogenesis. It would be of interest and relevant to the study of Volvox morphogenesis to clarify the role(s) of the extracted matrix components during development.

Inhibitor studies:

Inhibitors of protein glycosylation have been known to affect the synthesis of glycoproteins in numerous systems (Olden et al., 1978; Schneider et al., 1978) and have been implicated as such in Volvox as well (Duksin and Bornstein, 1977; Kurn and Duksin, 1982). It would be

significant to determine what effect inhibitors of protein glycosylation have on spheroid integrity. Indeed, in view of the fact that dissociator mutants of normal Volvox strains have altered matrix synthesis or deposition, it would be interesting to learn what role protein glycosylation plays in regulating Volvox spheroid integrity, and whether the inhibition of such is a factor in causing the aberrant development of matrix mutants such as DIS 111.

APPENDIX

I. Preparation of $\text{NaH}^{14}\text{CO}_3$

(Baye, 1977).

A specially designed Warburg trapping apparatus was used to convert barium carbinate ($\text{Ba}^{14}\text{CO}_3$) to sodium bicarbonate ($\text{NaH}^{14}\text{CO}_3$). A known amount of $\text{Ba}^{14}\text{CO}_3$ was placed onto the base of a 25 ml Warburg flask. One ml of 10 N NaOH was carefully pipetted into a center cell situated on the base of the flask. A 1 ml aliquot of lactic acid was introduced into the sealed sidearm via a disposable capillary pipette that had been bent by heat to an angle of 45° . A white silicone seal was applied to the glass surface surrounding the flask cap interface to insure full collection of the released $^{14}\text{CO}_2$ gas.

After a period of three days had elapsed, the contents of the center well, now containing trapped $^{14}\text{CO}_2$ in the form of $\text{NaH}^{14}\text{CO}_3$, were removed with a capillary pipette and diluted with distilled water to give a final concentration of 3 N NaOH. This served as the concentrated stock for $\text{NaH}^{14}\text{CO}_3$. Small aliquots of stock $\text{NaH}^{14}\text{CO}_3$ were diluted with SVM and adjusted to pH 8.0 for experimental purposes.

II. Composition of Volvox medium

(Provasoli and Pinter, 1959) modified by Darley
(unpublished).

For each liter of medium the following amounts of stock solutions were added to 980.8 ml of deionized water:

# of ml	Stock solution	per 100 ml
1	$\text{Ca}(\text{NO}_3)_2 \cdot 4 \text{H}_2\text{O}$	11.8 g
1	$\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$	4.0 g
1	$\text{Na}_2\text{glycerolphosphate} \cdot 5 \text{H}_2\text{O}$	5.0 g
1	KCl.....	5.0 g
*10	Glycylglycine.....	5.0 g
1	Biotin.....	10.0ug
1	B_{12}	10.0ug
3	P_{IV} trace metal solution.....	see below
0.2	Na_2CO_3	10.0g
Adjust pH to 8.0 with 1 N NaOH.		

P_{IV} Trace Metal Solution

The following amounts of salts and chelating agent were added to 500 ml of deionized water:

$\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$	0.097 g
$\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$	0.041 g
ZnCl_2	0.005 g
$\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$	0.002 g

Na_2MoO_4 0.004 g

Na_2EDTA 0.750 g

* 0.004 M Hepes buffer (0.953 g / l) was substituted for glycylglycine to lessen incidence of bacterial contamination (R. Starr, personal communication).

III. Protein assays:

A. Estimation of protein by the method of Lowry et al. (1951)

Protein content was assayed with the Folin - Ciocalteu phenol reagent using BSA as a standard. Absorbancy of samples was measured at 750 nm.

B. Biuret reagent

Assay: 1 ml protein solution was incubated with 4 ml biuret reagent (1.5 g $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ and 6.0 g $\text{NaKC}_4\text{H}_4\text{O}_6 \cdot 4 \text{H}_2\text{O}$ were dissolved in 500 ml H_2O , and 300 ml 2.5 N NaOH were added. Water was added to bring the total volume to 1 liter.) for 15-30 minutes. BSA was used as the protein standard. Absorbancy of samples was measured at 550 nm. (Assay accurate at 1-10 mg protein).

C. Microbiuret reagent (Zamenhoff, see Layne, 1957)

Eighty ml of a 1% CuSO_4 solution was added dropwise with stirring to 150 ml 40% NaOH. The reagent was filtered through a medium porosity glass filter. Absorbancy of samples was measured at 290 nm using BSA as a standard. 1 O.D.unit (A_{290}) = 362 mg protein. (Assay sensitivity: 50-500 ug protein).

D. Biuret protein assay for intact Volvox cells and broken cell fractions (modification of Tustanoff and Bartley, 1964, courtesy of Dr. S. Cottrell).

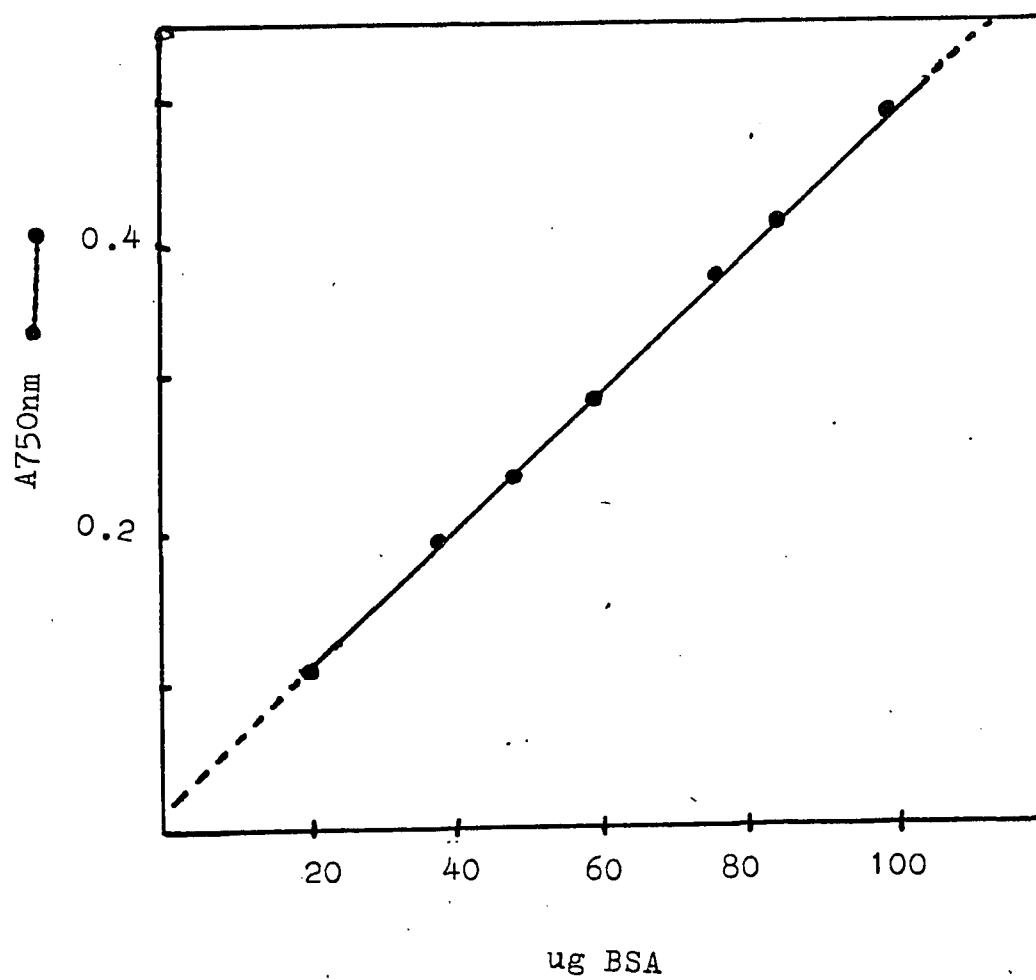
Intact cells:

