THE UPTAKE OF FREE FATTY ACIDS FROM SEA WATER BY A MARINE FILTER FEEDER, *CRASSOSTREA VIRGINICA*

By

TERRY ALLEN BUNDE

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Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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By

Terry Allan Runde

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The ability of the American oyster, *Crassostrea virginica*, to remove naturally occurring dissolved free fatty acids, in concentrations approximating those found in sea water, was investigated using radioactive isotopes of palmitate, stearate, and oleate.

Petroleum ether (30 - 60°C) extracts of the sea water from a Florida Gulf Coast estuary contained up to 280 μg of total lipid material per liter including 77 μg of free fatty acid. The fatty acids, separated by gas liquid chromatography, were predominately saturated with even carbon numbers. The major fatty acid present was palmitate.

The animals were shown to remove labeled palmitate from sea water by measuring the appearance of the radioactivity in the chloroform extractable material. The uptake process was shown to be physiological and not chemical adsorption onto shells. This assimilation was inhibited with 200 mM sodium cyanide. The temperature dependence of the uptake process was investigated at 20, 25, 30, and 35°C.

The rate of uptake of 50 μm celite particles carrying adsorbed radioactively labeled stearate and palmitate demonstrated that the process

of filtration feeding was not responsible for the removal of freely dissolved fatty acids. The rate of uptake of celite bound material was delayed by 30 minutes when compared to the uptake of an equal concentration of dissolved material.

The kinetics of the uptake into chloroform extractable material was investigated for palmitate, stearate, and oleate. Both palmitate and stearate showed saturable uptake systems as determined from reciprocal rate-concentration plots. The rate of uptake of both acids markedly increased when micellar concentrations of the fatty acids were reached. The rate of uptake of oleate was much less than that for palmitate and stearate, and was not saturable at natural concentrations.

The rate of uptake into isolatable lipid classes was investigated; the major species labeled were phosphatidyl choline, triglycerides, and cholesterol. The rates of incorporation of palmitate into phosphatidyl choline and stearate into the total polar lipids were determined.

Oleate was shown to effectively inhibit the uptake of stearate in competition experiments, but no effect was seen by oleate on the palmitate uptake. Increased oleate concentrations were shown to promote palmitate uptake.

Turnover rates for various lipid classes were determined by labeling with sodium [^{3}H]acetate, removing the label, and following the decrease in specific activity of each lipid with time.

The contribution of the uptake process to the total metabolic needs of the animal was estimated. The impact of such lipid uptake studies was discussed in light of municipal sewage and petrochemical pollution of natural oyster habitats as well as the selection of oysters as a possible aquaculture species.

INTRODUCTION

Metabolic Significance of Dissolved Organic Matter

The salt waters of the world contain relatively constant concentrations of inorganic compounds, evidencing only small changes in salinity, but they show orders of magnitude variation with time and location in concentrations of dissolved organic matter and dissolved particulate matter (Wagner, 1969; Duursma, 1961). Early investigations of dissolved organic substances were hampered by crude methods of sampling, analysis, and quantitation; but with newer, more refined techniques, it has become apparent that the oceans of the world contain more dissolved organic matter than that which is represented by the entire living biomass of the oceans (Duursma, 1961). All major classes of biologically important organic molecules are found in sea water: amino acids and peptides, simple and conjugated carbohydrates, nucleic acids, and lipids. These materials share the common property of being able to pass through a 0.45 µm cellulose acetate filter and are, therefore, distinguishable from the particulate matter which such filters retain. The concentrations of these molecules vary within fairly wide limits from one body of water to another depending upon the season, the metabolic activity of the ecosystem, the depth of the water, and the specific flora and fauna found in the water.

The methods by which these compounds have been analyzed involve techniques such as dialysis, adsorption, ion-exchange, solvent-extraction,

and co-precipitation (Wagner, 1969). The difficulties inherent in measuring mg/liter or μg/liter quantities of organic compounds in solutions containing g/liter quantities of inorganic salts have made quantitation difficult, but reliable data show total amino acid concentrations of 30 μg/liter of which 16 μg/liter is glycine (Hobbie *et al.*, 1968); carbohydrate concentrations of 0.5 mg/liter (Okaichi, 1967); and lipids in 1-10 mg/liter quantities (Jeffrey, 1966).

