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The ingestion, uptake, and metabolism of liposomes by juvenile Pacific oysters (Crassostrea gigas) were studied by several methods in an effort to assess their potential as encapsulating agents. Liposomes composed of egg phosphatidylcholine-cholesterol-stearylamine (7:1:2) formed readily and appeared stable in $20^{o}/oo$ seawater. Radiotracer studies with liposomes made with ^{14}C-labeled cholesterol or phosphatidylcholine showed uptake of up to 40% of the dose in 24 hrs, with the majority of uptake occurring in the visceral mass. Only slight amounts of label were observed in adductor muscle or mantle tissue. Absence of label in free fatty acids in oysters fed liposomes made with di[1-^{14}C] palmitoyl phosphatidylcholine indicated a lack of significant amounts of fatty acid hydrolysis from phospholipid in the stomach or lumen of the digestive diverticula. However, radioactivity was observed in lipid other than phosphatidylcholine, including triglyceride, phosphatidylethanolamine, and an unidentified polar lipid. Radioactivity in these lipids resided exclusively in the fatty acids, indicating breakdown of the ^{14}C-phosphatidylcholine via acyl transfer.

To examine metabolism of liposome-encapsulated substances, [1-^{14}C]glucose and [U-^{14}C]amino acids were entrapped and fed to oysters. Label from glucose appeared largely in a choloroform-methanol-insoluble fraction, with little radioactivity recovered in the lipid or soluble aqueous fractions. Most label from amino acids was recovered in trichloroacetic acid-precipitable protein. Control oysters given the same amounts of non-encapsulated [1-^{14}C] glucose or [U-^{14}C]amino acids as in liposome trials showed (1) the same uptake of label from free amino acids in comparison with encapsulated glucose, and (2) increased uptake of free amino acids in comparison with encapsulated amino acids. Label from free glucose or amino acids entered the same fractions as encapsulated label.

Evidence for intracellular uptake of liposomes was obtained with fluorescence microscopy after feeding oysters with liposomes containing bovine serum albumin conjugated with fluorescein isothiocyante (FITC). The appearance of small fluorescent inclusions within the apical portions of many of the ducts and tubules of the digestive diverticula suggest phagocytosis of intact liposomes. Uptake was not observed in other parts of the alimentary canal. The feeding of liposomes in which the stearylamine had been conjugated with FITC resulted in generalized fluorescence in most of the digestive diverticula and stomach epithelium, perhaps due to extracellular hydrolysis of FITC and its subsequent diffusion into epithelial cells. No fluorescence occurred in tissues other than those of the digestive tract. Autoradiography studies with liposomes containing di[1-^{14}C]palmitoyl phosphatidylcholine showed radioactivity dispersed throughout the epithelial cells of the ducts

and tubules of the digestive diverticula. Only slight radioactivity was observed in the intertubular connective tissue or the lumen of the tubules or stomach. This distribution of liposomal materials resembled that of fluorescence from feeding trials with FITC-tagged liposomes, and indicated uptake of intact liposomes followed by intracellular breakdown and dispersal of the liposomal components.

To investigate the process of particle selection in oysters, polyacrylamide beads ($2 \pm 1\mu$) with aminoethyl side groups, and beads with FITC-conjugated side groups were fed to oysters. Large quantities of both types of beads were observed in the stomach and intestine, but not in the digestive diverticula, indicating recognition as non-food particles despite their organic nature. The ingestion of such derivitizable particles suggests their use in studies of acceptance-rejection processes in the stomach of bivalves.

The ingestion, intracellular uptake, and breakdown of liposomes and their contents indicates a use for these particles in studies of nutrition or pollutant-food web relationships in bivalve molluscs or other filter-feeding organisms.

The Use of Liposomes as Encapsulating Agents for Feeding Juvenile Pacific Oysters (*Crassostrea gigas*)

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To the wild and undammed rivers which provided moments of clarity when they were most needed.

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I. INTRODUCTION

Although oysters have been cultured for many centuries, very little is known of the nutritional requirements of these or other marine bivalve molluscs. Knowledge of such requirements would be useful both in the assessment of the natural feeds and locations for oyster production, and in the design of artificial rations for large or laboratory scale operations. In many coastal areas, interest is growing in artificial culture systems due to rising levels of environmental contamination, and a decrease in availability of natural growing areas.

Shellfish culture in artificial systems has generally depended upon supplies of unicellular algae as food sources for both larvae and juveniles. However, adequate supplies of algae can be maintained only with considerable investment in trained manpower, equipment, and space. In addition, the most desirable species tend to be the more difficult to culture (Chanley and Normandin, 1967). Usually the growth of oysters fed single species or mixed algal diets in the laboratory has not approached that of oysters in the wild, and attempts to correlate differences in oyster growth with the chemical composition of algal species have seen little success (Castell and Trider, 1974). In some cases attempts have been made to alter the biochemical composition of single algal species by physical means

in order to obtain information of basic nutritional needs (Flaak and Epifanio, 1978).

Such studies have resulted in valuable background data, but precise information concerning specific nutritional requirements would be best obtained through controlled experiments with a well defined and manipulatable diet. The first attempt at maintaining oysters for extended time periods on a semi-purified diet (Castell and Trider, 1974) yielded information on effects of dietary carbohydrates and lipid. In a later study, Trider and Castell (1978) reported evidence for an n-3 fatty acid requirement in oysters. The diet utilized in both of these studies, however, was composed of both soluble and particulate phases, and actual ingestion of each dietary component could not be determined. The authors pointed out that encapsulation of dietary components in materials of low permeability could overcome some of the problems of feeding artificial diets.

Gabbott et al. (1976) attempted to encapsulate various substances in microcapsules formed from crosslinked nylon-protein or modified egg protein. These capsules proved to undergo only very slight digestion in the stomach of oysters and were not reported to have been phagocytosed intact by digestive epithelium, and thus appeared to have limited usefulness.

It is evident that one criterion for any method of diet encapsulation is that the capsular material be broken down, either extracellularly or intracellularly. In the case of marine animals,

phagocytosis and intracellular breakdown would be the more desirable during nutrition studies, since one could be reasonably assured that most encapsulated material would reach the tissues with minimal leakage to the exterior. An encapsulation method presently being investigated for use in intracellular delivery of compounds in mammals involves phospholipid vesicles, or liposomes. Liposomes are enclosed spheres composed of one or several concentric bilayer lamellae similar in structure to those of natural biomembranes (Bangham, 1972). In mammals they tend to accumulate in tissues which exhibit phagocytic activity, and some success in tissue targeting has been achieved through altering the lipid composition of the liposomes as reviewed by Kimelberg and Mayhew (1978).

Since several authors have suggested or presented evidence for a primary role of phagocytosis in bivalve digestion (Coe, 1948; Mathers, 1972; Owen, 1974), the applicability of liposomes in this system was investigated. Possible advantages of liposomes over synthetic microcapsules include near-neutral buoyancy, proper size range, and a composition of natural, non-toxic, digestible materials. All of these characteristics are desirable for any method of microencapsulation to be applied to the feeding of bivalve molluscs.

The objectives of the present study were first to examine liposomes with respect to their formation in seawater and subsequent uptake by juvenile oysters. The route of movement of ingested particles through the digestive tract would then be determined by fluorescence microscopy. The metabolic fate of both liposomal components or encapsulated sub-

stances would be determined by various radiotracer methods. Uptake and digestion of liposomes were then to be compared to inert, non-digestible particles of acceptable size.

II. LITERATURE REVIEW

Introduction

The study reported in this thesis combines information and principles from two different fields: invertebrate zoology and lipid chemistry. In order to afford a clearer understanding of the problems and the rationales behind the approaches employed, a review of pertinent literature from both fields is presented. The first part deals with digestive physiology of marine bivalve molluscs, feeding studies with live and artificial foods and the use of these animals in toxicological investigation. The concluding portion covers the preparation, physical characteristics, and current use of phosopholipid vesicles, or liposomes.

Collection and Movement of Food Through the Digestive Tract

Oysters are filter feeders, collecting food particles by the straining action of cilia of pairs of gills. Suspended particulates enter the shell cavity both passively in water currents through the open valves, and by currents induced by the adducting action of the valves.

Moore (1971) utilized both phase contrast and scanning electron microscopy to ellucidate the arrangement of cilia on the latero-frontal cirri of M. edulis (unreported size). It was found that a complete mesh of dimensions 0.6μ by 2.7μ was present, regardless of the filtration rate. Retained particles are carried in mucous strings to marginal grooves, the ciliary tracts of which conduct the particle strings via the labial palps to the mouth (Bernard, 1974). Mucous strings reaching the mouth are conducted through the ciliated esophagus to the stomach.

The rotating motion of the crystalline style may also assist in pulling these strings into the stomach. In the stomach these strings are broken up, with particles of potential food value entering the digestive diverticula and excess food or non-food particles conducted directly into the midgut and intestine for export. Mucous secreting cells aid in the reforming of fecal strings in the intestine. There is no peristaltic action in the gut since the required muscular layers are absent (Galtsoff, 1964).

In large C. virginica, the time required for food to pass through the entire digestive tract was 90 to 150 minutes (Galtsoff, 1964). Fecal matter is voided from the rectum as compact and slightly flattened ribbons often characteristic of the species of bivalve (Moore, 1931).

In oysters, and perhaps in other lamellibranchs, active valve adduction is not necessary for the maintenance of pumping and filtration activity. Drinnan (1964) observed a steady pumping rate of 4 l hr^{-1} in Ostrea edulis in the absence of shell movement.

Bernard (1974) examined the mechanism of particle sorting and the function of the labial palps in Crassostrea gigas. He proposed four possible fates of particles entering the mantle cavity: 1) sedimentation of particles of high specific gravity due to low water current velocity in the mantle cavity; 2) passage between the ciliary net of the gills and exit in the exhalent stream; 3) filtration by the gills and transportation on frontal mucous bands to the food grooves; 4) rejection in mucous masses by the gills. In addition, it was concluded that the function of the palps was to: 1) reduce mucus volume of the mucous strings, thus concentrating food particles prior to entering the mouth,

or 2) reject the entire load of mucous during abnormal states. Rejection of mucus by the gills (4, above) results in pseudofeces formation and can be caused by overstimulation of the filaments by large masses of food, which in turn causes secretion of a heavier rejectory mucus. The rejected material is removed from the gills either by muscular action or by its inability to enter the food grooves.

It is apparent that particle sorting and rejection may take place at two locations in the oyster: at the collection apparatus (gills), or in the stomach. Particle discrimination at the gills appears to be primarily a function of particle size. Vahl (1973) observed 100% retention of particles greater than 6μm in diameter in four species of lamellibranchs (Chlamys islandica, Mytilus edulis, crassostrea virginica, and Cardium edule). Retention decreased to 30-60% for particles of 2μm diameter. Particles below 1μm were retained by all species, but only to a limited degree. Particles too large to be ingested fall out of the ventral food grooves before reaching the lamellae of the labial palps. At the palps, small particles are retained for ingestion, and large particles are carried to the edge of the palps and drop off to the mantle. Rejected material from both gills and palps are expelled from the mantle cavity by the adducting action of the valves.

Relatively large particles are often filtered, but it is not clear whether or not the particles are ingested. For example, Chipman & Hopkins (1954) measured filtration rates in Pectens irradians by observing the rate of loss of ^{32}P-labeled diatoms (Nitzchia) and flagellates (Chlamydomonas). Results showed that Nitzchia with a

diameter of 56μm was filtered at a greater rate than Chlamydomonas, of 7μm diameter. However, whether the ingestion rate of both cell types were the same was not determined.

Rice and Smith (1958) and Allen (1962) fed ^{32}P-labeled Phaeodactylum to several species of lamellibranchs including Ostrea edulis. This algal species measures approximately 4 X 40μm, and was efficiently filtered out of suspension. In addition, assimilation, as reported by Allen, reached 94%.

Particle selection by the gills and palps appears to be minimal with regard to the nature of the filtered particles. Winter (1976) fed M. edulis suspensions of unicellular algae and ferric hydroxide, and observed no selection between the two types of particles. Foster-Smith (1975), using a mixed suspension of Phaeodactylum and alumina, demonstrated a lack of preference for one particle over the other over a range of concentrations, and that the ingested material always contained the same proportion of particles as in suspension.

A second level of particle discrimination occurs internally in the stomach. According to Barnes (1968) the lower pH of the stomach, along with mechanical activity of the crystalline style, dislodges particles from the incoming mucous strings. These particles are directed against a portion of the stomach wall composed of a series of ciliated grooves. Coarse, heavy particles are segregated out and are directed into the intestine. Finer particles and products of partial digestion by enzymes of the style are retained by the cilia and directed into the caecal ducts of the digestive glands.

Rejected mucous strings which have not gone through the digestive diverticula, and which may be termed pseudofeces (formed either by rejection at the gills or in the stomach), may also occur as a result of an excess particle concentration in the surrounding medium. Winter (1978) showed that both ingestion rate and filtration rate increase as particle concentration increases from lower levels. However, at some critical particle concentration ingestion rate remains constant, and filtration rate declines. Finally, at still higher concentrations, both filtration and ingestion rates decrease with concommitant pseudofeces production. The concentration at which this occurs has been termed the "pseudofeces-free cell density" (Schulte, 1975), the value of which may increase with decreasing particle size (Winter, 1978).

Bernard (1974) examined the ability of Crassostrea gigas to discriminate between algae and bacteria. He fed cultures of the bacteria Chromatium or Chromobacterium, mixed with either of two unicellular algal species, Chlorella or Tetraselma. The oysters were unable to sort the bacteria from the algae, ingesting both equally at loads less than 200 mg/l. However, bacteria were digested much more readily than the algae, as the oysters produced feces containing few bacteria but much undigested algal material. Thus a type of discrimination occured post-ingestion, based possibly on differences in digestibility (resistance to extracellular enzymes) or recognition of chemical or physical characteristics of ingested particles.

Digestion

A short esophagus leads from the mouth cavity to the stomach, which is located in the posterior portion of the visceral mass, and is

completely surrounded by tubules of the digestive diverticula. The stomach is comprised of two chambers, one anterior and the other a larger posterior chamber. Both chambers are ciliated with the posterior chamber containing a gastric shield or plate-like structure at the point of contact of the crystalline style with the stomach wall. Two out-pouchings in the posterior stomach form the style sac, containing the crystalline style, and the midgut, sometimes referred to as the tubular stomach. The midgut shortly becomes the ascending, median and descending portions of the intestine. A relatively short rectum terminates the digestive tract near the adductor muscle.

Ducts from both chambers of the stomach lead to the digestive diverticula. This organ consists of a mass of irregular lobules, surrounding the stomach which together with the esophagus and stomach constitute the main visceral mass. Owen (1955) described a continuous two-way flow within the main ducts of the digestive gland. A non-ciliated inhalent portion carries material into the gland and to the terminal tubules. Waste material is conducted out of the gland along a ciliated exhalent portion of the main ducts. An outward ciliary beat in the exhalent channel is thought to produce a countercurrent inward flow through the inhalent channel. A typhlosole, or extended fold in the stomach prevents waste materials leaving the diverticula from mixing with incoming food material before the waste is conducted into the midgut and intestine. (Refer to Appendix I.)

The digestive tract is ciliated throughout with the exception of the gastric shield region and possibly the epithelium of the terminal absorptive tubules of the digestive diverticula. No cilia have been

observed in the tubules of C. virginica but the cells in the diverticula of other bivalves (e.g. the boring mollusc Teredo) are ciliated (Galtsoff, 1964). Yonge (1926a, 1926b) suggested the cilia are present in the tubules of Ostrea edulis but retract too rapidly to be observed.

Coe (1948) maintained that the alimentary canal of bivalves in general is deficient in most extracellular enzymes required for primary digestion. The principal extracellular enzymes were believed to be amylase and glycogenase, released from the crystalline style as the style slowly dissolved. Cellulase was found in quantities believed to be inadequate for complete digestion of phytoplankton with cellulose walls, which constitutes a large part of the natural diet of these filter feeders.

Yonge (1926a, 1946) found that proteases which would be required for extracellular protein digestion could be detected only in very small amounts in the lumen of the alimentary canal. Free lipase was also observed to be present only in trace quantities, and it was concluded that the presence of these enzymes was due to cytolyzed phagocytic cells of the digestive diverticula.

In contrast, Mansour (1946a, 1946b) and Mansour-Bek (1946) reported that filtered, phagocyte-free stomach fluid of two species of bivalves do contain effective amounts of proteases, lipases, and carbohydrases. George (1952) claimed that hydrolysis of triglycerides could take place extracellularly in the stomach and that lipase could be extracted from the crystalline style. However, it is generally held by most authors that the majority of extracellular enzymes are liberated by frag-

mentation and cytolysis of cells in the digestive diverticula, where they are present intracellularly.

The picture which emerged from these earlier studies regarding digestion in oysters and other bivalves is one in which phagocytosis of intact or partially digested food particles by the cells of the digestive diverticula is the dominant process. Extracellular digestion of whole cells assumes a secondary or supporting role.

More recently, Van Weel (1961) reviewed aspects of digestion in molluscs, and noted tryptic proteases in Ostrea edulis. He also stated that esterases seemed to be common in the Mollusca, but that lipase activity was generally low. Kozlovskaya and Vaskovsky (1970) concluded that, as a class, bivalves exhibit relatively weak proteolytic ability, including that of digestive diverticula, compared to other marine invertebrates. Owen (1966) indicated that the greater part of protein hydrolysis in bivalves takes place intracellularly in the digestive tubules.