Volvox cells were centrifuged, the pellet washed in distilled water, resuspended in 3 ml 10% NaOH, and incubated at room temperature for at least 12 hours. A 0.4 ml aliquot of 1% CuSO_4 was added, the suspension mixed, and allowed to stand for 10 minutes. The suspension was centrifuged in a clinical centrifuge at top speed for 10 minutes. The supernat was decanted and absorbancy measured at 560 nm using BSA as a standard (2.5 mg BSA/ml of 10% NaOH).

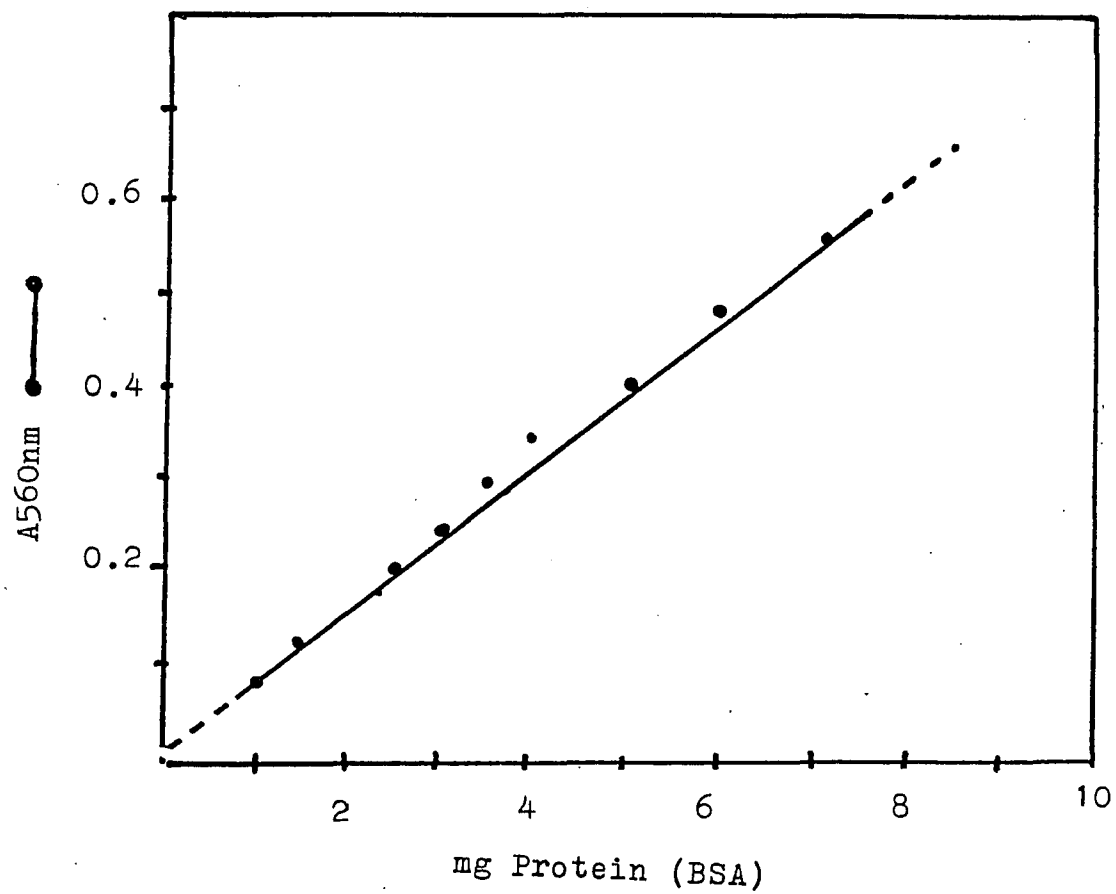
Broken cell fractions:

One ml of 10% NaOH was added to 2 ml of sample, mixed, and allowed to stand for at least 10 minutes. A 0.4 ml aliquot of 1% CuSO_4 was added to the suspension, the mixture allowed to stand for at least 10 minutes, and centrifuged at 12,000 x g for 10 minutes. The supernat was decanted and the absorbancy measured at 560 nm using BSA as the standard.

STANDARD CURVE -- LOWRY ASSAY



STANDARD CURVE -- BIURET REAGENT



sensitivity = 1-10 mg protein

IV. Carbohydrate assays:

A. A colorimetric method for determination of sugars was utilized as described by Dubois et al. (1956) One ml 5% phenol was added to 1 ml of sugar solution. Five ml concentrated reagent grade sulfuric acid was added rapidly and the contents of the tube mixed vigorously using a vortex mixer. The tubes were allowed to stand for 10 minutes, shaken, and placed for 10-20 minutes in a water bath at 25°C. The color is stable for many hours. The absorbance of the characteristic yellow - orange color is measured at 490 nm for hexoses (mannose was used as a standard) and 480 nm for pentoses (arabinose was used as a standard).

B. PAS Procedure: Slides were prepared with intact spheroids, broken cell fractions, or matrix smears. Slides were placed in periodic acid solution for 5 minutes at room temperature, and transferred to basic fuchsin Schiff reagent for 30 minutes at room temperature. Slides were placed in three changes of bleach and washed in tap water.

Periodic acid solution:

0.4 g periodic acid in 45 ml water. Add 5 ml 0.2 M sodium acetate.