The sources, and energy and matter interrelationships of this huge reservoir of organic matter are not specifically known, but several possible pathways have been investigated. The best description is derived from a figure in a review by Duursma (1961) which is Figure 1. This flow diagram depicts the dynamic nature of the pool of dissolved organic solutes and its relationships to the several pathways of decomposition, excretion, and leakage which result in these molecules. The primary producers in a salt water ecosystem, the phytoplankters, have been shown to lose a large amount of their photosynthetic products through leakage and overproduction, up to 1-40 mg Carbon/m^3 sea water/day depending upon the water depth and latitude (Thomas, 1971). The zooplankters which consume the primary producers, also leak organic molecules into the pools of both dissolved and particulate substances (Johannes and Webb, 1965). This complex relationship between the various organisms and the organic matter, and the probable importance of bacteria in processing dissolved organic matter, are outlined in a figure derived from Johnson (1974) which is Figure 2. The physical and environmental forces involved in the production and processing of organic matter by the benthic animal communities are as complex and as important as the biochemical interconversions that occur. The pools of detritus and dissolved organic matter are not static but in a constant dynamic state as are the organisms at each trophic level.

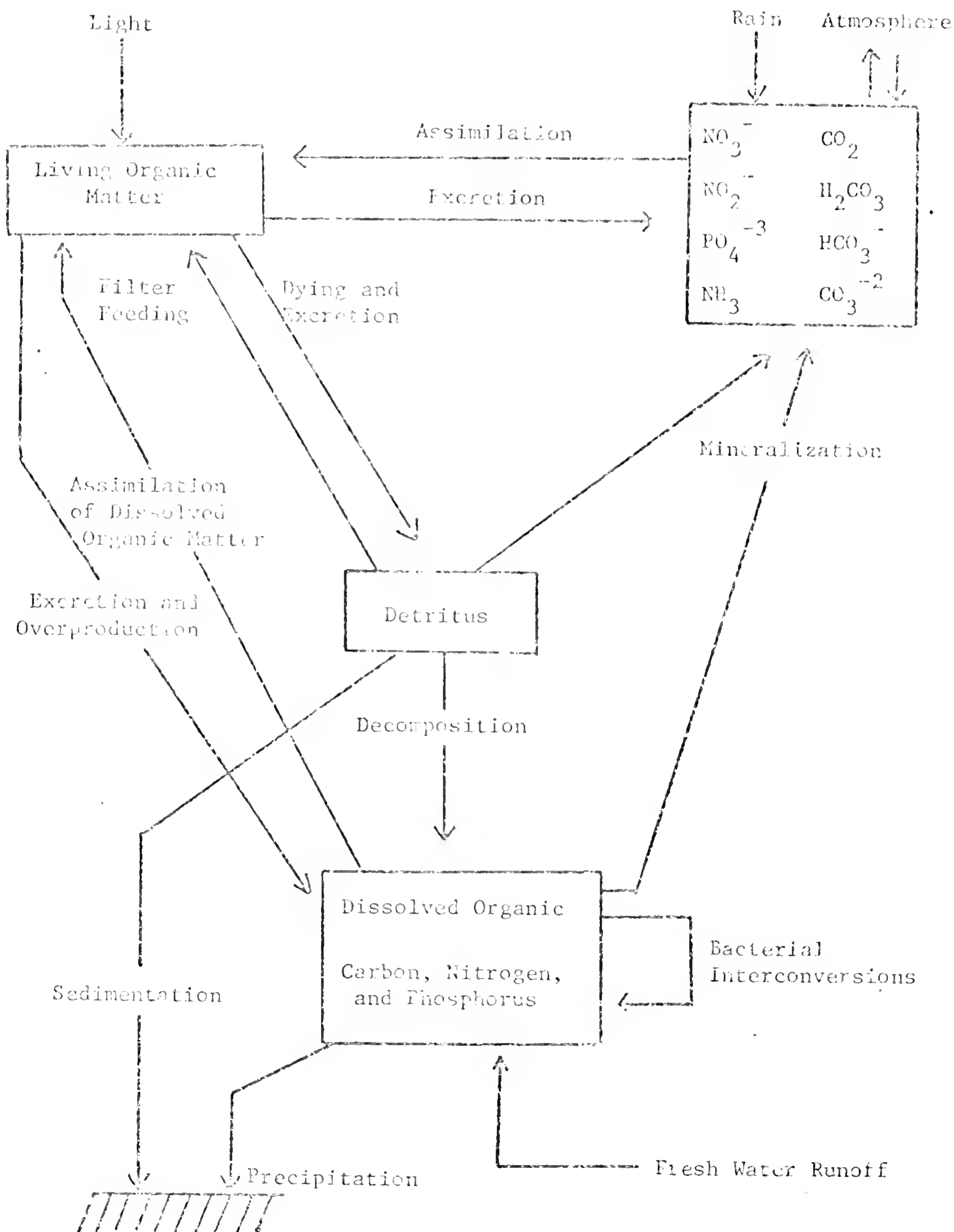


Figure 1. The Flow of Organic Compounds in the Marine Ecosystem.
Taken from: Duursma, E.K. *Netherlands J. of Sea Res.* 1:4 (1961).

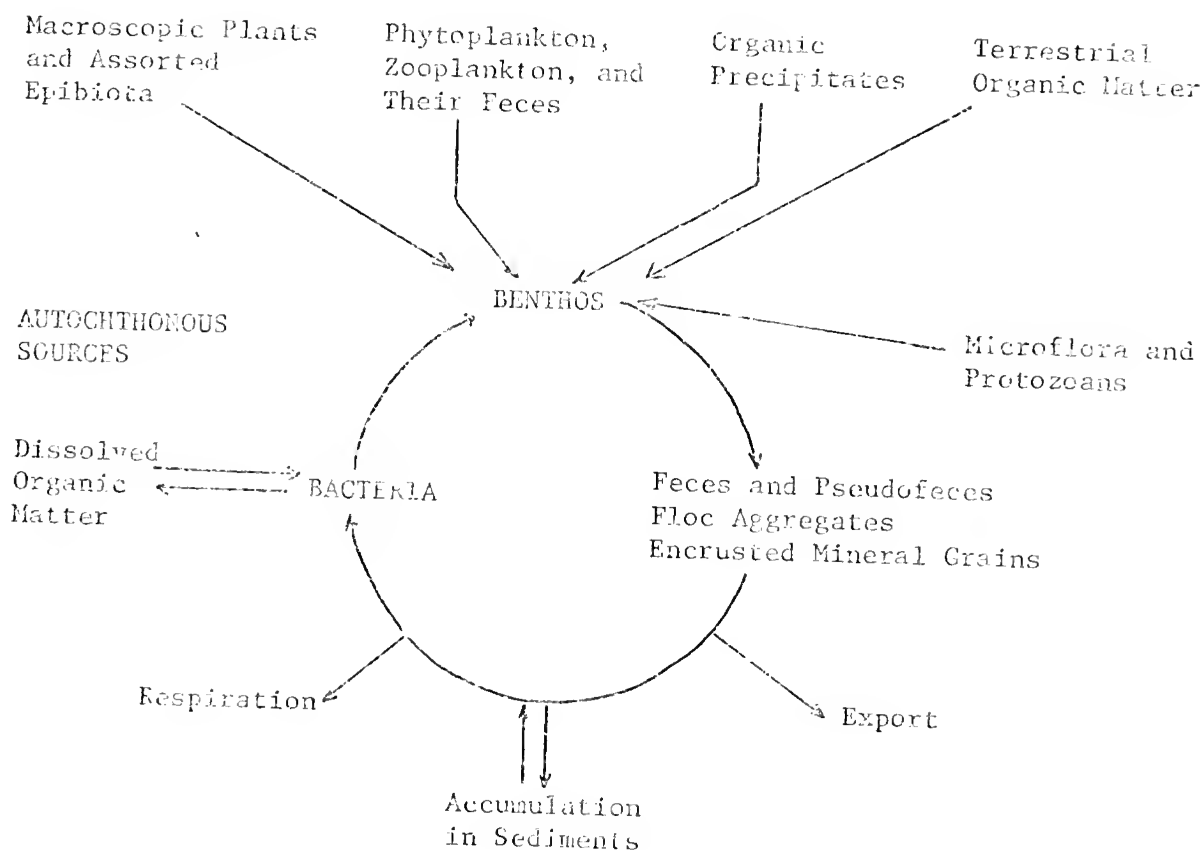


Figure 2. Cycling of Organic Matter by the Benthos.