Reid (1968) reported in several bivalves a distribution of esterase activity similar to that of proteases. Mathers (1973) found that the epithelia of the ducts of the digestive diverticula, stomach, midgut, and gastric shield showed the greatest nonspecific esterase activity in O. edulis and C. angulata. Esterase was also reported in the lumen of the digestive diverticula ducts of fed animals. Mathers (1972) observed the release of "excretory spherules" from the distal regions of the epithelia of the digestive tubules. Such spherules may contain various digestive enzymes which could be released in the stomach upon rupture by

the style or gastric enzymes. Thus, it would seem unlikely that all extracellular stomach enzymes secrete by the style or stomach epithelium.

Histochemical analyses of the digestive tract of bivalves indicate that hydrolytic enzymes are located primarily in the digestive diverticula, gastric and style sac epithelia, and the epithelial lining of the midgut (Reid, 1968; Sumner, 1969; Mathers, 1973). Other enzymes reported in the style and digestive diverticula include butyrase, lactase, and maltase. Proteolyic activity in O. edulis is absent in the gut but is present in extracts of diverticula, with pH optima of 3.7 and 8.5 for casein (Galtsoff, 1964). Purchon (1971) claimed that cellulase and possibly lipase are also found in the styles of lamellibranchs, but Galtsoff (1964) maintained that cellulase has not been found in digestive extracts of oysters. He observed, however, that some cellulase activity may be present in gut flora.

At any rate, the often-demonstrated occurrance of live algal or bacterial cells in fecal material (Loosanoff and Engle, 1947) attests to the weakness of the lytic power of the extra-cellular gastric fluids. Some cell types, however, may be more resistant than others to gastric enzymes. Dean (1958) exposed various species of algae to style extracts from C. virginia. Cryptomonas disintegrated rapidly and Monoisochrysis was immobilized, but Isochrysis was unaffected. Morton (1970, 1971) has proposed a rhythmic digestion pattern in lamellibranchiate bivalves, including O. edulis. According to his hypothesis, extracellular digestion in the stomach and intracellular digestion in the diverticula occurred in alternating phases. It was further proposed that the cycle was imposed by the tidal phase, with feeding occurring during high tide,

extracellular gastric digestion at ebb tide, and intracellular digestion and absorption during low tide and the succeeding rising tide. However, such rhythmic activity, if real, may not be widespread among lamellibranchs. Thompson and Bayne (1972) found that Mytilus edulis filters continuously, if food is present, provided that particle concentration is not excessive. Owen (1972) has proposed that digestive activity may simply be under the influence of available food levels rather than an endogenous rhythm of digestion. After studying the digestive diverticula of C. edule, it was suggested that following ingestion, food particles were transferred rapidly to the digestive tubules with little or no delay in the stomach. In the diverticula the particles were endocytosed by the epithelia and underwent intracellular digestion in lysosomes.

Absence of a lag between ingestion and food transfer to the diverticula was supported by Mathers (1972) who observed ^{14}C activity in the ducts and tubules of the diverticula of Ostrea edulis within 10 min of feeding ^{14}C-labeled algae. The brush-border epithelial cells of the tubules appeared to absorb soluble or finely particulate material, probably products of extracellular digestion in the stomach. In addition, these cells phagocytosed whole algal cells, which accumulated in large digestive vacuoles in the basal region of the epithelia. Amoebocytes were observed within these vacuoles. In the early stages of feeding, no ^{14}C activity was observed in the ciliated crypts of the ducts. Heavily labeled amoebocytes were observed, supporting the concept that these cells are important in the absorption and translocation of materials throughout the tissues. The rapid appearance of

free ^{14}C activity in the haemocoele also indicated that some vacuolar contents are released into circulation from the basal regions of the digestive epithelial cells. Radioactivity also increased steadily in the connective tissue surrounding the digestive diverticula, indicating that this tissue may contain storage sites. No radioactivity was observed in the crypt cells of the diverticular tubules; thus these may not be involved with intracellular digestion.

Feeding Trials and Nutrition Studies

Feeding trials with marine bivalves can be divided into two groups: those utilizing cultured live food, and those involving a synthetic or artificial food not normally considered part of the diet of such organisms. While the literature on the food requirements of bivalve larvae is extensive, as reviewed by Loosanoff and Davis (1963) and Walne (1964), knowledge of the food requirements of the post-larval forms (juveniles and adults) is relatively scant. Since the present study deals with post-larval forms, some of the more pertinent studies concerning these forms is reviewed here.

With few exceptions, most experimentation with live food sources has involved one or more species of unicellular algae. Loosanoff and Engle (1947) investigated the relative food value of varying concentrations of different algae. It was observed that an excessive concentration of algal cells adversely affects the feeding of oysters, and that the concentration above which this occurred varied inversely to the size of the algal cells. For example, above 450 X 10^6 Chlorella cells (6μm) per liter, oysters (Crassostrea virginica) produced pseudofeces and

ingestion decreased. The comparable value for Nitzschia closterium (40-50μm) was only 20-30 x 10^6 cells/liter, and for Euglena (60μm), 2 X 10^6 cells/liter. It was observed that the green alga Chlorella was a relatively poor food source for the oysters, and that even filtrates of cultures of this species resulted in depressed feeding rates.

Tenore and Dunstan (1973) observed the feeding and biodeposition (production of feces and pseudofeces) rates of juvenile and adult oysters (C. virginica) fed various species of live phytoplankton. The diatoms Thalassiosira pseudonana and Skeletonema costatum were consumed at the greatest rates, and the green alga Dunaliella tertiolecta at the least. However, T. pseudonana produced the lowest rate of biodeposition. With the exception of T. pseudonana, all species of algae resulted in increased rates of biodeposition at increased food levels. No feeding trials longer than one week were undertaken.

Epifanio (1979a) compared the nutritional effects of yeast and algal diets in four species of bivalves: Argopectens irradians, Mytilus edulis, Mercenaria mercenaria, and Crassostrea virginica. The algal employed was Thalassiosira pseudonana, and the yeast a non-living, food grade spray-dried Torula preparation. In all species except C. virginica yeast could be used as a replacement for up to 50% of the algae. However, mean growth was not well correlated with either gross dietary composition or amino acid composition.

Epifanio (1979b) examined the nutritional effects of the simultaneous feeding of two or more species of algae on both C. virginica and M. mercenaria. Growth was fastest when diets contained the highest proportion of Isochrysis galbana or Thalassiosira pseudonana, and slowest

with Carteria chui. Platymonas suecica proved to be a poor food source when utilized alone, but its food value was increased when fed concommitantly with either I. galbana or T. psuedonana. Attempts were made to correlate growth with biochemical composition of the dietary algae. Although the amino acid composition of the four algal species tested were nearly identical, some species promoted better growth than others, as pointed out above. There appeared to be no correlation between growth and chemical composition (protein, lipid, carbohydrate, or ash content). It was suggested that perhaps certain of the algal species were deficient in one or more trace nutrients. An alternative hypothesis was that differences existed in the digestibility of the various algal species. C. chui and P. suecica are bounded by a non-rigid theca composed of non-cellulose complex polysaccharides and protein. I. galbana and T. pseudonana lack this feature and therefore might be expected to undergo extracellular breakdown more easily. In other words, the exterior packaging of the food particle may determine its relative acceptance and food value.

In the absence of a formulated feed whose composition could be altered to allow evaluation of selective nutritional needs of bivalves, Flaak and Epifanio (1978) resorted to changing the biochemical composition of an alga, Thalassiosira pseudonana, by spectral means. By culturing the algae under different light regimes and by harvesting them from either exponential or stationary phase, several diets of differing protein content were obtained. Results from 10 week feeding trials with C. virginica indicated that the nutritional value of the algae was related to the ratio of protein to non-protein in the diet. Percent

change in whole weight, increase in shell height, and increase in glycogen content correlated with higher carbon: nitrogen ratios in the algal cultures.

Adequate supplies of algae can be maintained only with large investments in time, manpower, space, and equipment. In addition, most desirable algal species are difficult to culture (Chanley and Normandin, 1967). Consequently, attempts have been made to replace live food sources with artificial preparations. Only limited success has been achieved to date.

In an earlier study, Chanley and Normandin (1967) examined the efficacy of several artificial feeds in the culture larval of Mercenaria mercenaria. Ground fresh or frozen Ulva lactuca or Fucus appeared to be the most effective ration with respect to survival and metamorphosis when compared to unfed controls. It is interesting to note that fresh milk at 0.003 ml/l produced some growth but was generally unsatisfactory. Soluble solutions of amino acids and carbohydrates (unspecified content) also proved unsatisfactory, as did bacterial and yeast cells and corn starch.

Willis et al. (1976) attempted to improve the quality of market size oysters grown in the natural environment by feeding ground cornmeal or yeast preparations for 1-4 weeks. Cornmeal produced increases in glycogen, dry weight, and meat volume. Yeast supplementation did not improve oyster quality. No feeding trials were attempted with younger animals.

Gabbott et al. (1976) and Jones et al. (1976) described the preparation and preliminary feeding experiments with two types of synthetic

microcapsules, one made from crosslinked nylon and protein, and the other from a glycopeptide fraction of ovomucoid. Digestion experiments using stomach juice from Crassostrea gigas showed that neither type of capsule was broken down extracellularly, although encapsulated starch did appear to be digested (based on disappearance of positive iodine staining). Short term feeding trials showed that both types of capsules would be ingested by mussels and oysters if in the size range of 10 to 100μm. Larger particles were rejected in pseudofeces. Feeding trials lasting 21 days with oyster spat fed an encapsulated starch-cholesterol mixture showed significant differences between starved controls and those receiving microcapsules, although much less than those fed a standard algal diet. A primary problem was to maintain the microcapsules in suspension, as their density exceeded that of seawater. The study also showed that modified proteins undergo only limited digestion by the species tested. The authors presented desirable characteristics of any microencapsulation technique applied to the feeding of bivalves. These included small size, neutral buoyancy, lack of toxicity, low permeability, and digestibility.

Castell and Trider (1974) were the first to attempt to maintain juvenile oysters (Crassostrea virginica) on a semi-purified artificial diet for an extended period of time. The diets consisted of dissolved water-soluble nutrients mixed with suspended particulates ranging in size from less than 1μm to 50μm in diameter. In a twelve week feeding trial, it was observed that high dietary carbohydrate resulted in increased glycogen content. Inclusion of cod liver oil also promoted glycogen deposition, more so than corn oil. While this study showed

that oysters could be maintained on an artificial diet, growth rates were only one-tenth those of oysters in the natural environment, indicating that the quality and/or quantity of nutrients in the artificial rations was lacking.

In a more recent study, Trider and Castell (1980) fed several formulations of artificial diets containing different oils to oysters (C. virginica) eighteen months of age for thirty weeks. Diets with higher levels of n-3 fatty acids (linolenate series) resulted in better growth than those with high n-6 fatty acid content, possibly indicating a requirement for the former. Glycogen content increased in all dietary treatments, particularly those containing corn or cod liver oils. However, there were no significant differences among lipid levels of oysters on any of the treatments, and growth differences were not significant until 20-30 weeks. The authors observed that microencapsulation methods, not employed in their study, could prove beneficial in future experimentation in oyster nutrition with artificial diets.

Relevance of Marine Shellfish to Toxicological Studies

Bivalves and other estuarine invertebrates have been used with increasing frequency in recent years as indicators of environmental quality of near-shore marine ecosystems. Many of these organisms are sedentary, and spend their lives (excepting the larval stages) in the same location. They are thus exposed to any chemical insults which may occur in the waters they inhabit. Many such organisms also show a tendency to accumulate several chemical pollutants such as heavy metals,

insecticides, and petroleum wastes. There is considerable data concerning the accumulation and distribution of chemical pollutants in the marine environment (Fowler and Elder, 1978). These authors have also noted that the kinetics of bioaccumulation of toxicants in filter feeders and other invertebrates remains unclear due to a lack of adequate information about the relative importance of food and water in the uptake of these compounds. One of the main reasons for this deficiency has been the lack of a defined and manipulatable food for these types of organisms. Most of the studies on toxicants in shellfish have been concerned with uptake of soluble forms from seawater (e.g. Hamelink et al., 1971; Lee et al., 1972; Stegeman and Teal, 1973).

More recently, Couch et al. (1979) studied the rates of uptake from water, tissue accumulation, metabolism, and effects of two carcinogenic aromatic hydrocarbons, benzo [a]pyrene and 3-methyl-cholanthrene, in the oyster Crassostrea virginica. Test compounds were concentrated over 200-fold relative to the original concentration of carcinogen in the water. Autoradiography using ^{14}C-labeled compounds revealed that uptake occurred primariy in the digestive gland, particularly the distal portions of the digestive tubules. No significant uptake was observed by mantle or gill epithelium. Radioactivity appeared in vesicular connective tissue and gonads at later times (7 to 14 days).

Assessment of pollutant contamination in marine bivalve molluscs has attracted considerable interest, since these organisms are useful in determining the status of coastal areas with regard to petroleum pollution (Farrington and Quinn, 1973). Surveys of polyaromatic hydrocarbon lavels (Blumer et al., 1970; Erhart, 1972; Bravo et al., 1978)

and pesticide contamination (Ernst et al., 1976) have been reported for bivalves in several locations. Many pollutants may become associated with particulate detrital matter in coastal areas (Odum and Driftmeyer, 1978).

Absorption of heavy metals, radionuclides, and pesticides onto detritus has been recently reviewed by Odum and Driftmeyer (1978). All three of the above pollutants have been found to accumulate and, in some cases concentrate, in detrital matter. Animals whose diets include such particulate matter have also been reported to accumulate these contaminants, thus introducing them into the food web. Such organisms have included fish, worms, crabs, and bivalves. However, this review also noted that not all studies of detritus-food web transfer have proved positive, and the importance of this process in nature remains unresolved

Transfer of the insecticide Kepone from both water and food in laboratory food webs was demonstrated by Bahner et al. (1977). Oysters (Crassostrea virginica) accumulated the insecticide directly from water and from algae which had been contaminated (from water) prior to feeding to the oysters. In the natural environment, bioconcentration factors for DDT in plankton have been shown to exceed 4000 X (Cox, 1971).

Stegeman and Teal (1973), in studying the uptake and retention of petroleum hydrocarbons by the oyster C. virginica, also noted that it has not been established if the primary mechanism concerned with uptake of non-biogenic hydrocarbons involves consumption of contaminated food or equilibrium with hydrocarbons in the water. They also noted that uptake across outer membranes (gills) could be significant when pollutant concentrations in the water are high, but that over time the

concentration in the water should decrease by absorption to particulate phases. As this occurs, ingestion of contaminated food particles may become a major pathway of exposure.

Liposomes

Liposomes are enclosed synthetic vesicles which may be made from a variety of lipids, the major component of which is usually a phospholipid. In aqueous media these vesicles take the form of smectic mesophases of one or many concentric spheres (Bangham et al., 1965), each lamella being composed of a single lipid bilayer such as is found in most natural biological membranes (Bangham, 1972).

Over the past ten years, liposomes have been found useful for a wide variety of applications. These have included investigations of the in situ properties of lipid components of biomembranes such as acyl group composition (Kimelberg and Mayhew, 1978), cholesterol (Papahadjopoulos et al., 1973), and integral proteins (Kimelberg and Papahadjopoulos, 1971). Other studies have utilized liposomes as synthetic membranes in which to study membrane active compounds such as anesthetics (Papahadjopoulos, 1972), ionophores (Bangham, 1968), and cell surface receptor proteins (Kinsky and Nicolotti, 1977). Reconstitution studies with membrane-bound enzymes and cytochromes in purified form (Kimelberg and Papahadjopoulos, 1974; Vik and Capaldi, 1977) have yielded new and valuable information on how these enzymes function and the effect of membrane lipid microenvironment on their activity. Liposomes have also been used as artificial "cells" in the study of

proteins whose function is to transfer cholesterol or phospholipids from one membrane site to another (DiCorleto and Zilversmit, 1977).

Another use for which the applicability of liposomes has been examined is the internal delivery of encapsulated substances in animals. Such a use was prompted by the need for a more effective delivery system for therapeutic drugs. Liposomes have the advantages of versatility, ease of preparation, and are composed of non-toxic materials whose composition may be varied to more closely suit the needs of the application.

Liposomes may be taken up by cells via two mechanisms - endocytosis and fusion. Endocytosis (phagocytosis, pinocytosis) results in internalization of intact vesicles which usually accumulate in the lysosomal apparatus. Fusion may result in release of the entrapped aqueous volume to the cytosol (unilamellar liposomes) or loss of the outer lamella and entry of the remaining vesicle intact (multilamellar vesicle) (Kimelberg and Mayhew, 1978). Multilamellar liposomes have been observed by electron microscopy in hepatocytes and Kupffer cells following intravenous injection (Rahman and Wright, 1975). This may indicate that liposomes are endocytosed intact or partially intact and contained in cytoplasmic lysosomal vesicles. In addition, further studies (Rahmen et al., 1974) showed that compared to injection of free drug, liposome encapsulation resulted in much higher concentrations and in increased retention time of drug in liver, spleen, and marrow.

A comprehensive study of the trapping of globular proteins in several types of lipid vesicles was done by Adrian and Huang (1979).