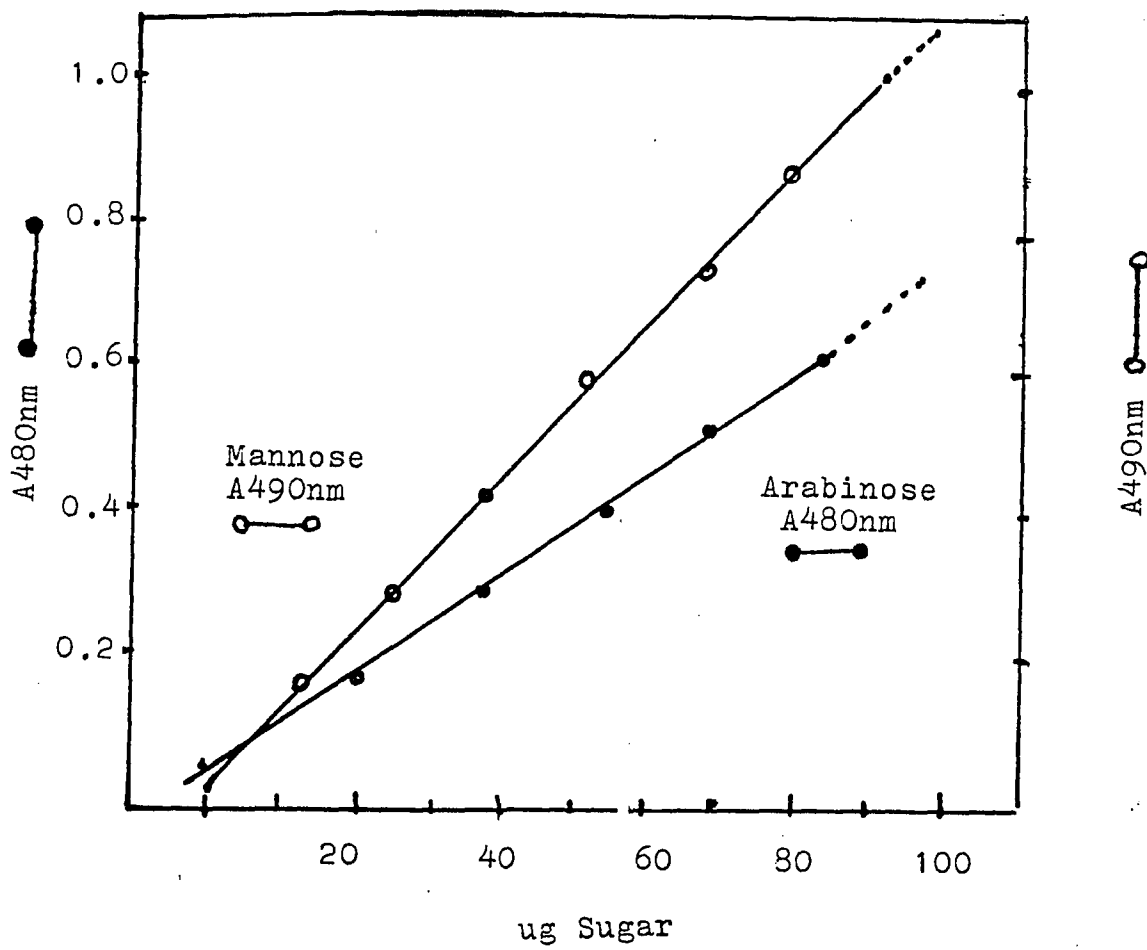
Basic Fuchsin Schiff reagent:

0.5 g basic fuchsin in 100 ml very hot water. Cool, add 10 ml 1 N HCl and 1 g anhydrous sodium or potassium metabisulfite. Let stand in glass stoppered bottle for 24 hours then add 0.25 g activated charcoal, shake and filter rapidly. Store in glass stoppered bottle.

Bleach:

Add 5 ml 1 N HCl, 5 ml 10% $\text{Na}_2\text{S}_2\text{O}_7$ and 90 ml water.

STANDARD CURVE — DUBOIS ASSAY



V. SDS polyacrylamide gel electrophoresis buffer system

Stock solutions:

a) Acrylamide : BIS, 30:0.8

300 g acrylamide

8 g N,N'-methylenebisacrylamide (BIS)

Make up to 1 liter with distilled water, filter, store at 4°C in a dark glass bottle.

b) 1.5 M tris-Cl, pH 8.8

18.15 g tris

50 ml distilled water

Adjust to pH 8.8 with HCl

Make up to 100 ml with distilled water.

c) 0.5 M tris-Cl, pH 6.8

3 g tris

20 ml distilled water

Adjust to pH 6.8 with HCL

Make up to 50 ml with distilled water.

Separating gel:

Acrylamide : BIS , 30:0.8.....5% gel.....10 ml

7.5% gel.....15 ml

10% gel.....20 ml

15% gel.....30 ml

1.5 M tris-Cl, pH 8.8 6 ml

10 % SDS.....0.6 ml

0.2 M EDTA 0.6 ml

TEMED 0.03 ml

Distilled water was added to give a total volume of 54.4 ml

Solutions were evacuated and 0.6 ml of 10% ammonium persulfate added before pouring gels.

Spacer gel:

A 5% spacer gel was used on all gels with the exception of gradient gels.

Acrylamide:BIS, 30:0.8.....5 ml

0.5 M tris-Cl, pH6.8.....3.75 ml

10% SDS.....0.3 ml

0.2 M EDTA.....0.3 ml

TEMED.....0.015 ml

Distilled water was added to give a total volume of 27.7 ml.

The solution was evacuated and 0.3 ml of 10% ammonium persulfate added before pouring gel.

Electrode Buffer:

6.0 g trizma base

28.8 g glycine

10 ml 10% SDS

10 ml 0.2 M EDTA

The buffer was made up to one liter with water.

Sample preparation:

Samples were suspended in a solution of the following composition, heated for 2 minutes in a boiling water bath, and allowed to remain an additional 2 minutes in the water bath with the flame turned off.

0.5 M tris-Cl, pH 6.8	1.0 ml
10% SDS.....	1.0 ml
0.2 M EDTA.....	0.1 ml
Mercaptoethanol.....	0.1 ml
Glycerol.....	1.0 ml
Distilled water.....	6.8 ml

Sample solvent was freshly prepared before each use.

VI. Isolation of Volvox cells with Ludox gradients.

(D.L. and M.M. Kirk, personal communication)

A. Purification of Ludox for cell separation gradients.

1. Adjust pH to 7.0 by slow addition of Biorad AG 50w-XB, 100-200 mesh resin, H^+ form.
2. Remove resin by filtration through Miracloth.
3. Add 25 g/l of activated charcoal from which fines have been removed by repeated suspension, settling, and decantation.
4. Stir overnight.
5. Filter through a 1 to 1.5 inch bed of diatomaceous earth (Sigma Grade 1). If necessary, filter through Whatman #5 paper to remove last traces of charcoal.
6. Dialyze exhaustively against SVM.
7. Measure specific gravity, interpolate silica content from Dupont graph of specific gravity vs. solids content.