This figure taken from Johnson (1974) depicts the function of the benthos or bottom dwelling organisms in the processing of organic matter. The external sources of organic matter are shown at the top and the sinks at the bottom. The bacteria are the major organisms involved in processing the material into freely dissolved matter.

Taken from: Johnson, R.G. *J. Mar. Res.* 32(2):326 (1974).

In 1908 a German biologist, Pütter, concluded, from the crude analytical data on the concentrations of dissolved organic matter in sea water which were available to him, that this pool of organic molecules was a valuable and even necessary resource in the nutrition of marine organisms (Pütter, 1908). His theory was considered valid until Krogh (1931) showed that Pütter's determinations of the concentrations of those materials erred significantly on the high side, and held that, although there were amino acids, carbohydrates, and lipids in sea water, they were not present in sufficient quantities to be a valuable energy source. In a later paper (as reviewed in Duursma, 1961) Krogh acknowledged that some organisms could remove these molecules but still held that they were not energetically significant. The current concepts of what is indeed metabolically significant to an organism were formulated in a series of papers by Lucas (1946, 1949, and 1961), whose views of utilization involve the idea of pools of molecules providing necessary metabolic intermediates and "essential molecules" for the organism, rather than functioning as significant sources of nitrogen, carbon, and phosphorous for metabolic energy. However, if an organism did possess pathways for assimilation of these molecules for anabolic or catabolic needs, then such pools of organic molecules in the sea could be very important.

With the presence of dissolved organic matter in sea water an undisputed fact, research was initiated into elucidating the physical and chemical forces that convert these organic compounds into particulate matter of sufficient size for filtration or adsorption methods to be used by marine organisms in their removal from the sea water. The initial studies employed fish hemoglobin as a substrate for coalescence of organic matter from sea water. This material was then shown to be important in

the nutrition of invertebrates in feeding experiments (Fox *et al.*, 1953). Studies into the geophysical forces involved in those sea surface currents known as Langmuir circulation and the production of foam lines at their interface led researchers to formulate the thesis that organic particulates formed at disturbed air-water interfaces (Baylor *et al.*, 1962; Riley, 1963; Sutcliffe *et al.*, 1963). The formulation of organo-phosphate containing particles at such disturbed surfaces, and the resultant accumulation of phosphate containing material when these particles were isolated and added to 0.45 μm filtered sea water are examples of the process whereby these particles may be formed and increase in size (Sutcliffe *et al.*, 1963).

Baylor and Sutcliffe isolated organic particulate matter from despumated sea water following filtration through a 0.45 μm filter and demonstrated the survival of *Artemia* cultures fed this material for 11-16 days (Baylor *et al.*, 1963). The cultures survived and grew as well as those fed yeast extracts. The data of these investigators seemed to support Pütter's original ideas and provided an impetus for further work based on the hypothesis that dissolved organics were metabolically important to at least some invertebrates.

The work of Fox, Baylor, Riley, and Sutcliffe indicated that particulate generation was required for feeding. They said nothing about areas in which no significant physical condensation of organic molecules could occur, but where freely dissolved molecules existed. Grover C. Stephens and co-workers showed in work published from 1961-1973 that dissolved free amino acids and carbohydrates, at naturally occurring concentrations, were removed from sea water solutions by several marine species (see Table 1). Based on results with radioactive tracers, the

Table 1. Amino Acids and Glucose Uptake.

Author	Date	Organic Compound	Organism	Concentration Tested
Collier *et al.*	1953	Glucose	*Crassostrea virginica*	1×10^{-3}
Stephens & Schinske	1958	Amino Acid (Glycine)	12 invertebrate phyla	2×10^{-3}
Stephens & Schinske	1961	Amino Acid (Glycine)	11 invertebrate phyla	2×10^{-3}
Stephens	1962	Glucose	*Fungia scutaria*	4.7×10^{-5}
Stephens	1963	Amino Acids (Ala, Gly)	*Clymenella torquata*	1×10^{-6}
Stephens	1964	Amino Acid (Glycine)	*Nereis limnicola* and *succinea*	2×10^{-5}
Stephens & Virkar	1966	Amino Acids	*Ophiactis arenosa*	2×10^{-8} 1×10^{-2}
Reish & Stephens	1969	Amino Acid (Glycine)	*Neanthes arenaceodentata*	1×10^{-7}
Anderson & Stephens	1969	Amino Acid (Glycine)	Crustaceans	4.3×10^{-7}
Taylor	1969	Glucose and Amino Acids	*Nereis virens* and *sars*	2×10^{-5}
Chien *et al.*	1972	Amino Acids	*Glycera*	1×10^{-6}
Shick	1973	Amino Acid (Glycine)	*Aurelia aurita*	1.27×10^{-6}