For two types of large vesicles (multilamellar vesicles and large unilamellar vesicles), proteins from 14,500 MW to 97,000 MW were entrapped with the same efficiency as that of lysosyme (14,500 MW). For the two types of small vesicles employed (small unilamellar vesicles prepared by sonication or ethanol injection), proteins up to 25,000 MW were entrapped with efficiencies similar to lysosyme. With larger proteins, however, trapping efficiencies decreased with increasing molecular weight. It was concluded that with large vesicles, the internal sampling volume is significantly larger than the size of the solute (protein), resulting in a representative sampling of the solution. If the internal volume approaches that of the solute, then deviation from ideal trapping behavior occurs.

III. EXPERIMENTAL

Animals

Cultchless juvenile Pacific oysters, Crassostrea gigas, measuring 15 to 25 mm in shell diameter, were obtained from International Shellfish, Inc. (AMFAC Foods), Moss Landing, CA. Oysters were kept at 7°C in 2% synthetic seawater on small trays in a 30 gallon polyethylene tank equipped with a bottom filter and a zeolite ammonium-sodium ion exchanger. Animals were not fed during holding, and were generally used within 2-3 weeks of receipt. No histological evidence of starvation, e.g. decrease in height of epithelium of digestive gland, was observed. Mortalities were rare. All animals used in feeding trials were acclimated to 18-20°C water for at least 6-8 hrs prior to the start of any experiment.

Chemicals

All solvents employed were of reagent grade quality. Synthetic seawater salt mix (Instant Ocean, Aquarium Systems, Inc., Eastlake, OH) was purchased from a local pet shop and reconstituted with distilled water. Fluorescein isothiocyanate (FITC), Isomer I, and FITC-bovine serum albumin (8 moles FITC per mole albumin) were purchased from Sigma, St. Louis, MO. Polyethylene glycols (200, 1000, and 4000) were obtained from J.T. Baker, Phillipsburg, NJ. Lipid mixtures of egg phosphatidylcholine, cholesterol, and stearylamine (molar proportion 7:1:2) were purchased from P-L Biochemicals, Inc. Milwaukee, WI. L-α-di-[1-^{14}C] palmitoyl phosphatidylcholine (114 mCi/mmol), [4-^{14}C]cholesterol

(57.8 mCi/mmol), [U-^{14}C]algal protein hydrolysate (57 mCi/m-atom C), and [1-^{14}C]glucose (50-60 mCi/mmol) were obtained from Amersham, Arlington Heights, IL.

Derivitization of Polyacrylamide Beads

Two milliliters of a 10% solids solution of aminoethyl derivatized polyacrylamide beads (Affi-Gel 701, Bio Rad, Richmond, CA) were mixed with 12 mls 0.15 M NaCl and 1.2 mls 0.20 M carbonate buffer, pH 9.2, and 200 μmoles FITC. The ratio of terminal amino groups to FITC in the reaction mixture was approximately 1:4. The mixture was shaken at room temperature for 1 hour. Unreacted FITC was removed by repeated centrifugation and decantation. Derivatized beads were given two rinses in 50% ethanol to remove traces of particulate unreacted FITC, and diluted in synthetic seawater. The final preparation consisted of a solution of highly fluorescent beads, 1-3μm in diameter, which exhibited minimal clumping and required several hours to settle out of suspension.

Fluorescent beads, underivatized beads, or a mixture (1:1) of the two were fed to oysters at regular intervals (every three hours during the day, once overnite) at 20°C for three days. Animals were then fixed in Davidson's fixative (Shaw and Battle, 1957) without glacial acetic acid, embedded in paraffin, and sectioned at 6μm. Some sections were stained with hematoxylin (beads stain positively); others were left unstained for examination by fluorescence microscopy.

Derivatization of Liposomes with FITC

A stock solution of FITC was made by dissolving 20 µmoles FITC in 90 mls synthetic seawater and 10 mls 200mM carbonate buffer, pH 9.2. Fifteen µmoles of lipid, consisting of egg phosphatidylcholine/cholesterol/stearylamine (7:1:2) was evaporated from chloroform to a thin film on the wall of a round bottom flask. Five milliliters of the above FITC solution and several glass beads were added and the flask swirled for 1 hr at 25°C. The ratio of terminal amino groups to FITC in the reaction mixture was approximately 3:1. Unreacted (or unentrapped) FITC was removed by allowing the liposomes to settle over several hours and replacing the supernatant with synthetic seawater. After several repetitions, no fluorescence was observed in the filtrate following filtration through a 0.45µm pore Millipore syringe filter.

Preparation and Feeding of Liposomes Containing FITC-labeled Bovine Serum Albumin

Twenty milligrams of lipid (egg phosphatidylcholine/cholesterol/stearylamine, 7:1:2) was evaporated from chloroform to form a thin film on the walls of a round-bottom flask. A solution of 5 mg FITC-albumin dissolved in 5 ml synthetic seawater was added and the solution agitated with the aid of glass beads for one hour in a shaker incubator at 25°C. The resulting suspension was diluted to 10 ml and allowed to settle for several hours. The supernatant was removed and the liposomes resuspended in fresh synthetic seawater. Washing was repeated several times until no fluorescence appeared in the supernatant.

Concentration of vesicles was determined using a hemacytometer and phase contrast optics.

Oysters were fed liposomes containing FITC-protein at regular intervals for three days, after which they were fixed in Davidson's fixative for paraffin embedding or embedding in polyethylene glycol (refer to section on Light Microscopy).

Preparation of [4 -^{14}C] Cholesterol Liposomes

A chloroform solution of 20 mg lipid (egg PC-cholesterol-stearylamine, 7:1:2) containing 1.5 μCi [4-^{14}C] cholesterol was evaporated to a thin film in a round bottom flask. Five milliliters of synthetic seawater and several glass beads were added and the mixture swirled in a shaker-incubator at 25°C under nitrogen for 1 hr. The resulting suspension of liposomes was dialyzed against synthetic seawater for 6 hr at 6°C. Negligible loss of radioactivity occurred as a result of dialysis, indicating that most ^{14}C-cholesterol was associated with the liposomes. Concentration of liposomes, determined optically under phase contrast in a hemacytometer, was 1.6 x 10^8 vesicles per milliliter. At 2-4 hr intervals, 100 μl of the above suspension was fed to pairs of oysters in 40 ml synthetic seawater. One pair received a total of 400,000 dpm (Experiment 1), the other pair a total of 780,000 dpm (Experiment 2). Oysters were fixed in Davidson's fixative and dissected into various tissues. Tissue samples were digested in Soluene-350 (Packard, Downer's Grove, IL) and counted by liquid scintillation (LSC) in Toluene-PPO-POPOP fluor.

Preparation and Feeding of ^{14}C-Glucose and ^{14}C-Amino Acid Liposomes

Liposomes were prepared by the solvent evaporation technique using 15 μmoles of lipid (egg phosphatidylcholine/cholesterol/stearylamine, (7:1:2). Twenty μCi of ^{14}C-amino acids mixture prepared from an algal hydrolyzate, or 10 μCi of ^{14}C-glucose in 1.0 ml synthetic seawater with glass beads was added to the lipid film and swirled for 1 hr at 5°C. The resulting milky suspension was diluted to 4.0 ml and added to a 1/4 inch diameter dialysis bag which had been previously hydrated and rinsed in synthetic seawater. Liposomes were dialyzed against several 1 liter portions of synthetic seawater until no further counts were obtained in the dialyzate. Liposomes were then removed from the bag with a Pasteur pipette, brought to a known volume with synthetic seawater, and used immediately. Storage was at 4°C. Ten microliter samples of each preparation were dissolved in 1.0 ml Soluene-350 and counted by liquid scintillation in 16 ml Toluene fluor. Percent entrapment of label, calculated from loss by dialysis, was 13.7% for ^{14}C-glucose and 14.3% for ^{14}C-amino acids.

Suspensions of either ^{14}C-glucose or ^{14}C-amino acid-containing liposomes were fed to six oysters in 120 mls synthetic seawater at 2-3 hr time intervals. Seawater was replaced every eight hours. Three oysters were sampled at 24 hrs and the remaining three at 60 hours.

As a positive control, additional oysters were exposed to ^{14}C-glucose or ^{14}C-amino acids without liposomes (free in autoclaved seawater) in identical fashion as described above.

Analysis of ^{14}C-Glucose and ^{14}C-Amino Acid Metabolism

Oysters fed liposomes containing 1-^{14}C glucose or exposed to free ^{14}C-glucose were removed from their shells, weighed, and individually ground in a mortar with chloroform-methanol (2:1). Lipids were extracted according to the method of Folch (1957). Insoluble particulate material was filtered, solubilized in Soluene-350, and counted by LSC in Toluene-PPO-POPOP fluor. Samples of both lipid and aqueous phases were counted in Aquasol.

Oysters fed liposomes containing ^{14}C-labelled amino acids or free ^{14}C-amino acids were removed from shells, weighed, and individually homogenized in 0.9% NaCl with a Potter-Elveheim homogenizer. Protein was precipitated with 10 percent trichloroacetic acid (TCA), centrifuged, washed with 5% TCA, and the resulting pellet solubilized in Soluene. Both pellet and aqueous supernatant were analyzed for radioactivity.

Preparation and Feeding of Liposomes Containing ^{14}C-Phospholipid

[1-^{14}C]dipalmitoyl phosphatidylcholine was purchased from Amersham, Arlington Heights, IL. A solution containing 16.5 μCi of [1-^{14}C] dipalmitoyl phosphatidylcholine and 40 mg of lipid (egg phosphatidylcholine-cholestrol-stearylamine, 7:1:3, was evaporated to a thin film in a round bottom flask and prepared as for ^{14}C-glucose-containing liposomes (previous section), except that swelling was done in 3 ml synthetic seawater. Recovery of label was 99.9% in the final liposome preparation, which was adjusted to 5.0 ml.

Labelled liposomes were administered at the rate of 0.2 ml (0.1 μCi) per 120 mls synthetic seawater in each of two aerated beakers which initially contained 6 animals each. At 24 and 80 hrs, 3 oysters were sampled for lipid analysis, and 2 oysters for autoradiography. Two animals were allowed to depurate for seven days before sacrifice for lipid analysis. Total exposure was 0.5 μCi per oyster at 24 hrs and 2.25 μCi per oyster at 80 hrs, assuming equal access of oysters to label.

Lipid Extraction and Analysis

Oysters were removed from the shell, weighed, and ground in a mortar with chloroform-methanol (2:1). Extraction of lipids was done by the method of Folch (1957). Material insoluble in chloroform-methanol was collected on a filter pad, digested in Soluene-350, and counted for radioactivity. Samples of both aqueous phase and lipid extract were also measured for radioactivity.

Samples of total lipid were run on thin layer chromotography (TLC) plates to separate individual phospholipids. The solvent employed was chloroform-methanol-water (70:30:4). Neutral lipids were separated on different plates in the solvent hexane-diethylether-glacial acetic acid (85:15:1). Bands 0.5 cm in width were scraped into vials, hydrated with water, and counted by liquid scintillation in Aquasol (New England Nuclear, Boston, MA).

Neutral lipids, phosphatidylcholine, phosphatidylethanolamine and an unidentified lipid band were analyzed for distribution of label between the acyl portion and the glycerol-base backbone. Samples of each lipid were separated by TLC in hexane-diethyl ether-glacial acetic

acid (70:30:4), scraped into vials, and heated for 90 min in 4% H_2SO_4 in methanol. The solution was extracted several times with hexanediethyl ether (90:10) to remove fatty acid methyl esters. Samples of both aqueous and organic phases were taken for liquid scintillation counting.

Autoradiography

Oysters exposed to ^{14}C-phosphatidylcholine liposomes for 24 or 80 hrs were used in this study. Two oysters at each time period were removed from the shell and fixed in Davidson's fixative for at least 48 hours. Tissues were then dehydrated in graded ethanol solutions and xylene, and embedded in paraffin. Triplicate sections were cut (6μm thickness) at each of 2 planes through the oyster. One section was stained with hematoxylin and eosin (reference section), and the other 2 were coated with NTB-3 liquid nuclear track emulsion (Eastman Products, Research Div., Special Products, Rochester, NY) according to the procedure of Lillie (1965). Slides were exposed in a refrigerator at 6°C for 3 weeks after which they were developed and photographed.

Electron Microscopy

Digestive diverticula from oysters given one of the following treatments were examined by electron microscopy for evidence of uptake: (1) untreated; (2) oysters fed 8 hrs with a 0.013% solids solution of polystyrene latex beads, 1.0μm diameter (Sigma, St. Louis, MO); (3) oysters fed 3 hrs with a preparation of sonicated liposomes (egg phosphatidylcholine-cholesterol-stearylamine, 7:1:2 formed in a

ferritin-containing solution and subjected to ultrafiltration to remove unentrapped ferritin.

Animals were dissected while bathed in 3% glutaraldehyde in 0.1M phosphate buffer, pH 7.2, and containing 7% sucrose (Giusti, 1970). Small pieces of digestive diverticula were fixed for 2 hrs at 4°C. The tissues were then rinsed in buffer and dehydrated in a graded ethanol series, and embedded in Bojax media (Hayat, 1972, Method II). Silver sections were cut on a Sorvall MT-1 ultramicrotome and collected on Formvar coated copper grids protected with evaporated carbon. Grids were then stained with Reynold's lead citrate (diluted 1:3 with distilled water) for 4 min at room temperature, dried, and examined in a RCA EMU-3H electron microscope (50 kv). Sections were photographed on a 70 mm roll of film. Magnification is given in the legend for each figure. Visualization of liposomes with transmission electron microscopy utilizing the negative staining method of Bangham and Horne (1964) was attempted. A drop of liposome suspension in seawater was placed on a Formvar-coated copper grid, and the excess removed with filter paper. A drop of stain (phosphotungstic acid, 2% in seawater, or ammonium molybdate, 2% in seawater) was then placed on the grid, excess removed, and allowed to dry before examination. Addition of phosphotungstic acid to the liposome suspension prior to placement on the grid caused precipitation of the vesicles.

Light Microscopy

Oysters used for fluorescence microscopy were those fed FITC-labeled polyacrylamide beads, liposomes containing FITC-labeled

bovine serum albumin, or untreated oysters. Animals were removed from the shell and fixed 48 hrs in Davidson's fixative. Tissues were dehydrated in ethanol and xylene and embedded in paraffin using an automated Tissue Tek II tissue processor, Sakura Finetechnical, Tokyo, Japan. Duplicate sections were cut at 6μm at 2-3 planes through the oyster. One set was cleared in xylene, stained with hematoxylin, and mounted with a cover slip. The other set was left as a paraffin section, and covered with a coverslip using a glycerol (pH 8.5) wet mount. Cover slips were sealed in place with fingernail polish.

Alternatively, oysters fed FITC-albumin-containing liposomes were embedded in polyethylene glycol (PEG) according to the method of Humason (1967). Details of the procedure may be found in Appendix II. This procedure avoids treatment with non-polar solvents. Blocks were kept desiccated until cut at 6μm. Sections were floated on a solution containing 400 mls distilled water, 100 ml diethylene glycol, 7 mls 37% formalin, and 1.0 ml PEG 200. Sections were picked up on slides and either stained with methylene blue or left unstained. Coverslips were mounted over glycerol (pH 8.5) and sealed in place with fingernail polish.

Fluorescence microscopy was performed using a Zeiss Universal microscope. Film employed was either Kodak Tri-X Pan 400 ASA/27 DIN (black and white) or Ektachrome 400 ASA/27 DIN (color). Normal light microscopy was performed on either the above microscope or a Zeiss Standard GFL microscope, using Kodak Ektachrome 64 (color) or Panatomic-X 32 (black and white) films.

IV. RESULTS AND DISCUSSION

Formation and Characterization of Liposomes

Liposomes produced by the solvent evaporation technique appeared to exhibit the characteristics of intact lipid vesicles in aqueous solution. Formation appeared unhindered by the presence of relatively high concentrations of divalent cations in the synthetic seawater employed in this study, particularly calcium and magnesium. It has been noted (Kimelberg and Mayhew, 1978) that liposomes consisting solely of negatively charged lipids are sensitive to divalent cations which may act to neutralize the negative charges on the polar head groups, resulting in instability and increased permeability of the lipid bilayer. Optical evidence indicates that this was not the case in the present study with liposomes composed of egg phosphatidylcholine cholesterol, and stearylamine. The latter lipid presumably confers a positive charge to the lipid bilayer, thus minimizing effects of divalent cations.

Figure 1a shows liposomes in aqueous media (synthetic seawater) visualized by phase contrast microscopy. A wide range of vesicle diameters were produced by the technique employed, and non-spherical shapes were often observed. The latter phenomenon could have been due to heating under the microscope beam. Figure 1b shows a similar preparation viewed by plane polarized light. Cross-shaped birefringent patterns typical of multilamellar spheres can be seen. The size range of vesicles measured from negative enlargements of birefringence patterns was 0.4μm to 8.0μm. Mean diameter was 1.9μm, with a standard

Figure 1. Optical characteristics of liposomes composed of egg phosphatidylcholine-cholesterol-stearylamine (7:1:2). (a) phase contrast, bar equals 10μm; (b) polarized light, bar equals 10μm.