B. Cell separation.

1. Disrupt spheroids in SVM with Dounce homogenizer using the "A" ball.
2. Add Ludox to give a 1.5% silica content.

3. Centrifuge 2-5 minutes at 1,000 x g ; matrix floats, free gonidia and somatics form pellet.

4. Re"dounce" supernat and recentrifuge.

5. Treat supernat from step 4 for 2 minutes in cold Waring blender. Recentrifuge.

6. Repeat step 5 until matrix is essentially free of somatics.

7. Pool gonidial-somatic cell pellets and wash once with 1.5% Ludox in SVM, centrifuge.

8. Prepare gradients by centrifuging 27 ml of 6.5% Ludox in SVM 35 minutes at 25,000 rpm in a Spinco #25.1 rotor.

9. Remove top 2 ml of each gradient. Add cells that have been resuspended in SVM with Dounce homogenizer; 5 ml per gradient.

10. Spin 15-30 minutes at 7,500 x g.

11. Polyethylene glycol (Carbowax 6000, Union Carbide) that has been dialyzed against SVM may be added to all Ludox containing solutions at a level of 1% to improve viability, but centrifugation times must be increased to compensate for increased viscosity.

VII. Extraction buffer for Volvox cellular protein.

Isolated Volvox cells (from protease treatment, mechanical disruption or Ludox gradients) were resuspended in extraction buffer and lysed in the omnimixer or by sonication, as described in section III.

Extraction buffer:

0.05 M tris-Cl pH 7.2, 0.05 M sucrose, 0.01 M MgCl_2 ,
0.005 M 2-mercaptoethanol.

VIII. Sepharose 4B exclusion gel chromatography buffers.

Tris buffer was originally used to fractionate Volvox extracellular matrix on chromatography columns of Separose 4B.

0.02 M tris-Cl, 0.01 M NaCl, pH 8.0 (0.02% sodium azide).

It was determined that tris buffers interfere with the Lowry protein assay, producing a blue background color, which was routinely used to assay the protein content of each fraction. 0.1 M sodium phosphate buffer, pH 7.8, was therefore substituted routinely with no change in the elution profiles.

IX. Composition of Chlamydomonas reinhardi normal medium

(Sueoka, 1960).

Per liter of distilled water:

0.5 g NH_4Cl

0.02 g $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$

0.01 g $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$

1.44 g K_2HPO_4

0.72 g KH_2PO_4

1 ml ferric chloride solution:

2.4 g $\text{FeCl}_3 \cdot 6 \text{H}_2\text{O}$ in EDTA (18.6 g/l).

1 ml microelement solution:

per liter of distilled H_2O ;

40 mg ZnCl_2

600 mg H_3BO_3

15 mg $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$

40 mg $\text{CuCl}_2 \cdot 2 \text{H}_2\text{O}$

400 mg $\text{MnCl}_2 \cdot 2 \text{H}_2\text{O}$

27 mg

$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$

X. Filtration of Volvox spheroids and cells.

I. Harvesting synchronous cultures:

<u>Nitex Pore Size</u>	<u>Retained</u>	<u>Filtered</u>
300 um	pre-release spheroids	all others
125 um	pre-release spheroids	newly released
80 um	newly released "	free cells

II. Isolation of cells from disrupted spheroids:

<u>Nitex Pore Size</u>	<u>Retained</u>	<u>Filtered</u>
100 um	large masses of sheaths	gonidia
	intact spheroids	somatic cells
80 um	sheaths	gonidia
	embryos	somatic cells
40 um	gonidia	small gonidia
		somatic cells
15-20 um	small gonidia	somatic cells
5-7 um	somatic cells	matrix

LITERATURE CITED

- Ames, G. F. (1974).
Resolution of bacterial proteins by
polyacrylamide gel electrophoresis on slabs.
J. Biol. Chem. 249: 634-644.
- Avigad, G. (1978).
Lectin-bound alcohol and lactic dehydrogenase as
a reagent for the visual detection of
glycoproteins.
Anal. Biochem. 86: 443-449.
- Bause, E. and Jaenicke, L. (1979).
Formation of lipid-linked sugar compounds in
Volvox carteri f. nagariensis Iyengar.
FEBS letters 106: 321-324.
- Baye, L.A. (1977).
Regulation of DNA biosynthesis during cyanophage
infection.
M.S. Thesis, Brooklyn College, CUNY.
- Bell, G. (1985).
The origin and early evolution of germ cells as
illustrated by the Volvocales. In: Origin and
Evolution of Sex (eds. H.O Halvorson and A. Monroy)
New York: Alan R. Liss. pp.221-256.
- Bisalputra, T. and Stein, J.R. (1966).
The development of cytoplasmic bridges in
Volvox aureus.
Canad. J. Bot. 44: 1697-1702.
- Bonner, W.M and Laskey, R.A. (1974).
A film detection Method for tritium-labelled
proteins and nucleic acids in polyacrylamide gels
Eur. J. Biochem. 46: 83-88.
- Bradley, D.M., Claybrook, J.R., and Goldin, H.H.
(1974)
Analysis of histones in V. carteri.
Genetics 77 suppl. 6.
- Bradley, D.M., Goldin, H.H., and Claybrook, J.R.
(1974) Histone analysis in Volvox.
FEBS letters 41 (2): 219-222.

- Brysk, M.M. and Chrispeels, M.J. (1972).
Isolation and partial characterization of a
hydroxyproline-rich cell wall glycoprotein and
its cytoplasmic precursor.
Biochim. Biophys. Acta. 257: 421-432.
- Burr, F.A. and McCracken, M.D. (1973).
Existence of a surface layer on the sheath
of Volvox.
J. Phycol. 9: 345-346.
- Burridge, K. (1975).
Changes in cellular glycoproteins after
transformation: Identification of specific
glycoproteins and antigens in sodium dodecyl
sulfate gels. Proc. Natl. Acad. Sci. 73: 4457-4461.
- Caplan, H.W. and Blamire, J. (1980).
Polyadenylated RNA of Volvox: Isolation and
partial characterization.
Cytobios 29: 115-128.
- Cathala, N., Ghorbal, M.H., Lamant, A., and
Salsac, L. (1978).
Obtention de parois cellulosiques a l'aide d'un
detergent: etude preliminaire de leur composition
minérale. C.R. Acad.Sc. Paris. 286: 1025-1027.
- Catt, J.W., Hills, G.J., and Roberts, K. (1976).
A structural glycoprotein, containing
hydroxyproline, isolated from the cell wall of
Chlamydomonas reinhardtii.
Planta 131: 165-171.
- Cheung, W.Y. (1982).
Calmodulin.
Scientific American, June 1982, pp.62-70.
- Chrispeels, M. (1976)
Biosynthesis, intracellular transport, and
secretion of extracellular macromolecules.
Ann. Rev. Plant Physiol. 27: 19-38.
- Coggin, S.J., Hutt, W., and Kochert, G. (1979).
Sperm bundle-female somatic cell interaction in the
fertilization process of Volvox carteri
f. weismania.
J. Phycol. 15: 247-251.

- Cooper, J.B., Adair, W.S., Mecham, R.P.,
Heuser, J.E., and Goodenough, U.W. (1983).
Chlamydomonas agglutinin is a hydroxyproline-rich
glycoprotein. Proc. Natn. Acad. Sci. 80: 5898-5901.
- Crayton, M.A. (1980).
Presence of a sulfated polysaccharide in the
extracellular matrix of Platydorina caudata
(Volvocales, Chlorophyta).
J. Phycol. 16: 80-87.
- Crayton, M.A. (1982).
A comparative cytochemical study of Volvocacean
matrix polysaccharides.
J. Phycol. 18: 336- 344.
- Curling, J. (1970).
The use of Sephadex in the separation,
purification, and characterization of biological
materials.
Exp. in Physiol. and Biochem. 3: 417-484.
- Darden, W.H. (1966).
Sexual differentiation in Volvox aureus.
J. Protozoology 13: 239-255.
- Darden, W.H. (1973)
Formation and assay of a Volvox factor-histone
complex.
Microbios 8: 167-174.
- Dauwalder, M., Whaley, W.G., and Starr, R.C. (1980)
Differentiation and secretion in Volvox.
J. Ultrastruct. Res. 70: 318-335.
- Davies, D.R. and Lyall, V. (1973).
The assembly of a highly ordered component of the
cell wall: The role of heritable factors and of
physical structure.
Molec. Gen. Genet. 124: 21-34.
- Dorfman, A. (1983).
Proteoglycan biosynthesis.
In: Cell Biology of Extracellular Matrix,
ed. E.D. Hay (Plenum Press, N.Y. and London)
pp.115-138.

- Dubois, M., Gilles, K.A., Hamilton, J.K.,
Rebers, P.A., and Smith, F. (1956).
Colorimetric methods for determination of sugars
and related substances.
Anal. Chem. 28: 350-356.
- Duksin, D. and Bornstein, P. (1977).
Changes in surface properties of normal and
transformed cells caused by tunicamycin, an
inhibitor of protein glycosylation.
Proc. Natl. Acad. Sci. 74: 3433-3437.
- Edelman, G. (1976).
Surface modulation in cell recognition and cell
growth.
Science 192: 218-226.
- Ericson, M.C. and Elbein, A.D. (1980).
Biosynthesis of cell wall polysaccharides and
glycoproteins.
In: The Biochemistry of Plants, Vol. 3.
Carbohydrates: Structure and Function pp. 589-616.
- Fincher, G.B. and Stone, B.A., Clarke, A. (1983).
Arabinogalactan-proteins: Structure,
biosynthesis, and function.
Ann. Rev. Plant Physiol. 34: 47-70.
- Fry, S.C. (1986).
Cross-linking of matrix polymers in the growing
cell walls of angiosperms.
Ann. Rev. Plant Physiol. 37: 165-186.
- Fulton, A.B. (1978).
Colonial development in Pandorina morum
I. Structure and composition of the extracellular
matrix. Dev. Biol. 64: 224-235.
- Fulton, A.B. (1978).
Colonial development in Pandorina morum
II. Colony morphogenesis and formation of the
extracellular matrix. Dev. Biol. 64: 236-251.
- Gilles, R., Gilles, C., and Jaenicke, L. (1983).
Sexual differentiation of the green alga
Volvox carteri; Involvement of extracellular
phosphorylated proteins.
Naturwissenschaften 70, S. 571-572.
- Gilles, R., Gilles, C., and Jaenicke, L. (1984).
Pheromone-binding and matrix mediated events in
sexual induction of Volvox carteri.
Z. Naturforsch. 39c: 584-592.

- Gilles, R., Moka, R., Gilles, C., and Jaenicke, L. (1985).
Cyclic AMP as an intraspheroidal differentiation signal in Volvox carteri.
FEBS letters 184: 309-312.
- Goodenough, U.W. and Heuser, J.E. (1985).
The Chlamydomonas cell wall and its constituent glycoproteins analyzed by the quick-freeze, deep etch technique.
J. Cell Biol. 101: 1550-1568.
- Goodenough, U.W. (1985).
Origins and evolution of eukaryotic sex. In: Origin and Evolution of Sex (ed. H.O. Halvorson and A. Monroy) New York: Alan R. Liss.
pp.123-140.
- Goodenough, U.W., Adair, W.S., Collin-Osdoby, P., and Heuser, J.E. (1985).
Structure of the Chlamydomonas agglutinin and related flagellar surface proteins in vitro and in situ. J. Cell Biol. 101: 924-941.
- Gottlieb, B. and Goldstein, M.E. (1977).
Colony development in Eudorina elegans.
J. Phycol. 13: 358-364.
- Green, K. and Kirk, D.L. (1981).
Cleavage patterns, cell lineages, and development of a cytoplasmic bridge system in Volvox embryos.
J. Cell Biol. 91: 743-755.
- Green, K. and Kirk, D.L. (1982).
A revision of the cell lineages recently reported for Volvox carteri embryos.
J. Cell Biol. 94: 741-742
- Green, K.J., Viamontes, G.I., and Kirk, D.L. (1981)
Mechanism of formation, ultrastructure, and function of the cytoplasmic bridge system during morphogenesis in Volvox.
J. Cell Biol. 91: 756-769.
- Grobstein, C. (1974).
Developmental role of intercellular matrix: Retrospective and prospective. In: Extracellular Matrix Influences on Gene Expression (eds. H.C Slavkin and R.C. Greulich) Academic Press, pp. 9-15