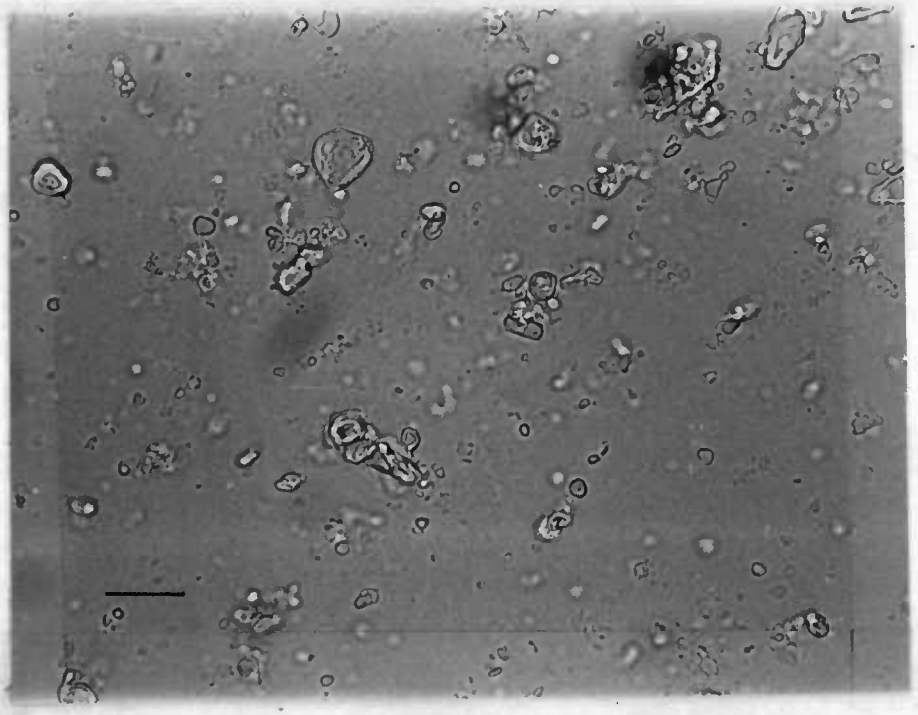

Figure 1a

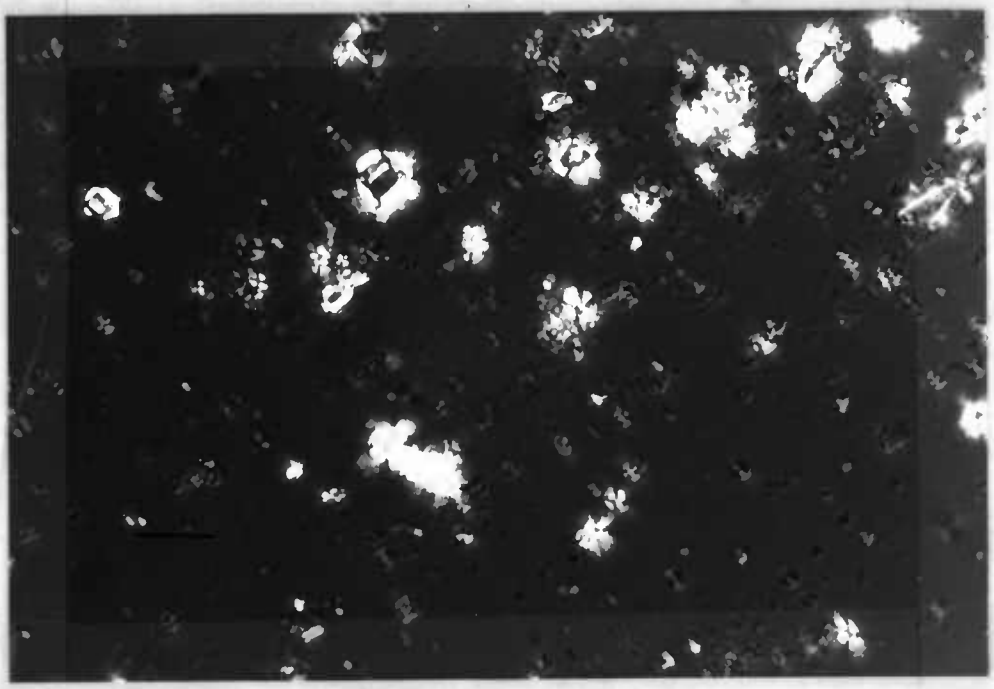

Figure 1b

deviation of 1.8μm.

Attempts to confirm the multilamellar structure by electron microscopy using negative staining techniques were not successful, due to precipitation of salts on the grid upon drying. However, the strong birefringence patterns observed by light microscopy indicate a probable multilamellar composition (Papahadjopoulos and Miller, 1967).

Formation and Uptake of Liposomes Containing ^{14}C-Cholesterol

Microscopic appearance of liposomes prepared with 4-^{14}C-cholesterol was identical to those shown in Figures 1a, b. This was to be expected since the molar amount of radioactive cholesterol added was negligible compared to non-radioactive cholesterol present in the lipid mixture. Cholesterol has a very limited solubility in water (< 0.2 mg/100 ml H_2O), and therefore would be expected to associate with the lipid phase in aqueous solution. This proved to be the case, since less than one percent of the radioactivity was lost to dialysis of the liposome preparation. Molecular weight cutoff for the dialysis tubing was 12,000, sufficiently low to prevent loss of small liposomes.

Feeding of ^{14}C-cholesterol liposomes to oysters for 72 hours resulted in the tissue distribution shown in Table 1. As expected, most of the radioactivity was recovered in the visceral mass, which included stomach, intestine, digestive diverticula, and associated connective tissue. In Experiment 1, oysters were allowed to depurate for 3 hrs, and in Experiment 2 for 14 hours. This increase in

Table I

Distribution of Label in Tissues of Oysters Fed [4-^{14}C] Cholesterol-Containing Liposomes

Experiment 1.

tissue	Oyster A, dpm	% Dose[1]	Oyster B, dpm	% Dose
visceral mass	40,000	10.00	28,300	7.10
adductor muscle	200	0.05	200	0.05
mantle	750	0.20	600	0.15
remainder	2,100	0.52	2,900	0.73

Experiment 2.

tissue	Oyster A, dpm	% Dose[1]	Oyster B, dpm	% Dose
visceral mass	46,300	5.91	36,300	4.65
adductor muscle	630	0.08	440	0.06
remainder[2]	5,000	0.64	3,700	0.47

[1]Calculated on basis of dose per oyster(Experiment 1, 0.1μCi; Experiment 2, 0.2μCi).

[2]Includes mantle tissue.

depuration time resulted in a decrease in recovered label, probably indicating clearance of lumenar radioactivity from stomach and intestine. However, considerable radioactivity remained after the longer depuration period, and probably reflects uptake of label into intracellular compartments by the digestive diverticula.

Tissues spatially removed from the digestive diverticula (adductor muscle) accumulated very small amounts of radioactivity. This is not surprising since in higher animals cholesterol carbon, in contrast with other lipids, does not enter the common biosynthetic pathways as a result of degradation of the parent molecule. Instead it could be expected to enter storage pools, probably in the lysosomal compartment of the digestive epithelium.

Small amounts of radioactivity were recovered in the mantle and remainder of the carcass, which consisted mostly of gill tissue. It is not known whether the radioactivity was intracellular, or adhering to the exterior and not able to be removed by rinsing.

Uptake and Metabolism of ^{14}C-Glucose and ^{14}C-Amino Acids Entrapped in Liposomes

Percent entrappment, calculated by loss to dialysis (since not all radioactivity could be recovered from the dialysis bag) was approximately the same (14%) for preparations of liposomes containing either [1-^{14}C]-glucose or [U-^{14}C] amino acids. This trapping percent is near that reported by Naoi *et al.*, (1977), who observed that liposomes containing stearylamine (positively charged) had an increased trapping

efficiency of amino acids. Diffusion of non-polar amino acids generally occurred more rapidly than polar amino acids (Naoi et al., 1977; Klein et al., 1971), thus the final intraliposomal amino acid composition is unknown.

The distribution of radioactivity in lipid, polar phase, and insoluble fraction in oysters fed liposomes containing [1-^{14}C]glucose is shown in Table II. At the earlier time period (24 hrs) oysters contained more radioactivity in the water-soluble portion than in the lipid extract. However, the latter still accumulated 2-3% of the dose per oyster. Radioactivity in the aqueous phase probably represents free glucose, bicarbonate (as a result of respiration), or soluble metabolic intermediates in various biosynthetic pathways. At 60 hrs, radioactivity in both lipid and aqueous fractions doubled, but the percent dose incorporated decreased. Material insoluble in the chloroform-methanol (2:1) solvent used in the lipid extraction was found to contain appreciable radioactivity. This fraction would contain primarily protein, but since glycogen is only slightly soluble in alcohol, at least some could be expected to remain in this fraction. It is clear, however, that free glucose would not be found in this fraction or in the lipid fraction. Thus, radioactivity in insoluble fractions would represent either incorporation into large carbohydrates (e.g. glycogen, mucopolysaccharide) or degradation and resynthesis of glucose carbon by other biosynthetic routes.

Table III shows the incorporation into protein of radioactivity from liposomes containing uniformly labeled amino acids. By 24 hrs considerable label had already accumulated in TCA-precipitable protein,

Table II

Distribution of Label in Oysters Fed Liposomes Containing [1-^{14}C] Glucose

Duration	Oyster	Wet wt(g)[3]	Lipid		Aqueous fraction		Insoluble	
			dpm	% Dose[1]	dpm	% Dose	dpm	% Dose
24 hr	1	0.22	460	2.3	1,175	5.9	ND[2]	
24 hr	2	0.37	500	2.5	2,421	12.1		
24 hr	3	0.49	580	2.9	2,824	14.2		
60 hr	1	0.43	1,060	1.0	2,650	2.5	17,900	17.2
60 hr	2	0.37	1,120	1.1	5,900	5.7	17,600	16.9
60 hr	3	0.40	1,440	1.4	11,650	11.2	24,300	23.4

[1]Calculated on basis of dose per oyster (24 hrs, .01μCi; 60 hrs, .05μCi).

[2]Not determined at 24 hr.

[3]Minus shell

Table III

Distribution of Label in Oysters Fed Liposomes Containing ^{14}C-Amino Acids

Duration	Oyster	Wet wt(g)[2]	Protein precipitate		Supernatant		Ratio
			dpm	% Dose[1]	dpm	% Dose	Protein : Supernatant
24 hr	1	0.25	1,880	6.6	710	2.5	2.6
24 hr	2	0.35	4,830	17.1	2,370	8.4	2.0
24 hr	3	0.41	7,850	27.7	3,100	11.0	2.5
60 hr	1	0.37	22,350	12.8	14,460	8.3	1.5
60 hr	2	0.55	26,000	14.9	14,400	8.2	1.8
60 hr	3	0.32	31,830	18.2	16,360	9.3	1.9

[1]Calculated on basis of dose per oyster (24 hrs, .013μCi; 60 hrs, .080μCi).

[2]Minus shell.

more so than in the corresponding supernatant fractions. Considerable variation was seen between individual oysters at the earlier time period, presumably due to variation in filtration rate or condition of the animals. However, the ratio of protein to supernatant radioactivity remained relatively constant. By 60 hrs, the protein fraction had accumulated 5-10 times the amount of radioactivity observed at 24 hrs, and the supernatant followed suit. Variability was less at 60 hrs, and the protein:supernatant ratio slightly lower. It is apparent that protein synthesis is taking place, even in partially starved oysters. In addition, data from Table II indicate that lipid synthesis and storage are not active in oysters in this state, at least from glucose carbon skeletons.

As positive controls, pairs of oysters were exposed to free [1-^{14}C] glucose and [U-^{14}C] amino acids in an identical fashion to oysters fed liposome-encapsulated label. Data showing uptake and incorporation of free ^{14}C-glucose is presented in Table IV. Percent uptake into lipid, aqueous, and insoluble fractions at both 24 and 60 hrs is remarkably similar to those for encapsulated glucose shown in Table II. This observation has two possible explanations. First, both free glucose and encapsulated glucose are internalized by the same mechanism, e.g. pinocytosis. This would imply that liposomes do not decrease the efficiency of absorption, and that internalized encapsulated glucose is released for incorporation into complex molecules in the same fashion as free glucose. Second, the data may indicate that liposomes are

Table IV

Distribution of Label in Oysters Exposed to [1-^{14}C] Glucose in Seawater

Duration	Oyster	Wet wt(g)[1]	Lipid		Aqueous fraction		Insoluble fraction	
			dpm	% Dose	dpm	% Dose	dpm	% Dose
24 hr	1	0.17	546	2.1	1,745	6.7	5,900	22.7
24 hr	2	0.16	580	2.2	3,030	11.7	11,400	43.9
60 hr	1	0.17	1,730	1.5	5,400	4.8	29,100	26.0
60 hr	2	0.18	2,660	2.4	3,670	3.3	34,970	31.2

[1]Minus shell.

[2]Calculated on basis of dose per oyster(24 hrs, .01μCi; 60 hrs, .05μCi).

either broken down or rendered permeable to glucose extracellularly in the stomach or lumen of digestive tubules resulting in the ultimate absorption of free glucose. There have been earlier reports of uptake of dissolved sugars from seawater by oysters (Yonge, 1928; Galtsoff, 1964; Owen, 1966). However, it has been unclear whether the uptake has been of free sugars or of bacteria which had incorporated the sugars prior to ingestion. Since the present study used autoclaved synthetic seawater with frequent changes, uptake was probably of the free carbohydrate. Galtsoff (1964) and Owen (1966) have proposed that pinocytosis of simple sugars may take place on the mantle.

Table V shows uptake and metabolism of ^{14}C-labeled amino acids dissolved in seawater. In comparison to uptake of liposome-encapsulated amino acids (Table III), a much higher percentage of the dose appeared in the protein fraction at 24 hrs, as reflected in the protein: supernatant ratio. A mortality occurred during the 60 hr trial, so only one value appears in Table V.

The greatly increased efficiency of uptake of free amino acids compared to encapsulated forms may reflect a stimulation of pumping and filtration rate by these organic compounds. It is interesting to note that species of fish have been shown to be extremely sensitive to low ($10^{-8}M$) concentrations of free amino acids, (Belghaung and Doving, 1977), and it is theorized that chemoreception of amino acids could be involved with specific behavioral responses (migration, alarm reaction). These authors also demonstrated that carbohydrates, including glucose, were less potent than α-amino acids. Further experimentation is needed to determine the effect of free amino acids on filtration rate in

Table V

Distribution of Label in Oysters Exposed to ^{14}C-Labeled Amino Acids in Seawater

Duration	Oyster	Wet wt(g)[1]	Protein precipitate		Supernatant		Ratio
			dpm	% Dose	dpm	% Dose	Protein : Supernatant
24 hr	1	0.18	17,050	48.7	4,670	13.3	3.6
24 hr	2	0.16	28,600	81.7	5,670	16.2	5.0
60 hr	1	0.31	68,850	39.3	12,170	6.9	5.6

[1]Minus shell.

[2]Calculated on basis of dose per oyster(24 hrs, .013μCi; 60 hrs, .080μCi).

bivalve molluscs. At any rate, differences in uptake between oysters fed free and encapsulated amino acids indicates that either 1) different mechanisms of uptake are operating, or 2) stimulation of uptake by free amino acids was masked by encapsulation in a lipid bilayer, though presumably free amino groups (from stearylamine) are exposed on the exterior of the liposomes.

Uptake and Metabolism of Liposomes Labeled with ^{14}C-Phosphatidylcholine

Analysis of purity of purchased di[1-^{14}C] palmitoyl phosphatidylcholine (DPPC) showed 100% recovery in the thin layer chromatography spot corresponding to standard phosphatidylcholine. Dialysis of liposomes prepared with this label showed that 99% of the initial radioactivity was associated with the particulate lipid phase.

Distribution of radioactivity in lipid, aqueous, and insoluble fractions of oysters fed ^{14}C-DPPC liposomes for 24 and 80 hrs is shown in Table VI. With the exception of one animal at each time, the magnitude of total uptake was similar to that observed for ^{14}C-cholesterol liposomes (Table I). This is to be expected since in both cases the radioactive label resides in the vesicle wall.

Distribution of label between the various biochemical compartments was quite different from that of glucose or amino acids. Whereas label from the latter markers was incorporated primarily into the insoluble phase, label from ^{14}C-DPPC appeared predominantly in the lipid phase. No increase in label in either aqueous or insoluble fractions was

Table VI

Distribution of Label in Oysters Fed di-[1-^{14}C]Palmitoyl Phosphatidylcholine-Containing Liposomes

Duration	Oyster	Wet wt[1] (gm)	Lipid		Aqueous		Insoluble		Ratio[3]
			dpm	% Dose[2]	dpm	% Dose	dpm	% Dose	
24 hr	1	.19	116,000	10.0	3,250	0.3	7,170	0.6	91.8:2.6:5.7
24 hr	2	.28	180,000	15.5	12,400	1.1	21,880	1.9	84.0:5.8:10.2
24 hr	3	.33	380,000	32.8	23,750	2.0	49,700	4.3	83.0:5.2:10.9
80 hr	1	.35	208,300	4.2	6,250	0.1	22,940	0.5	90.3:2.7:9.9
80 hr	2	.23	317,000	6.3	9,750	0.2	40,530	0.8	86.3:2.7:11.0
80 hr	3	.37	822,100	16.4	28,500	0.6	128,900	2.6	83.9:2.9:13.2

[1]Tissue wet weight, minus shell.

[2]Calculated on basis of dose per oyster; 0.5μCi/oyster at 24 hrs. and 2.25 μCi/oyster at 80 hrs.

[3]Ratio; dpm. lipid: aqueous: insoluble

observed at the later time period, indicating that only a small portion of the labeled palmitate underwent catabolism and subsequent resynthesis into non-lipid compounds. The ratio between compartments was constant.

An important question concerning any method of capsular feeding is the degree to which the capsular material is degraded in the subject animals. In the case of the liposomes used in the present study, egg phosphatidylcholine was the major component, and was thus examined as to evidence of its metabolism. It is probable that even partial breakdown of the lipid bilayer would result in release of entrapped materials. Digestion of capsular material would also result in an increased caloric value of the food, since both capsular material and encapsulated nutrients could be utilized.

Figure 2 shows the distribution of radioactivity in phospholipids of oysters fed ^{14}C-DPPC-liposomes for 24 or 80 hours. It can be seen in all individuals that radioactivity appears in phosphatidylcholine (Rf approx. 0.2), an unknown lipid (Rf approx. 0.3), and phosphatidyl-ethanolamine (Rf approx 0.5). In addition, label also appears in neutral lipids (includes triglyceride, di-and monoglycerides, sterols, and steryl esters: Rf approx. 0.9.). While the majority of the label was retained in PC, considerable amounts were observed in the other three lipids, which together with PC were the only major spots visualized by iodine vapor on the TLC plate. There were no striking differences in the distribution patterns between 24 and 80 hours. A slight trend toward increased label in the unknown lipid (Rf $\sim$ 0.3) was evident. The nature of this lipid is being investigated. Specific TLC sprays indicate the absence of choline or free amino groups.

Figure 2. Distribution of radioactivity in total lipid extract of oyster fed ^{14}C-DPPC-containing liposomes for 24 or 80 hours, separated by thin layer chromatography. A, B, and C represent individual oysters. O, origin; F, solvent front; PC, phosphatidylcholine; X, unidentified lipid; PE, phosphatidylethanolamine; NL, neutral lipids. 25,000 dpm spotted per plate.

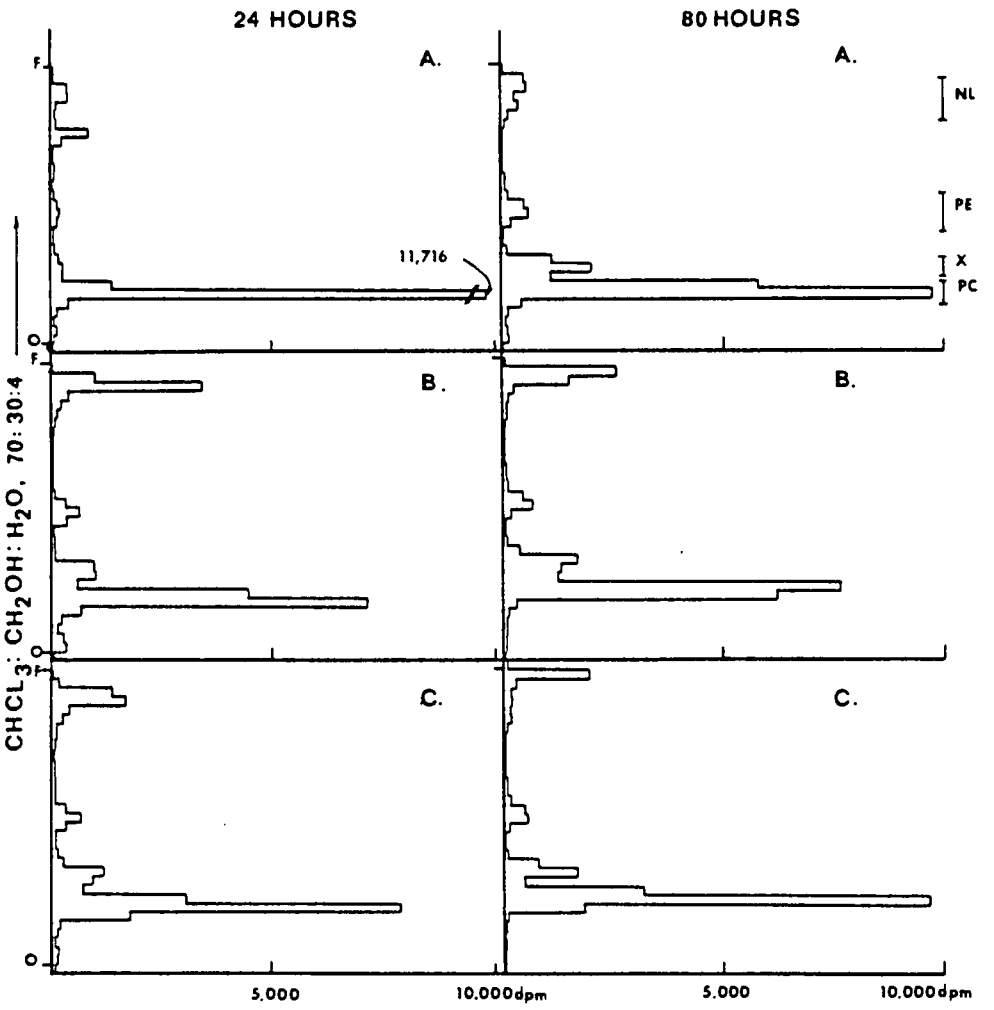
24 HOURS
80 HOURS
A.
B.
C.
11,716
NL
PE
X
PC
$CHCL_3$: CH_2OH: H_2O, 70:30:4
5,000
10,000dpm
5,000
10,000dpm

Figure 2

Figure 3 shows the distribution of label in phospholipids of an oyster allowed to depurate in clean synthetic seawater for seven days. A mortality during the depuration period resulted in only one oyster available for analysis at the seven day time period. Most radioactivity still resides in PC. Less activity was observed in neutral lipids, and little change occurred in PE, but a large increase was seen in the unidentified lipid at Rf 0.3. Whether this increase was due to an increase in specific activity or mass is not known. The decrease in radioactivity in neutral lipids may have been due to depletion of triglycerides during the depuration period.

Confirmation that the radioactivity observed in the neutral lipid fraction resides exclusively in triglyceride is shown in Figure 4. In the solvent system employed, polar lipids (including phospholipids) remain at the origin. Location of standards of common components of neutral lipids (mono-,di-, and triglycerides, cholesterol, free fatty acid, and cholesteryl esters are pointed out in the figure. It is apparent that the triglycerides are the only neutral lipids which are labeled in either the 24 or 80 hr feeding trials. The lack of radioactivity in mono- and diglycerides, free fatty acid, or lysophosophatidylcholine (having one acyl group removed) could be meaningful. It could reflect a very low level of phospholipase or non-specific esterase activity in the lumen of the stomach or digestive diverticula, since the action of these enzymes is to produce free fatty acids.

To provide information about the routes by which radioactivity from ^{14}C-DPPC is incorporated into other lipids, samples of PC, PE, triglyceride, and the unidentified lipid were examined for intramolecular

Figure 3. Distribution of radioactivity in total lipid extract of oyster fed ^{14}C-DPPC-containing liposomes for 80 hours and allowed to depurate for seven days. Abbreviations as in Figure 2.

Figure 3

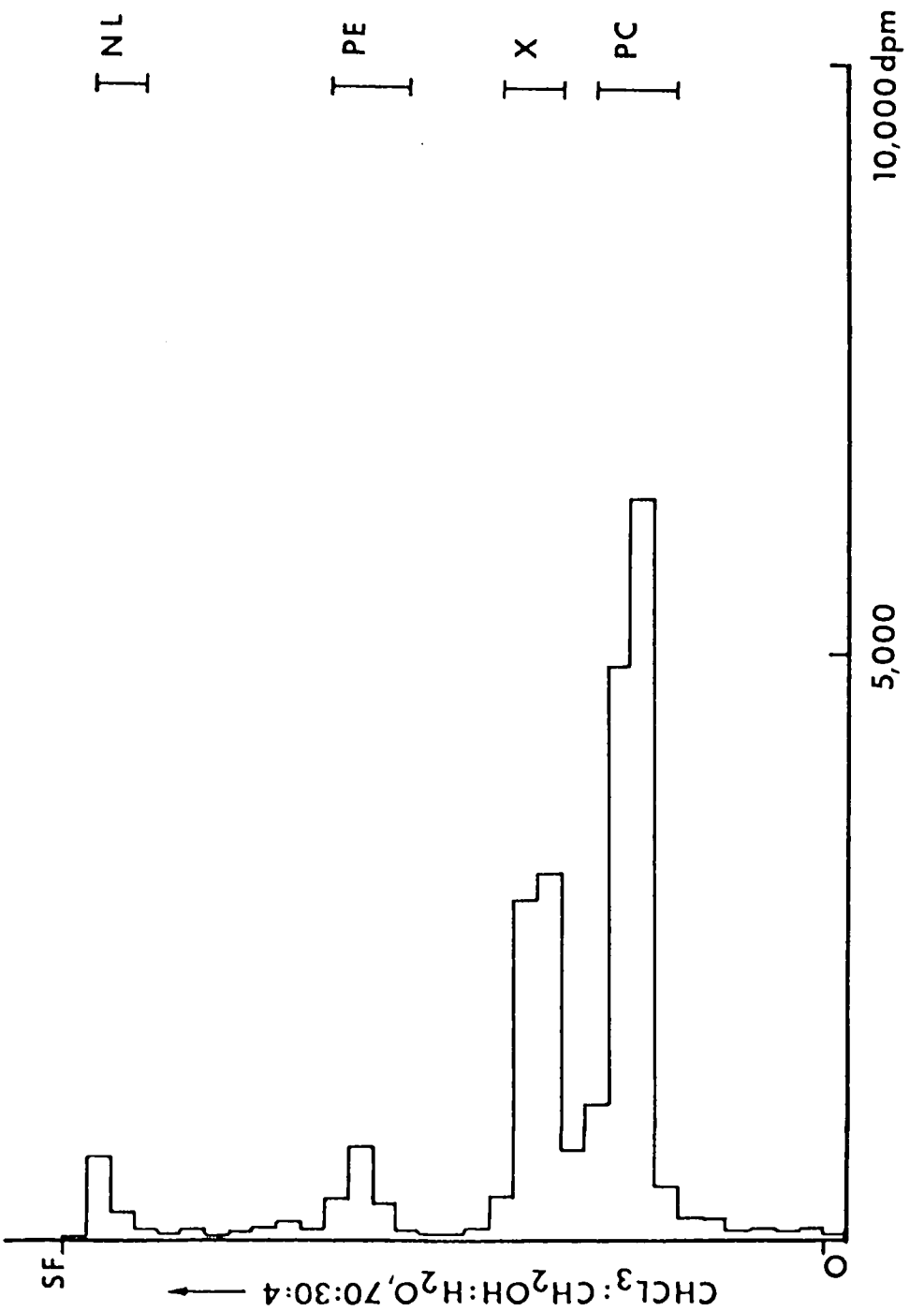

Figure 4. Distribution of radioactivity in neutral lipids of oysters fed ^{14}C-DPPC-containing liposomes for 24 hours (A), or 80 hours (B). PL, polar lipid; TG, triglyceride. Also marked are positions of TLC standards of cholesteryl esters (CE), free fatty acids (FA), cholesterol (C), and diglyceride (DG).

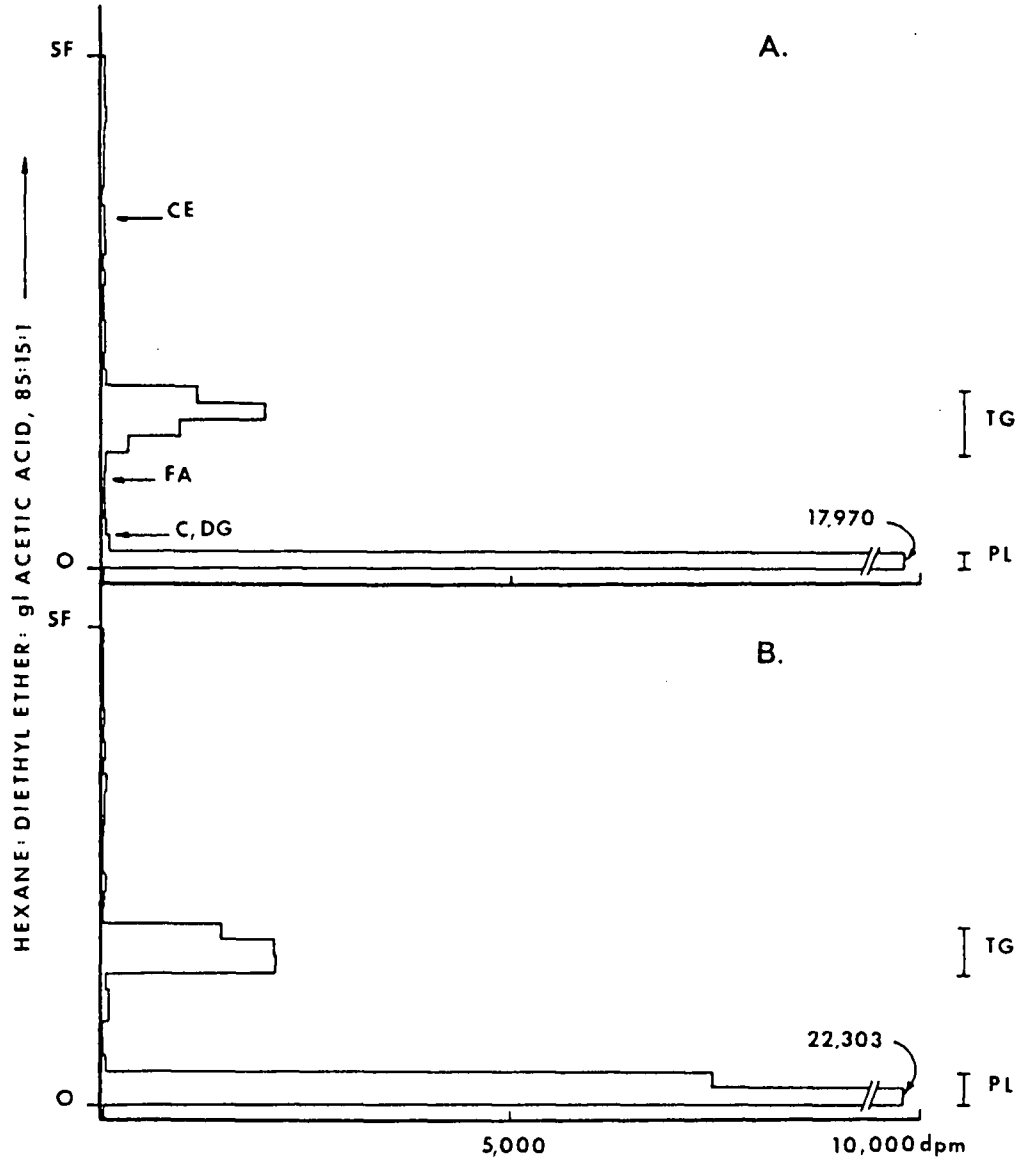
SF
A.
CE
TG
FA
C, DG
17,970
PL
O
SF
B.
TG
22,303
PL
O
HEXANE: DIETHYL ETHER: gl ACETIC ACID, 85:15:1
5,000
10,000 dpm

Figure 4

location of label. After transesterification and extraction, 100% of the label was recovered in the methyl ester (hexane) fraction. The lack of label in the triglyceride and phospholipid backbones indicates that the major process at work is transacylation of fatty acids from DPPC to endogenous lipids.

Microscopic Evidence of Uptake of Liposomes Containing a Fluorescent Protein

In an effort to further clarify the fate of ingested liposomes, a protein (bovine serum albumin (BSA), MW 60,000) covalently linked to a fluorescent ligand (fluorescein isothiocyanate, FITC) was encapsulated and fed to oysters. The location of ingested fluorescence was then observed in tissue sections with fluorescence microscopy.

Preparations of FITC-BSA-containing liposomes resembled those of liposomes made in the absence of protein. Figure 5a shows such a preparation viewed under fluorescence, and Figure 5b is the same field viewed with polarized light. Most fluorescence appears to be intravesicular, but some liposomes are fringed with a brighter fluorescence, perhaps indicating some absorption of protein to the outside of the liposome. Exterior absorption should be reduced due to the relatively high ionic strength of the aqueous medium (synthetic seawater), as observed by Sweet and Zull (1970).

Characteristic FITC fluorescence (yellow green) was observed in tissue sections of whole animals fed liposomes containing FITC-BSA for 72 hours. Figure 6a is a fluorescence micrograph of the lumen and epithelial lining of the posterior stomach. The lumen contained

Figure 5. Optical characteristics of liposomes containing FITC-derivitized bovine serum albumin. (a) fluorescence, bar equals 10μm; (b) polarized light, bar equals 10μm.

Figure 5a

Figure 5b

considerable fluorescence in discreet particles and masses, which probably represented liposomes carried in mucus. No fluorescence was observed in the stomach epithelium. The only portion of the alimentary canal to contain intracellular FITC fluorescence was the ducts and tubules of the digestive diverticula. Figure 6b shows ducts and tubules from an untreated section. Note the lack of fluorescence. In contrast, many ducts and tubules from oysters fed FITC-BSA in liposomes exhibited discreet patches of FITC fluorescence in the apical third of the digestive epithelium (Figure 6c, d). Fluorescent areas were of variable diameter, most were small, and no large vesicles such as appear in the initial preparation (Figure 5a) were observed intracellularly. Fluorescence was not observed in the basal portions of the cells, and only rarely in the crypt areas of the tubules (see Figure 6c). (Mathers, 1972, also observed a lack of uptake of ^{14}C-algae in the crypt cells of the ducts, but label moved basally with time.) On the other hand, ducts (Figure 6d) appeared to contain fluorescent particles in a more general distribution around the perifery of the lumen.