- Hearing, V.J., Klinger, W.G., Ekel, and Montague, Paul, M. (1976).
Molecular weight estimation of Triton X-100 solubilized proteins by polyacrylamide gel electrophoresis. *Anal. Biochem.* 72: 113-122.
- Heath, M.F. and Northcote, D.H. (1971).
Glycoprotein of the wall of sycamore tissue culture cells.
Biochem. J. 125: 953-961.
- Hascall, C.V. and Hascall, G.K. (1981).
Proteoglycans. In: *Cell Biology of Extracellular Matrix*, ed. E.D. Hay
(Plenum Press, N.Y. and London) pp. 39-60.
- Helenius, A. and Simons, K. (1975).
Solubilization of membranes by detergents.
Biochimica et Biophysica Acta 415: 29-79.
- Holloway, P.W. (1973).
A simple procedure for removal of Triton X-100 from protein samples.
Analytical Biochemistry 53: 304-308.
- Hook, M. (1984).
Cell-surface glycosaminoglycans.
Ann. Rev. Biochem. 53: 847-869.
- Hori, H. and Sato, S. (1977).
Extracellular hydroxyproline-rich glycoprotein of suspension-cultured tobacco cells.
Phytochemistry 16: 1485-1487.
- Hori, H. (1978).
The demonstration of galactosamine in extracellular hydroxyproline-rich macromolecule purified from the culture media of tobacco cells.
Plant and Cell Physiol. 19: 501-505.
- Huskey, R.J. and Griffin, B. (1979).
Genetic control of somatic cell differentiation in Volvox.
Dev. Biol. 72: 226-235.
- Irvine, D.E.G. and John, D.M., eds. (1984).
Systematics of the Green Algae.
The Systematics Association Special Volume no.27.
- Iwasa, K. and Murakami, S. (1968).
Palmelloid formation of Chlamydomonas
I. Palmelloid induction by organic acids.
Plant Physiol. 21: 1224-1233.

- Iwasa, K. and Murakami, S. (1969).
Palmelloid formation of Chlamydomonas
II. Mechanism of palmelloid formation by organic
acids.
Plant Physiol. 22: 43-50.
- Jaenicke, L. and Waffenschmidt, S. (1979).
Matrix-lysis and release of daughter spheroids
in Volvox carteri - a proteolytic process.
FEBS letters 107: 250-253.
- Jaenicke, L. and Waffenschmidt, S. (1981).
Liberation of reproductive units in Volvox and
Chlamydomonas: Proteolytic processes.
Ber. Deutsch. Bot. Ges. Bd. 94: 375-386.
- Jaenicke, L. (1982).
Volvox biochemistry comes of age.
Trends Biochem. Sci. 7: 60-64.
- Kirk, D.L. and Kirk, M.M. (1978).
Carrier-mediated uptake of arginine and urea by
Chlamydomonas reinhardtii.
Plant Physiol. 61:556-560.
- Kirk, D.L. and Kirk, M.M. (1986).
Heat shock elicits production of sexual inducer
in Volvox.
Science 231: 51-54.
- Kirk, D.L. and Kirk, M.M. (1976).
Protein synthesis in Volvox carteri f. nagariensis
Dev. Biol. 50: 413-427.
- Kirk, D.L. and Kirk, M.M. (1983).
Protein synthetic patterns during the asexual
life cycle of Volvox carteri.
Dev. Biol. 96: 493-506.
- Kirk, D.L., Baran, G.J., Harper, J.F., Huskey, R.J.,
Hudson, K.S., and Zagris, N. (1987).
Stage-specific hypermutability of the regA locus
of Volvox, a gene regulating germ-soma dichotomy.
Cell 48: 11-24.
- Kirk, D.L., Birchem, R., King, N. (1986).
The extracellular matrix of Volvox: A comparative
study and proposed system of nomenclature.
J. Cell Sci. 80: 207-231.

- Kirk, M.M. and Kirk, D.L. (1978)
Carrier-mediated uptake of arginine and urea by
Volvox carteri f. nagariensis.
Plant Physiol. 61: 549-555.
- Kirk, M.M. and Kirk, D.L. (1985)
Translational regulation of protein synthesis in
response to light, at a critical stage of Volvox
development. Cell 41: 419-428.
- Kochert, G. (1971).
Ribosomal RNA synthesis in Volvox.
Arch. Biochem. Biophys. 147: 318-322.
- Kochert, G. and Olson, L.W. (1970).
Endosymbiotic bacteria in Volvox carteri.
Trans. Amer. Microsc. Soc. 89: 475-478.
- Kochert, G. and Olson, L.W. (1970).
Ultrastructure of Volvox carteri
I. The asexual colony.
Arch. Mikrobiol. 74: 19-30.
- Kochert, G. and Yates, I. (1970).
A UV-labile morphogenetic substance in
Volvox carteri.
Dev. Biol. 23: 128-135.
- Kochert, G. and Yates, I. (1974).
Purification and partial characterization of a
glycoprotein sexual inducer from Volvox carteri.
Proc. Natl. Acad. Sci. 71: 1211-1214.
- Kochert, G. (1968).
Differentiation of reproductive cells in
Volvox carteri.
J. Protozool. 15: 438-452.
- Kochert, G. (1975).
Developmental mechanisms in Volvox reproduction.
in The 33rd Symposium of the Society for
Developmental Biology, Academic Press, N.Y.
pp. 55-90.
- Kopan, R., Kurn, N., and Ovadia, M. (1985).
Synthesis and possible role of proteoglycans
during Volvox development.
Cell Differentiation 16: 119-132.
- Kurn, N. and Duksin, D. (1982).
Effects of tunicamycin on protein glycosylation
and development in Volvox carteri.
Wilhelm Roux's Archives 191: 169-175.

- Kurn, N. and Sela, B.A. (1981).
Altered calmodulin activity in fluphenazine-resistant mutant strains; pleiotropic effect on development and cellular organization in Volvox carteri. Eur. J. Biochem. 121: 53-57.
- Kochert, G. and Jaworski, A.J. (1972).
Isolation and characterization of Volvox DNA. J. Phycol. 8 suppl. 16.
- Kurn, N. and Sela, B.A. (1979).
Surface glycoproteins of the multicellular alga Volvox carteri. Developmental regulation, exclusive Con A binding and induced redistribution. FEBS letters 104: 249-252
- Kurn, N., Colb, M., and Shapiro, L. (1978).
Spontaneous frequency of a developmental mutant in Volvox. Developmental Biology 66: 266-269.
- Laemmeli, U.K. (1970)
Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. Nature (London) 227: 680-685.
- Lamport, D.T.A. and Miller, D.H. (1971).
Hydroxyproline arabinosides in the plant kingdom. Plant Physiol. 48: 454-456
- Lamport, D.T.A. (1967).
Hydroxyproline-O-glycosidic linkage of the plant cell wall glycoprotein extensin. Nature 216: 1322-1324.
- Lamport, D.T.A. (1970).
Cell wall metabolism. Ann. Rev. Plant Physiol. 21:235-270.
- Lamport, D.T.A. and Catt, J.W. (1984).
Glycoproteins and enzymes of the cell wall. In: Encyclopedia of Plant Physiology, vol. 13 B (eds. H.F.Linskens and J.Heslop-Harrison pp. 133-165.
- Lamport, D.T.A. (1980).
Structure and function of plant glycoproteins. In: The Biochemistry of Plants, vol.3 (Academic Press).