Polyethylene glycol-embedded sections from oysters fed the same preparation of FITC-BSA liposomes (Figures 7a,b) showed a similar fluorescence pattern as the paraffin sections discussed above. Treatmemt of the paraffin-embedded oysters with xylene prior to embedding apparently had no effect on intra- or extra-cellular fluorescence.

Most of the intracellular fluorescence seen in FITC-BSA liposome-treated oysters appeared as solid areas of fluorescence similar to those in the original preparation. These may represent phagocytosed intact liposomes. Other particles appear as halos (e.g. Figure 7b) which may

Figure 6. Paraffin-embedded sections from oysters fed (FITC-BSA)-containing liposomes viewed with fluorescence microscopy. (a) stomach, bar equals 10μm; (b) tubules, untreated oyster, bar equals 40μm; (c) ducts and tubules from (FITC-BSA) liposome-fed oyster, bar equals 10μm; (d) higher magnification of ducts in treated oyster, bar equals 5μm.

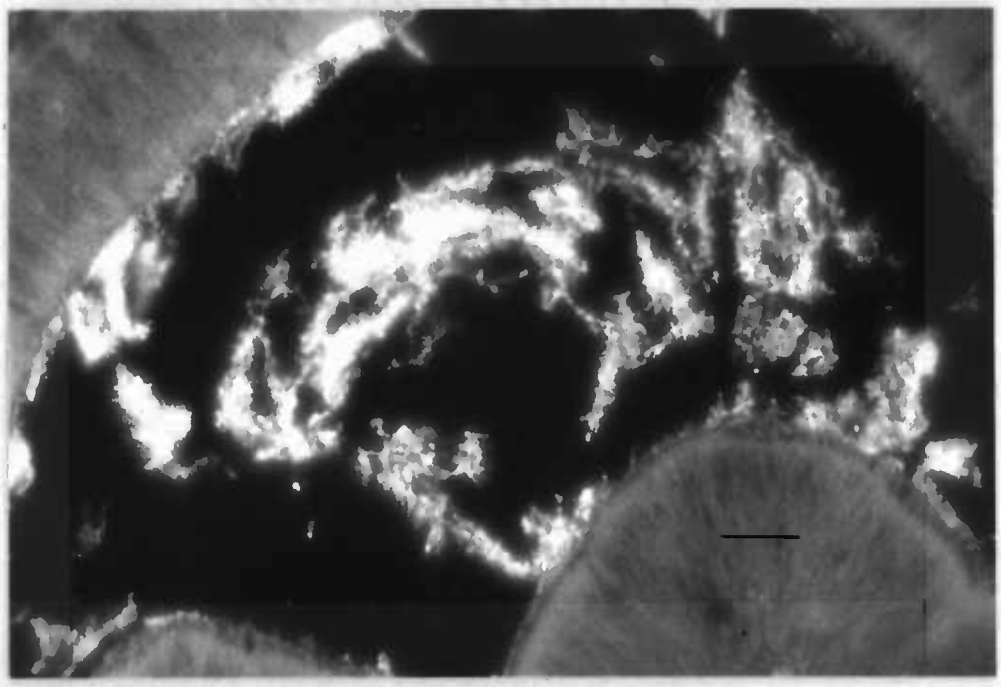

Figure 6a

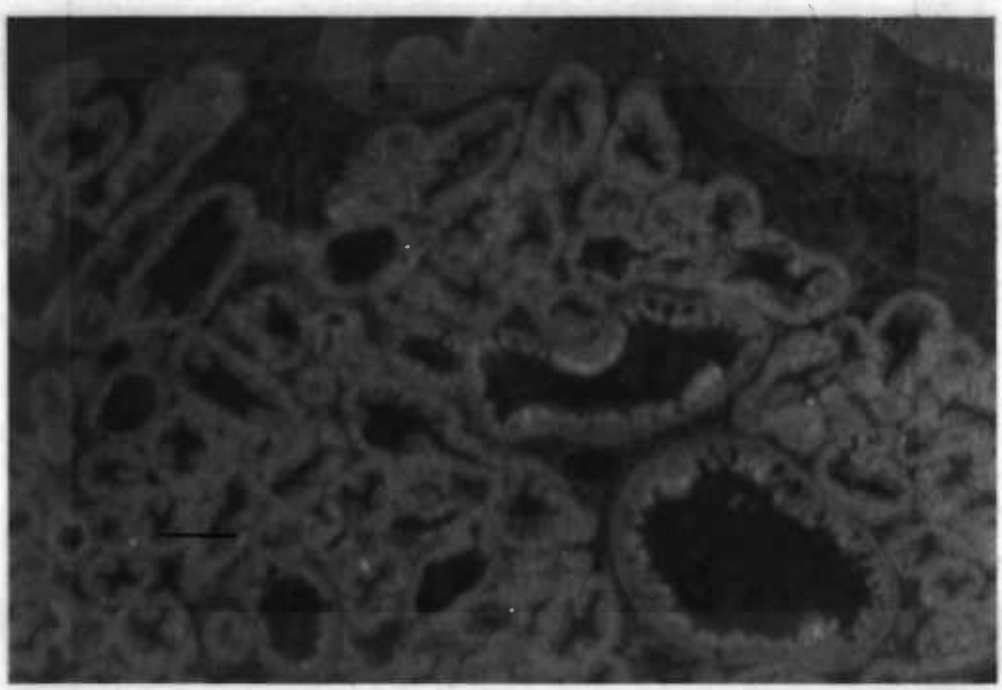

Figure 6b

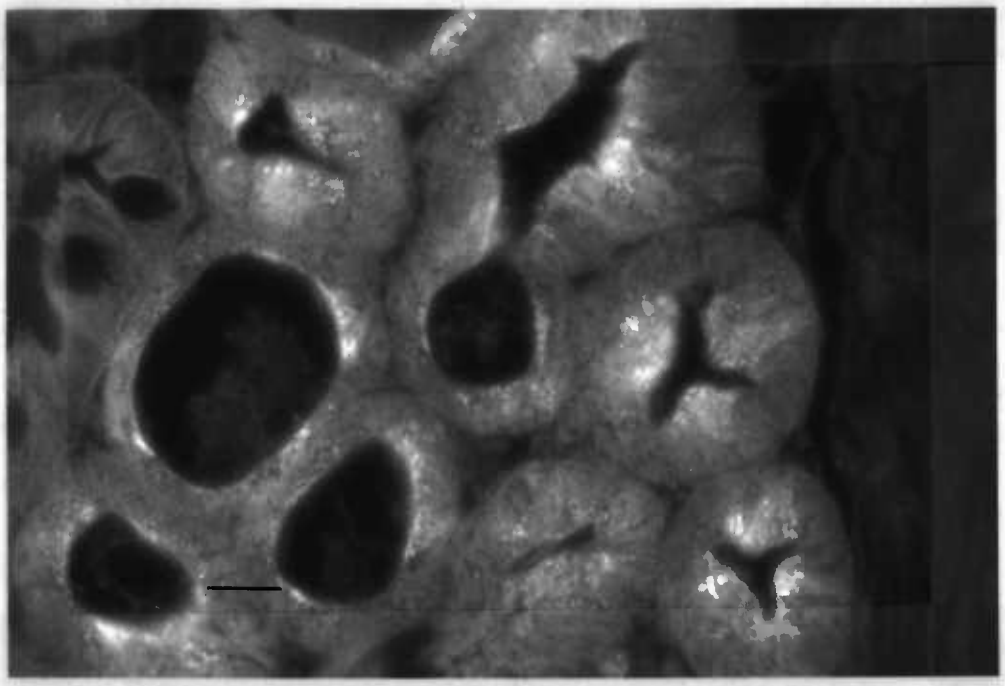

Figure 6c

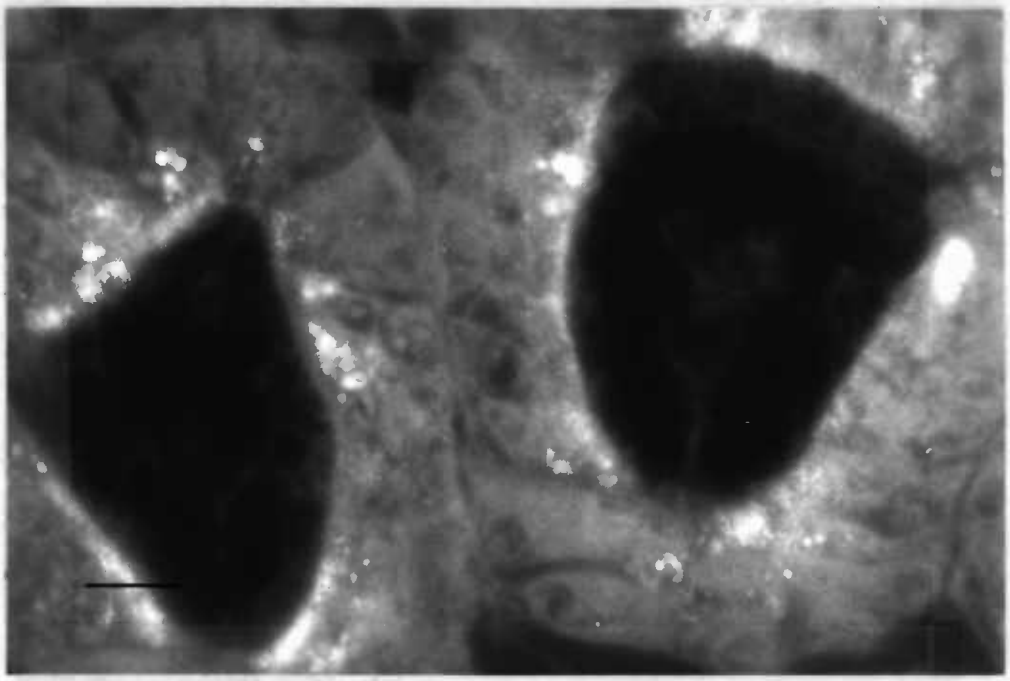

Figure 6d

Figure 7. Polyethylene glycol-embedded sections of digestive tubules from oysters fed (FITC-BSA)-containing liposomes viewed with fluorescence microscopy. (a) Bar equals 10μm; (b) bar equals 5μm. Arrows indicate fluorescent inclusions.

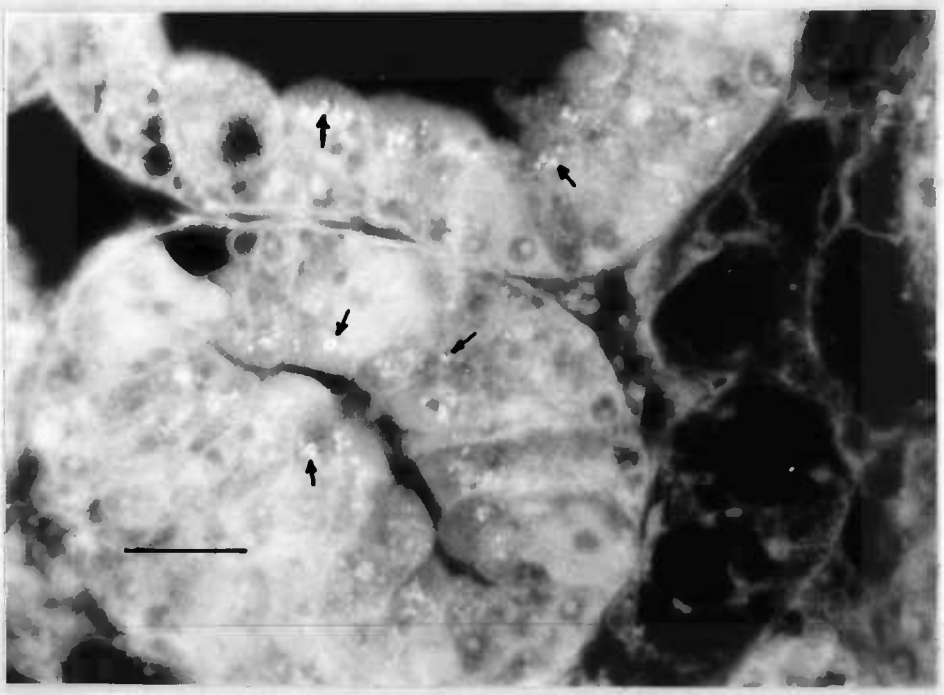

Figure 7a

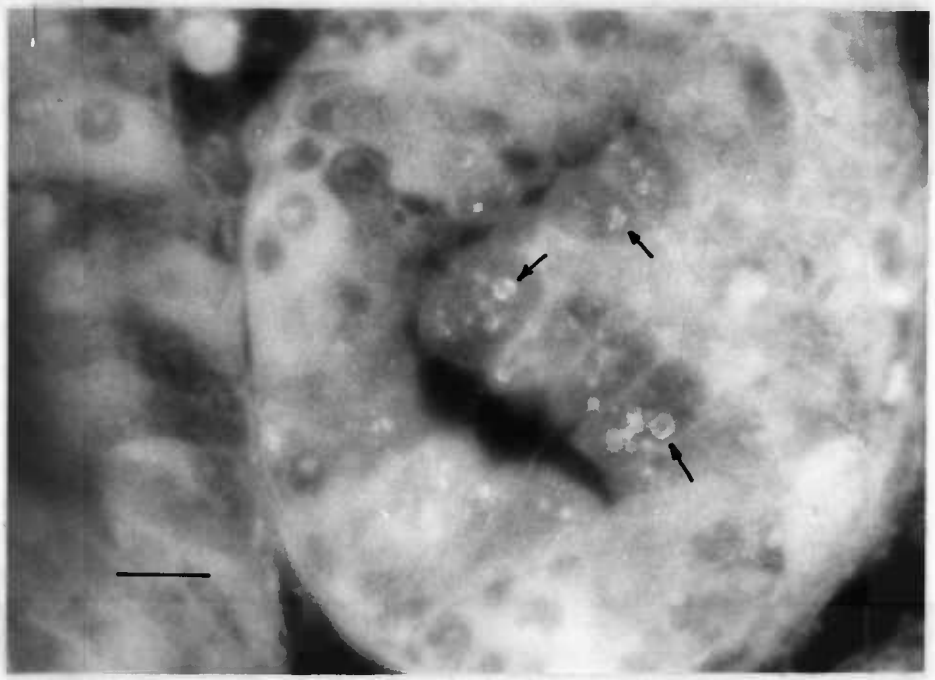

Figure 7b

be the result of liposomal degradation and subsequent incorporation of stearylamine-FITC into the limiting membranes of phagocytic vesicles or other organelles, the former of which are very common in the apical portions of the cells (refer to Appendix I for electron micrographs).

Not all ducts and tubules of the digestive diverticula contain fluorescent vesicles, rather those containing fluorescence tended to be aggregated. This may indicate the presence of ciliary currents in the branched caecal ducts (leaving the stomach) which route incoming food particles into some parts of the digestive gland before others. It also suggests that the observed intracellular fluorescence is not artifactual.

Feeding Trial with Liposomes Labeled with FITC

Derivatization of liposomes with fluoresein isothiocyanate (FITC) appeared successful. The final preparation consisted of highly fluorescent vesicles (Figure 8a) which exhibited typical birefringence patterns (Figure 8b). No change in the optical characteristics occurred over time, and the FITC appeared bound as no leakage or solubilization of fluorescence was observed over several days of storage. The probable reaction between stearylamine and FITC is shown in Figure 9 below.

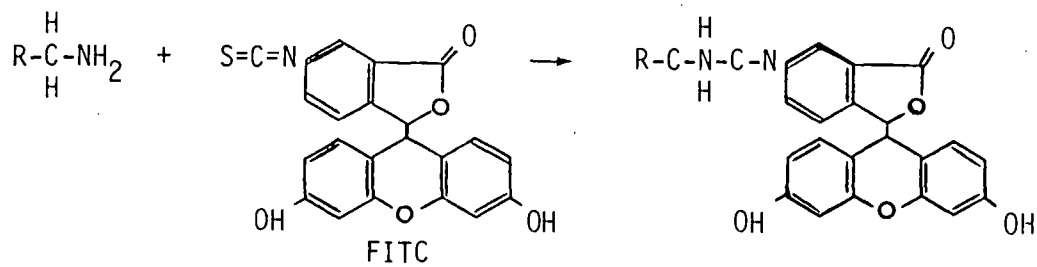

Figure 9. Reaction of stearylamine with FITC.

Figure 8. Optical characteristics of liposomes derivatized with FITC. (a) fluorescence, bar equals 10μm; (b) polarized light, bar equals 10μm.