- Lang, N.J. (1965).
Electron microscopy of the Volvocaceae and
Astrephomenaceae.
Am. J. Bot. 50: 280-300.
- Lang, W.C. and Chrispeels, M.J. (1976).
Biosynthesis and release of cell wall-like
glycoproteins during the vegetative cell cycle
of Chlamydomonas reinhardtii.
Planta 129: 183-189.
- Laskey, R.A. and Mills, A.D. (1975).
Quantitative film detection of ^3H and ^{14}C in
polyacrylamide by fluorography.
Eur. J. Biochem. 56: 335-341.
- Layne, E. (1957).
Spectrophotometric and turbidometric methods for
measuring proteins. In: Methods in Enzymology,
vol.III
(eds. S.P. Colowick and N.O. Kaplan) Academic Press
pp. 447-454.
- Lowry, O.H., Rosebrough, N.H., Farr, A.L., and
Randall, R.J. (1951).
Protein measurement with the folin phenol reagent
J. Biol. Chem. 193: 265-275.
- Margolis-Kazan, H. and Blamire, J. (1979).
Effect of Δ^9 -tetrahydrocannabinol on cytoplasmic
DNA metabolism in a somatic-cell regenerator
mutant of Volvox.
Microbios letters 11: 7-13.
- Margolis-Kazan, H. and Blamire, J. (1976)
The DNA of Volvox carteri: a biophysical and
biochemical characterization.
Cytobios 15: 201-216.
- Margolis-Kazan, H. and Blamire, J. (1979)
Effect of tetrahydrocannabinol and ethidium
bromide on DNA metabolism and embryogenesis in
Volvox. Cytobios 26: 75-95.
- Margolis-Kazan, H. (1978).
DNA metabolism in Volvox.
Ph.D. Thesis, The City University of New York.
- Mazia, D., Schatten, G., and Steinhardt, R. (1975)
Turning on of activities in unfertilized sea
urchin eggs: Correlation with changes of the
surface.
Proc. Natl. Acad. Sci. 72: 4469-4473.

- McCandless, E.L. and Craigie, J.S. (1979).
Sulfated polysaccharides in red and brown algae.
Ann. Rev. Plant Physiol. 30: 41-53.
- McCracken, M.D. and Barcellona, W.J. (1976).
Electron histochemistry and ultrastructural
localization of carbohydrate containing
substances in the sheath of Volvox.
J.Histochem. Cytochem. 24: 668-673.
- McNeil, M., Darvill, A.G., Fry, S.G., and
Albersheim, P. (1984).
Structure and function of the primary cell walls
of plants.
Ann. Rev. Biochem. 53: 625-663.
- Miller, D.H. and Lamport, D.T.A. (1972).
Hydroxyproline heterooligosaccharides in
Chlamydomonas.
Science 176: 918-920.
- Mitchison, J.M. (1971).
The Biology of the Cell Cycle,
Cambridge University Press.
Chapter 3: Synchronous Cultures.
- Muller, T., Bause, E., and Jaenicke, L. (1981).
Glycolipid formation in Volvox carteri
f. nagariensis.
FEBS letters 128: 208-212.
- Nielsen, B.L. and Brown, L.R. (1984).
The basis for colored silver-protein complex
formation in stained polyacrylamide gels.
Anal. Biochem. 141: 311-315.
- Northcote, D.H. (1972).
Chemistry of the plant cell wall.
Ann. Rev. Plant Physiology. 23: 113-132.
- Northcote, D.H. and Pickett-Heaps, J.D. (1966).
A function of the Golgi apparatus in polysaccharide
synthesis and transport in root cap cells of wheat.
Biochem. J. 98: 159-167.
- Olden, K., Pratt, R.M., and Yamada, K.M. (1978).
Role of carbohydrates in protein secretion and
turnover: Effects of tunicamycin on the major
cell surface glycoprotein of chick embryo
fibroblasts. Cell 13: 461-473.

- Olson, L.W. and Kochert, G. (1970).
Ultrastructure of Volvox carteri
II. The kinetosome.
Arch. Mikrobiol. 74: 31-40.
- O'Kelley, J.C. and Herndon, W.R. (1961).
Alkaline earth elements and zoospore release and
development in Protosiphon botryoides.
Amer. Jour. Bot. 48: 796-802.
- Pall, M.L. (1973).
Sexual induction in Volvox carteri
A quantitative study.
J. Cell Biol. 59: 238-241.
- Pall, M.L. (1974).
Evidence for the glycoprotein nature of the
inducer of sexuality in Volvox.
Biochem. Biophys. Res. Comm. 57: 683- 688.
- Percival, E. and McDowell
Algal Walls - composition and biosynthesis.
In: Encyclopedia of Plant Physiology, vol. 13 B.
pp. 277-331.
- Pickett-Heaps, J.D. (1970).
Some ultrastructural features of Volvox, with
particular reference to the phenomenon of
inversion.
Planta 90: 174-190.
- Pickett-Heaps, J.D. (1975).
The Green Algae
Sunderland, MA: Sinauer.
- Piez, K.A. and Reddi, A.H., eds. (1984).
Extracellular Matrix Biochemistry.
New York: Elsevier.
- Polysciences, Inc. (1983).
Silver Stain Kit, Data Sheet #293.
- Pommerville, J. and Kochert, G. (1982).
Effects of senescence on somatic cell physiology
in the green alga Volvox carteri.
Exp. Cell Res. 140: 39-45.
- Provasoli, L. and Pintner, I.J. (1959).
Artificial media for freshwater algae;
problems and suggestions.
In: The Ecology of Algae (Tyron, C.A. and
Hartmann, R.T. eds.) Spec. Publ. no. 2, pp84-96.
Pymatuning Laboratory of Field Biology,
University of Pittsburgh.