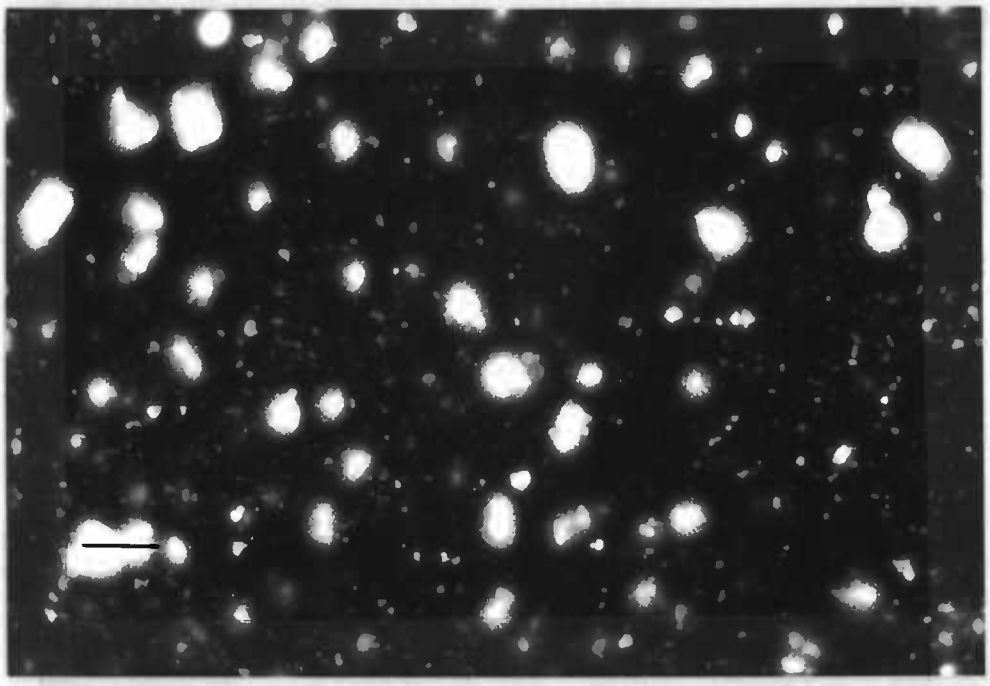

Figure 8a

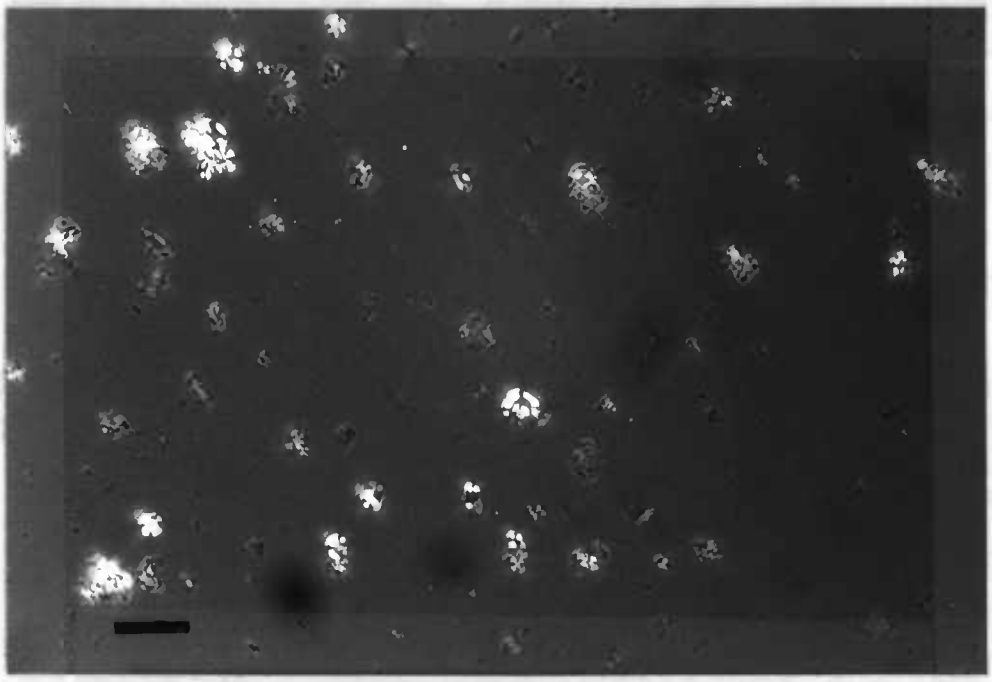

Figure 8b

Derivatized stearylamine molecules would presumably occur on the interior bilayers of multilamellar liposomes as well as the outside, since FITC was present in the aqueous medium in which the vesicles were formed.

FITC-liposomes exhibited greater fluorescence than did FITC-BSA liposomes discussed previously, but were generally the same size. Feeding trials of 26 and 75 hr duration resulted in a much different distribution of fluorescence in the digestive tract compared to FITC-BSA liposomes. Instead of discreet patches of fluorescence, there appeared generalized fluorescence throughout much of the digestive diverticula (Figure 10a). Caecal ducts tended to contain less fluorescence, especially toward the apical end of the cells (Figure 10b). The degree of fluorescence increased from 26 to 75 hours.

A possible explanation for the above observations is that FITC was cleaved from the liposomes extracellularly and was then taken up in a generalized fashion in many parts of the digestive tract. Movement of fluorescence to other organs (e.g. adductor muscle or gonads) did not occur, possibly due to intracellular binding of FITC to protein. Uptake of discreet liposomes may have taken place in the tubules, but the evidence was masked by the high generalized intracellular fluorescence.

Uptake of Polyacrylamide Beads

In an effort to learn more about the basis of discrimination between food and non-food particles by oysters, undigestible polyacrylamide beads ($2 \pm 1\mu m$) were fed for 72 hrs. Half the beads were derivitized with FITC on aminoethyl side chains, the other half unde-

Figure 10. Tissue sections of oysters fed FITC-derivatized liposomes, viewed with fluorescence microscopy. (a) digestive diverticula, 75 hr feeding trial. Note lack of fluorescence in caecal duct, CD, and bright fluorescence in ducts and tubules at left. Bar equals 40μm. (b) stomach epithelium. Note bright fluorescent fringe in apical portion of cells (arrow). Bar equals 10μm.

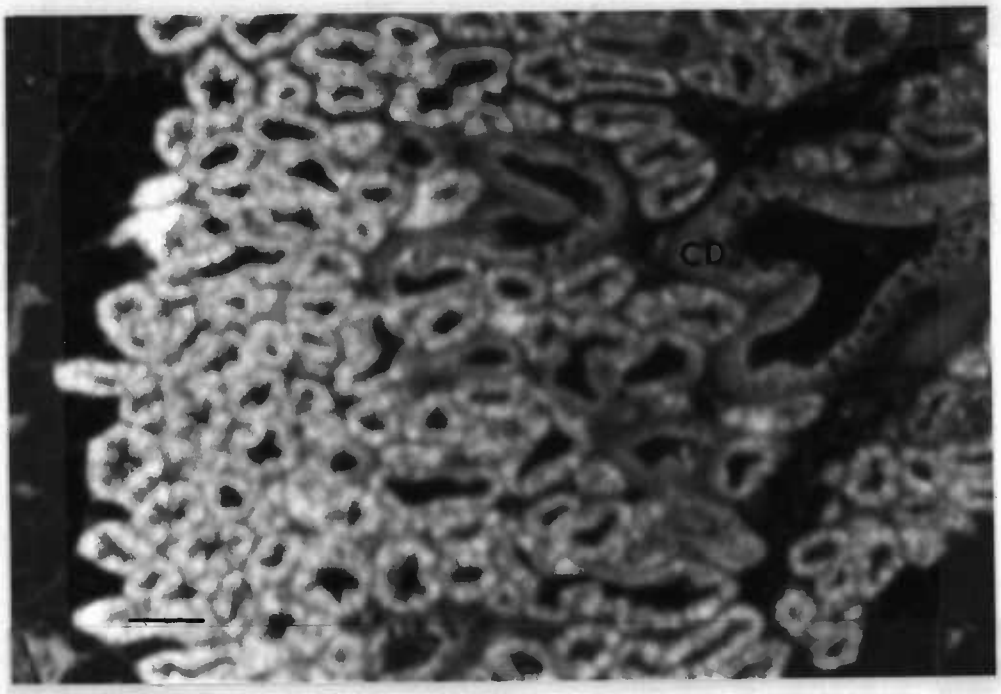

Figure 10a

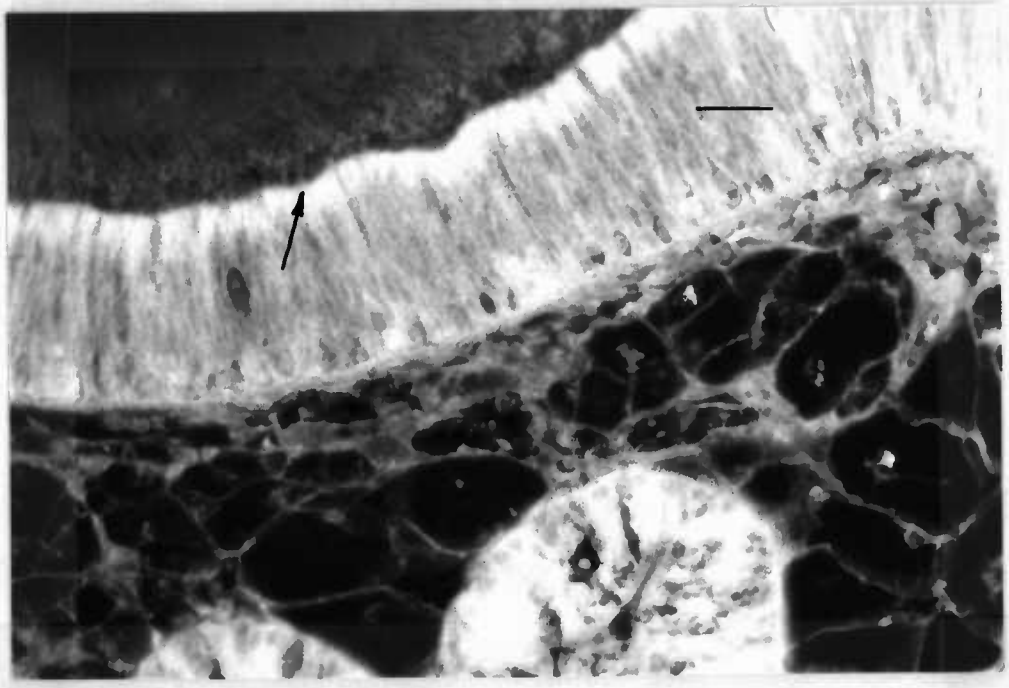

Figure 10b

rivatized, with free amine groups. Suspensions of hematoxylin-stained beads and FITC-beads under fluorescence are shown in Figures 11a and 11b, respectively.

Results of feeding trials showed no difference in the distribution of derivatized and underivatized beads in the digestive tract. Both were present in the stomach (Figure 12a) and intestine (Figures 12b, c), but were not observed in the ducts or tubules of the digestive diverticula. Despite the presence of free amine groups (underivitized beads) and an organic matrix, the beads were apparently identified in the stomach as non-food particles and shunted into the intestine for expulsion. Possible reasons for rejection may have been: 1) texture, or 2) lack of recognizable chemical characteristics (e.g. amino acids, lipids). Since it is evident that the beads were ingested in large amounts, the potential exists for the use of this type of particle to study internal selection. One possibility is to derivitize the beads with various organic substances (proteins, carbohydrates, nucleotides) to investigate possible recognition cues.

Little background fluorescence appeared as the result of feeding FITC-beads, indicating no breakdown of the thiourea linkage in the lumen of the stomach or intestine. This does not necessarily imply that the generalized fluorescence observed with FITC-liposomes was not due to extracellular hydrolysis of FITC since the beads may cause stearic hindrance of hydrolytic enzymes.

Autoradiography

Autoradiography studies using oysters fed liposomes made with

Figure 11. Optical characteristics of polyacrylamide beads. (a) beads derivatized with FITC and viewed with fluorescence microscopy. Bar equals 10μm. (b) beads stained with hematoxylin. Bar equals 10μm.

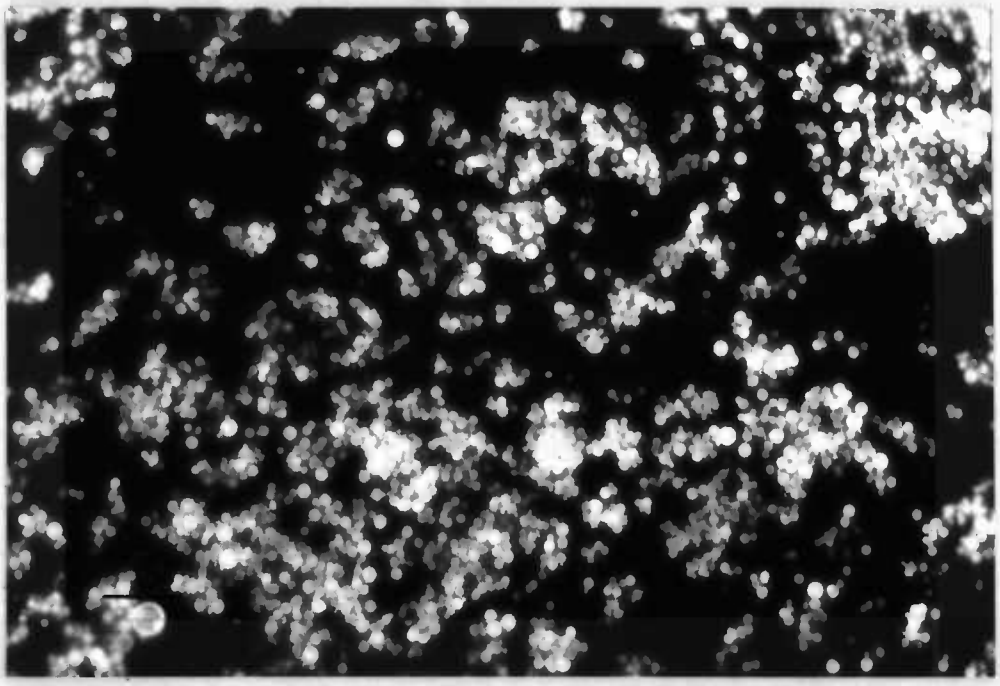

Figure 11a

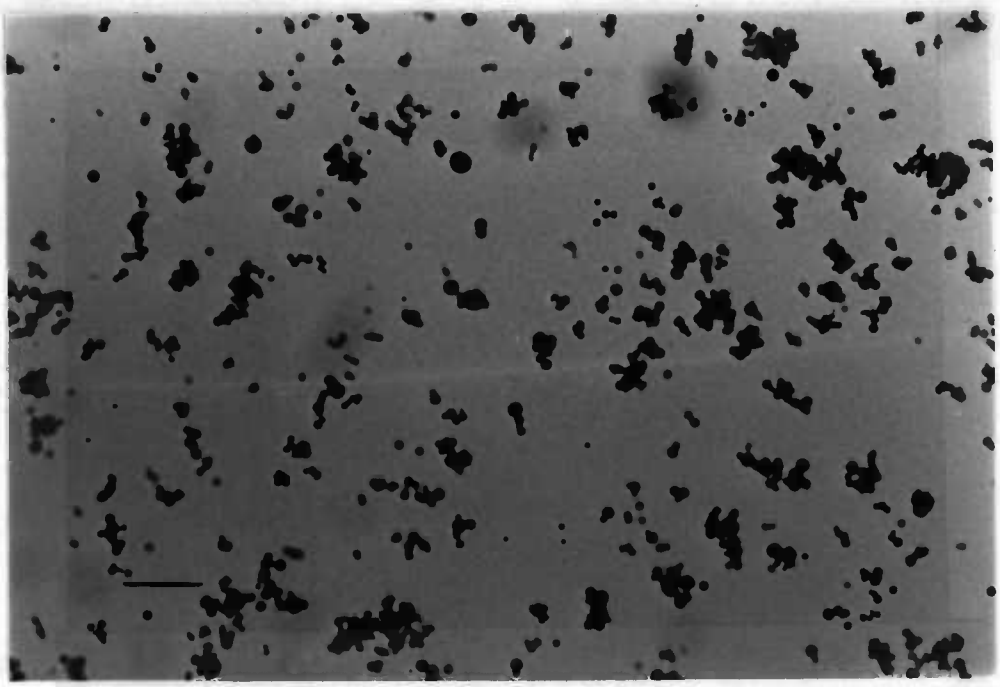

Figure 11b

Figure 12. Tissue sections of oysters fed polyacrylamide beads. (a) FITC-derivatized beads in stomach, bar equals 40μm; (b) FITC-beads in intestine, bar equals 10μm; (c) beads in intestine (cross section), stained with hematoxylin. Bar equals 20μm.