- Roberts, K. and Hills, G.J. (1976).
The crystalline glycoprotein cell wall of the
green alga Chlorogonium elongatum: A structural
analysis.
J. Cell Sci. 21: 59-71.
- Roberts, K. (1974).
Crystalline glycoprotein cell walls of algae:
their structure, composition, and assembly.
Phil. Trans. R. Soc. Lond. B, 268: 129-146.
- Robinson, N.C. and Capaldi, R.A. (1977).
Interaction of detergents with cytochrome c
oxidase.
Biochemistry 16: 375-380.
- Roseman, S. (1973).
Complex carbohydrates and intercellular adhesion.
In: The Cell Surface in Development (ed. Moscona)
- Schlipfenbacher, R., Wenzl, S., Lottspeich, F.,
and Sumper, M. (1986).
An extremely hydroxyproline-rich glycoprotein is
expressed in inverting Volvox embryos.
FEBS letters 209: 57-62.
- Schlosser, U.G., Sachs, H., and Robinson, D.G.
(1976).
Isolation of protoplasts by means of a "species-
specific" autolysine in Chlamydomonas.
Protoplasma 88: 51-64.
- Schlosser, U.K. (1981).
Algal wall-degrading enzymes - autolysines.
In: Encyclopedia of Plant Physiology, vol. 13
(ed. H.F. Linskens, J. Heslop-Harrison)
pp. 332-351.
- Schneider, E.G., Ngyen, H.T., and Lennarz, W.J.
(1978).
The effect of tunicamycin, an inhibitor of
protein glycosylation, on embryonic development
in the sea urchin. J. Biol. Chem. 253: 2348-2355.
- Sessoms, A.H. and Huskey, R.J. (1973).
Genetic control of development in Volvox:
Isolation and characterization of morphogenetic
mutants.
Proc. Natl. Acad. Sci. 70:1335-1338.

- Sharon, N. and Lis, H. (1972).
Lectins: Cell-agglutinating and sugar-specific proteins.
Science 177: 949-959.
- Snell, W.J. (1985).
Cell-cell interactions in Chlamydomonas.
Ann. Rev. Plant Physiol. 36: 287-315.
- Sueoka, N. (1960).
Mitotic replication of deoxyribonucleic acid in Chlamydomonas reinhardtii.
Proc. Natl. Acad. Sci. U.S. 46: 83-91.
- Starr, R. (1984).
Colony formation in algae.
In: Encyclopedia of Plant Physiology, new series vol. 17 (eds. H.F. Linskens and J. Heslop-Harrison) pp. 261-290.
- Starr, R.C. (1970).
A working model for the control of differentiation during development of the embryo of Volvox carteri f. nagariensis.
Soc. Bot. Fr., Memoires 1972: 175-182.
- Starr, R.C. and Jaenicke, L. (1974).
Purification and characterization of the hormone initiating sexual morphogenesis in Volvox carteri f. nagariensis Iyengar.
Proc. Natl. Acad. Sci. 71: 1050-1054.
- Starr, R.C. (1969).
Structure, reproduction, and differentiation in Volvox carteri f. nagariensis Iyengar, strains HK 9 and 10.
Arch. Protistenk. III 204-222.
- Starr, R.C. (1970).
Control of differentiation in Volvox.
Developmental Biology (Suppl.) 4:59-100.
- Stohlman, S.A., Eylar, O.R., and Wisseman, Jr., C.L. (1976). Isolation of the dengue virus envelope glycoprotein from membranes of infected cells by concanavalin A affinity chromatography.
J. Virology
- Studier, F. W. (1973).
Analysis of bacteriophage T7 early RNAs and proteins on slab gels.
J. Mol. Biol. 79: 237-248.

- Sumper, M. and Wenzl, S. (1980).
Sulphation-desulphation of a membrane component
proposed to be involved in control of
differentiation in Volvox carteri.
FEBS letters 114: 307-312.
- Sumper, M. (1979).
Control of differentiation in Volvox carteri.
A model explaining pattern formation during
embryogenesis.
FEBS letters 107:241-249.
- Tautvydas, K.T. (1978)
Isolation and characterization of an
extracellular hydroxyproline-rich glycoprotein
and a mannose-rich polysaccharide from Eudorina
californica (Shaw). Planta 140: 213-220.
- Toole, B.P. (1981).
Glycosaminoglycans in morphogenesis. In: Cell
Biology of the Extracellular Matrix, ed. E.D.Hay
(Plenum Press, N.Y. and London) pp. 259-288.
- Viamontes, G.I. and Kirk, D.L. (1977).
Cell shape changes and the mechanism of inversion
in Volvox.
J. Cell Biol. 75: 719-730.
- Viamontes, G.I., Fochtman, L.J., and Kirk, D.L.
(1979).
Morphogenesis in Volvox: Analysis of critical
variables.
Cell 17: 537-550.
- Voigt, J. (1985).
Macromolecules released into the culture medium
during the vegetative cell cycle of the
unicellular green alga Chlamydomonas reinhardtii.
Biochem. J. 226: 259-268.
- Weber, K. and Osborn, M. (1975).
Proteins and sodium dodecyl sulfate: Molecular
weight determination on polyacrylamide gels and
related procedures.
In: The Proteins, 3rd edition. (eds. H. Neurath
and R. Hill) Academic Press pp. 179- 223.
- Weber, K. and Osborn, M. (1969).
Proteins and sodium dodecyl sulfate: Molecular
weight determination on polyacrylamide gels and
related procedures.
J. Biol. Chem. 244: 4406.

- Weisshaar, B., Gilles, R., Moka, R., and Jaenicke, L. (1984).
A high frequency mutation starts sexual reproduction in Volvox carteri.
Z. Naturforsch. 39c: 1159-1162.
- Wenzl, S. and Sumper, M. (1986).
A novel glycosphingolipid that may participate in embryo inversion in Volvox carteri.
Cell 46: 633-639.
- Wenzl, S. and Sumper, M. (1981).
Sulfation of a cell surface glycoprotein correlates with the developmental program during embryogenesis of Volvox carteri.
Proc. Natl. Acad. Sci. 78: 3716-3720.
- Wenzl, S. and Sumper, M. (1982).
The occurrence of different sulphated cell surface glycoproteins correlates with defined developmental events in Volvox.
FEBS letters 143: 311-315.
- Wenzl, S. and Sumper, M. (1979).
Evidence for membrane-mediated control of differentiation during embryogenesis of Volvox carteri.
FEBS letters 107: 247-249.
- Wenzl, S., Thym, D., and Sumper, M. (1984).
Development-dependent modification of the extracellular matrix by a sulphated glycoprotein in Volvox carteri.
The EMBO Journal 3: 739-744.
- Willadsen, P. and Sumper, M. (1982).
Sulphation of a cell surface glycoprotein from Volvox carteri; evidence for a membrane-bound sulfokinase working with PAPS.
FEBS letters 139: 113-116.
- Wray, W., Boulikas, T., Wray, V., and Hancock, R. (1981).
Silver staining of proteins in polyacrylamide gels.
Anal. Biochem. 98: 231-237.
- Yates, I., Darley, M., and Kochert, G. (1975)
Separation of cell types in synchronized cultures of Volvox carteri.
Cytobios 12: 211-223.