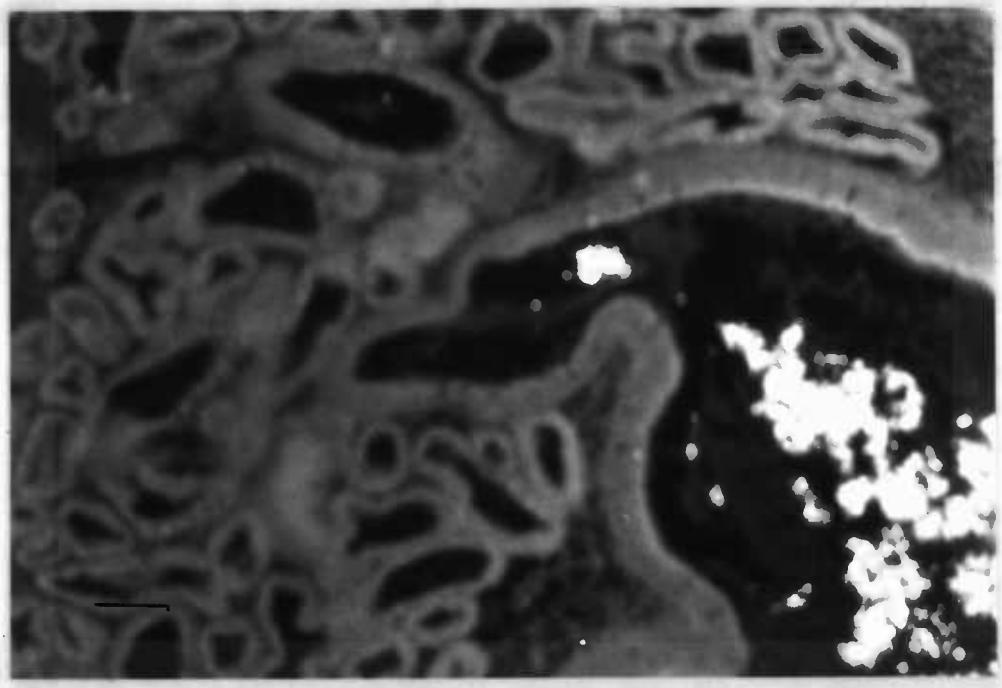

Figure 12a

Figure 12b

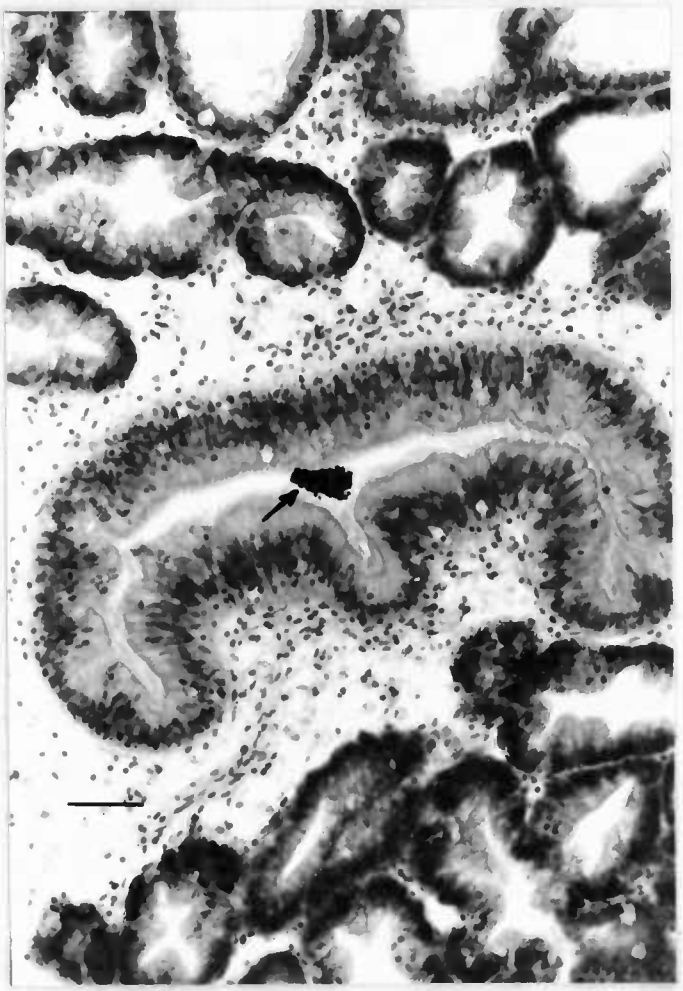

Figure 12c

di[1-^{14}C]palmitoyl phosphatidylcholine showed radioactivity dispersed throughout the epithelial cells of the ducts and tubules of the digestive diverticula (Figure 13). Slight amounts of radioactivity were also observed in the lining of the intestine, but only very little in the lumen of the tubules, ducts, or stomach. Some label appeared in the intertubular connective tissue, particularly at 80 hours. There were no striking differences in the distribution of radioactivity between 24 and 80 hours. This distribution of label from phosphatidylcholine resembled the distribution of fluorescence after feeding FITC-tagged liposomes, as would be expected since both are components of the liposome walls. Absence of both label and fluorescence in the lumen of the various organs indicates that liposomes were taken up intact, broken down intracellularly, and the constituents redistributed throughout the epithelial cells with little dispersal into other tissues.

Figure 13. Similar sections through digestive diverticula of oysters fed 80 hrs with liposomes made with di[1-^{14}C]palmitoyl phosphatidylcholine. (a) section stained with hematoxylin; bar equals 20μm. (b) Autoradiogram of similar section to (a), but left unstained to show silver grains resulting from exposure to ^{14}C. Bar equals 20μm.

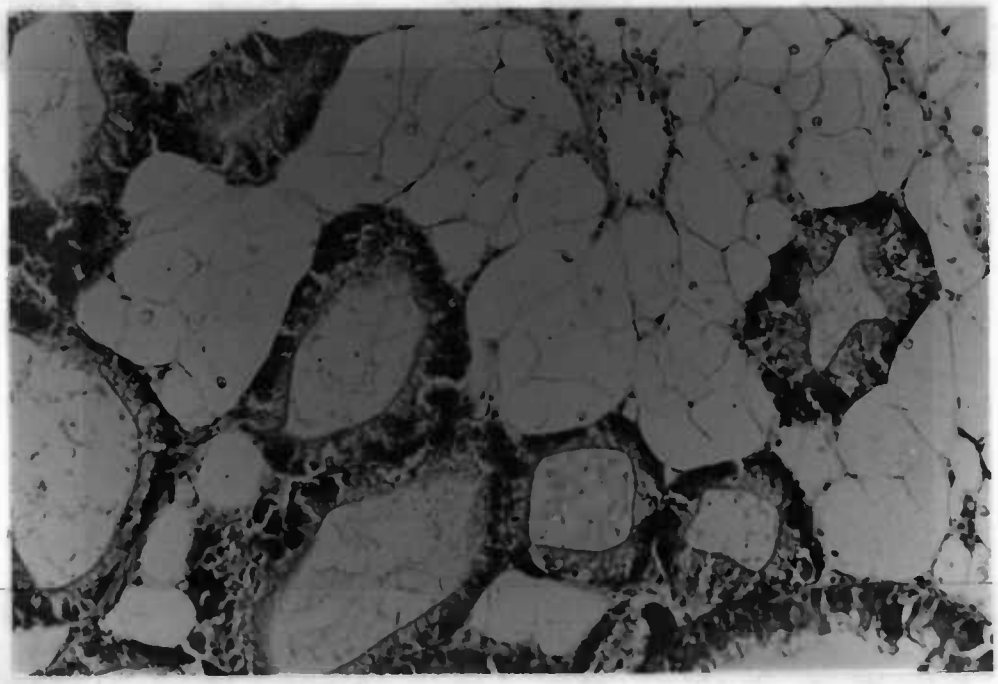

Figure 13a.

Figure 13b.

V. SUMMARY AND CONCLUSIONS

Several analytical methods were utilized to study the ingestion and digestion of liposomes and liposome-encapsulated substances in juvenile Pacific oysters (Crassostrea gigas). Liposomes composed of egg phosphatidylcholine-cholesterol-sterylamine (7:1:2) formed readily and appeared stable in 2% synthetic sea salts.

Radiotracer studies with labels positioned in the bilayer walls of the liposomes showed uptake and retention of up to 40% of the dose in 24 hours. The absence of label in free fatty acids in oysters fed liposomes containing di[1-^{14}C] palmitoyl phosphatidylcholine indicates a lack of significant amounts of cleavage of fatty acids from phospholipids in the stomach or lumen of the digestive diverticula in these animals. Metabolism of this tracer did occur, however, as radioactivity was found in fatty acids of triglycerides and phospholipids other than phosphatidylcholine. Intramolecular distribution of radioactivity in these lipids indicated that metabolism of ^{14}C-phosphatidylcholine was the major constituent of the liposomes, it is likely that such acyl hydrolysis would result in sufficient destabilization to release substances entrapped in the interior aqueous compartments.

To obtain evidence of the above hypothesis, small organic molecules (glucose, amino acids) were encapsulated in liposomes, fed to oysters, and examined for incorporation into more complex molecules. Such evidence was obtained for both ^{14}C-glucose, most label of which appeared in a chloroform-methanol-insoluble fraction, and ^{14}C-amino

acids, from which most label was recovered in a trichloroacetic acid-precipitable protein fraction. Oysters exposed to labeled glucose or amino acids in seawater readily incorporated radioactivity into these same fractions. Uptake of free amino acids exceeded that of encapsulated amino acids, possibly as a result of chemoreceptory processes.

Additional evidence of intracellular uptake of liposomes was obtained with fluorescence microscopy after feeding liposomes containing fluorescein isothiocyanate-labeled bovine serum albumin. The appearance of discreet patches of fluorescence within cells of tubules and ducts of the digestive diverticula suggest phagocytosis of liposomes. Liposomes which themselves had been derivatized with FITC resulted in vivid, generalized fluorescence in digestive diverticula and stomach epithelium, but not in the lumen of either of these organs. Autoradiography studies with liposomes containing di[1-^{14}C]palmitoyl phosphatidylcholine showed radioactivity dispersed throughout the epithelial cells of the ducts and tubules of the digestive diverticula. Only slight radioactivity was observed in the intertubular connective tissue or the lumen of the tubules or stomach. This distribution of liposomal materials resembled that of fluorescence from feeding trials with FITC-tagged liposomes, and indicated uptake of intact liposomes followed by intracellular breakdown and dispersal of the liposomal components.

Little is known of how oysters and other bivalve molluscs discriminate food from non-food particles, other than on the basis of size. To investigate this process, polyacrylamide beads ($2 \pm 1\mu m$) with aminoethyl side groups, and similar beads derivatized on the side group

with FITC were fed to oysters. Large quantities of both types of beads were seen in the stomach and intestine, but not in the digestive diverticula. Recognition of beads as non-food particles apparently took place in the stomach, despite the organic nature and free amine groups of beads. However, the ingestion of such particles of proper diameter and with derivatizable groups suggests their use for the study of the basis for acceptance-rejection processes in the stomach. Beads conjugated with proteins, lipids, and nucleotides could be used for this prupose.

The ingestion, intracellular uptake, and breakdown of liposomes shown in this study indicates a use for these particles for feeding oysters or other filter-feeders. The feasibility of large-scale preparation of these particles is questionable, but the potential for their use as a research tool in bivalve nutrition or pollutant-food web relationships is real. Both of these areas have been hampered by the lack of defined and manipulatable dietary preparations. The encapsulation method reported in this study could help to fill this gap.

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APPENDICES

APPENDIX I

A. Gross morphology of the digestive tract of C. gigas. Refer to Figure 14 for locations of organs and structures discussed in the Literature Review.

B. Ultrastructure of the ciliated epithelium of digestive tubules of C. gigas. Refer to Figure 15a,b.

Figure 14. Morphology of digestive tract of Crassostrea gigas. G, gills: LP, labial palps; E, esophagus; AS, anterior stomach; T, typhlosole; PS, posterior stomach; SS, style sac; CD, caecal duct; DD, digestive diverticula; I, intestine; BV, blood vessel. Bar equals 200μm.

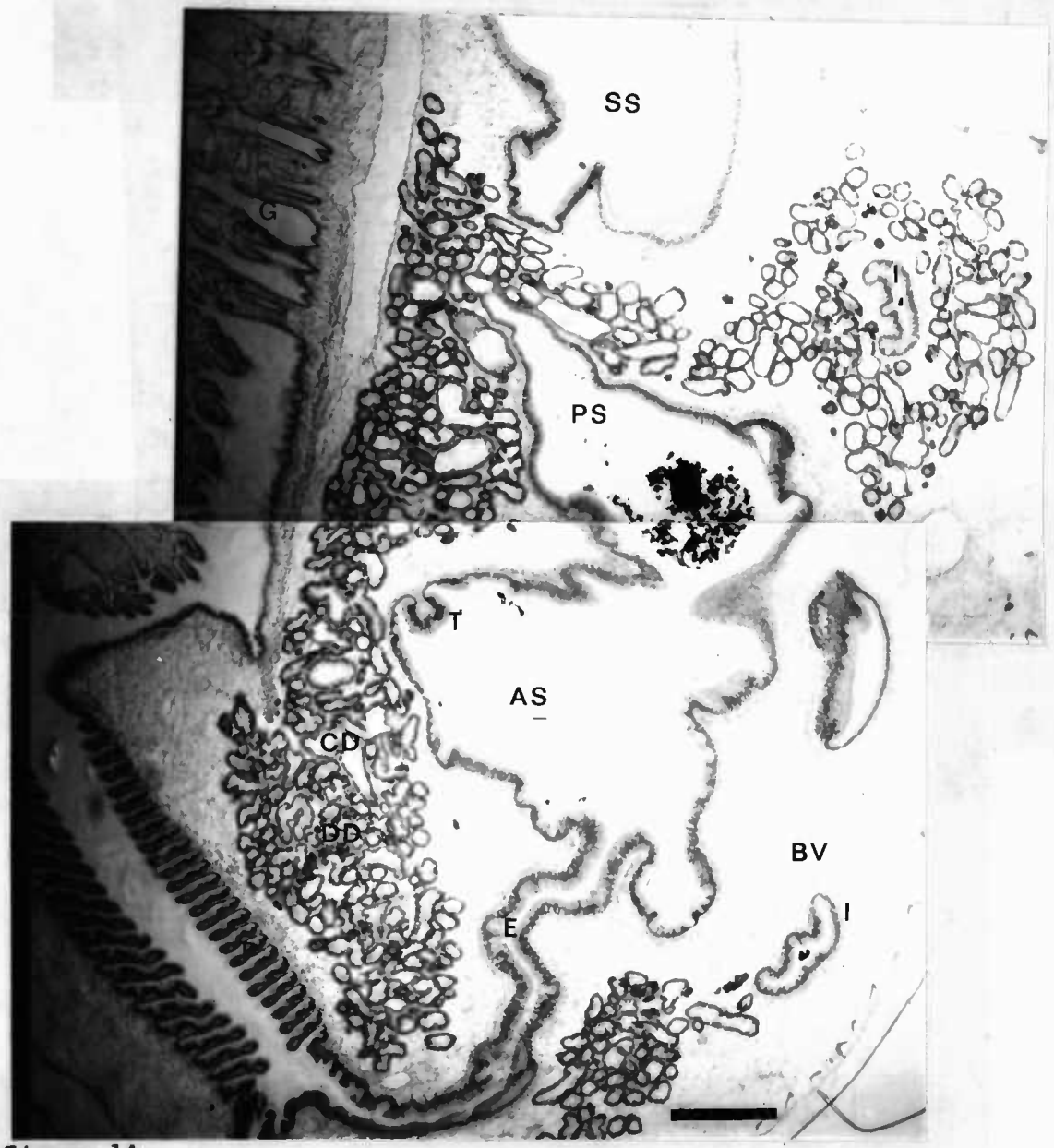

Figure 14

Figure 15. Ultrastructure of ciliated epithelium of digestive tubules of C. gigas. Prepared as in Experimental Section. (a) apical portion, (b) basal portion. C, cilia; M, mitochondria; N, nucleus; MV, microvilli; V, vacuoles. Note numberous tiny vacuoles in apical region. Bar equals 1μm.

Figure 15a

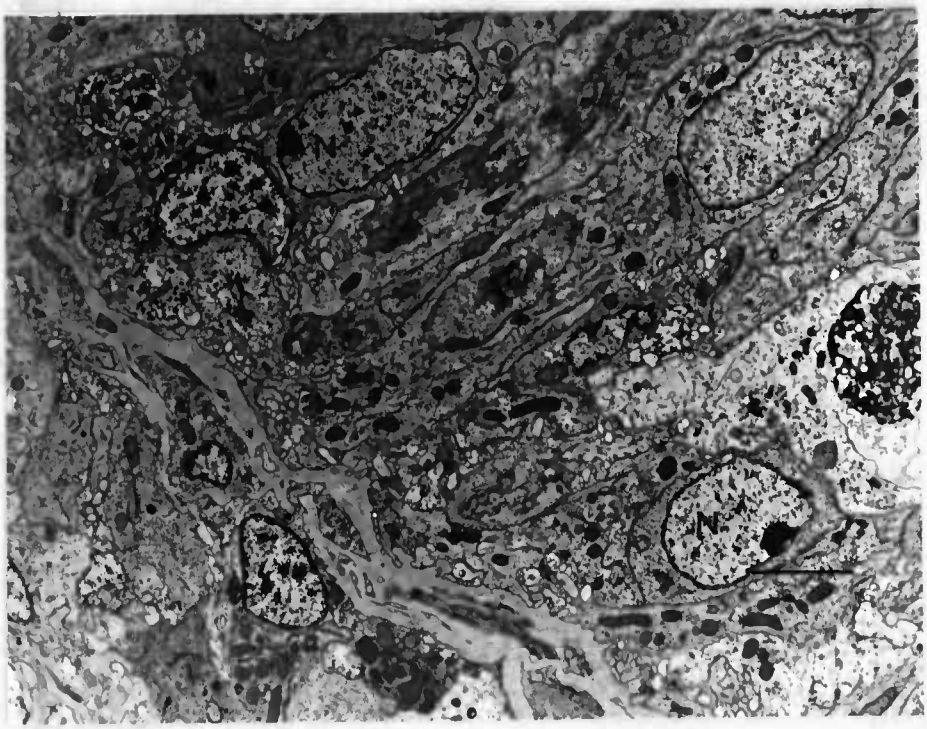

Figure 15b

APPENDIX II

Embedding of Tissue in Polyethylene Glycol

1. Infiltrate 6 hrs in Polyethylene Glycol (PEG) 200. (Two changes.)
2. Infiltrate 12 hrs in 2:1 mixture of PEG 200/PEG 1000-4000 (3:1). (Two changes.)
3. Infiltrate 6 hrs in PEG 1000-4000 (3:1). (Two changes.)
4. Infiltrate 6 hrs in PEG 4000-1000 (4:1). (Two changes.)
5. Embed in fresh PEG 4000-1000 (4:1). Keep dessiccated until sectioned.