dissolved organic matter could partially meet the energetic needs of these organisms. By the use of radioactive tracer techniques Stephens has surveyed several invertebrate phyla: coelenterates, annelids, crustaceans, molluscs, and echinoderms, and showed that at least some capacity to remove dissolved free amino acids or carbohydrates exists in all of them. In these studies the disappearance of a tracer molecule from sea water was monitored as was the appearance of label in the whole animal digest or extract. The rate at which this process proceeded at naturally occurring concentrations was used to determine the maximum amount of assimilation into the organism with time. Knowing the metabolic oxygen consumption of an experimental animal, the percentage of total carbon influx that was represented by dissolved organic solutes was determined.

Ferguson, using an autoradiographic technique to study the uptake of amino acids by starfish, has shown that these animals could remove the label from sea water and that the amino acids, first localized at the surface of the animal around the pseudopods, were later transported throughout the water vascular system. The uptake was quantitated by counting silver grains in the photographic emulsions (Ferguson, 1970, 1971). Other work with starfish species (Péquignat, 1972) demonstrated amino acid uptake into an isolated arm of *Henricia sanguinolenta* by autoradiographic techniques. The labeled amino acid, as in Ferguson's studies, could be seen to be incorporated almost exclusively into the ambulacra and aboral wall of the arm placed in the sea water. Time course studies revealed major incorporation of the amino acid into proteins of the gonadal tissue, indicating significant utilization of this dissolved material assimilated from sea water.

It is apparent from these studies that animals with soft body tissue surfaces exposed to the sea water can remove and assimilate dissolved material in a manner different from their normal feeding habits. Polychaetes are detritus feeders, bivalve molluscs are filter-feeders, starfish and urchins are herbivores, and coelenterates are carnivores, but all appear to have pathways for direct assimilation.

With the discovery and biological characterization of the pogonophorans, much attention was given to the possible mechanism of nutrition in these benthic invertebrates which possess no digestive system (Little and Gupta, 1968, 1969; Southward and Southward, 1970, 1971, 1972). Amino acid uptake from ambient sea water concentrations of 10^{-6} - 10^{-8} M was shown, followed by autoradiographic studies of its localization (Little and Gupta, 1968; Southward and Southward, 1968). Further work yielded data concerning the uptake of several different amino acids, hydrolysates of algal proteins (peptides), glucose, and fatty acids (Little and Gupta, 1968; Southward and Southward, 1970, 1971, 1972). Uptake of such compounds by the pogonophorans apparently differs only from that in the polychaetes (Taylor, 1969; Stephens, 1964) in that the efficiency with which pogonophorans remove dissolved substances is much better, i.e., they are better adapted to environments wherein concentrations of amino acids and fatty acids are less than 10^{-5} M (Southward and Southward, 1971). The studies of these animals indicate that as much as 50 percent of their metabolic needs can be met by the dissolved organics in the sea water around them. In pogonophorans, therefore, the ability to remove dissolved molecules is not accessory but is necessary for their basic nutrition. They have developed mechanisms that are finely tuned to ambient organic concentrations so that optimum usage of such pools can be maintained.

The basic *in vivo* experimental techniques of Stephens and indeed of all others who have looked at uptake of dissolved material from sea water, i.e., the use of tracer methods to yield some indication of the percentage of the metabolic needs met by these substances, have been challenged by Johannes *et al.* (1969). In experiments with the marine turbellarian *Bdelloura*, these workers found that this animal leaked amino acids into the medium at a faster rate than it removed them from solution; therefore, any discussion of uptake satisfying net metabolic needs is incorrect. However, Stephens, in a later paper (Chein *et al.*, 1972), showed that when a section of body wall of the blood worm *Glycera* was removed and placed in a Ussing chamber in which the flux of amino acids into and out of the organism could be measured, the net flux was into the animal.

The metabolic significance of the work with amino acid uptake is complicated by the function of the molecules as osmoregulators in marine and estuarine invertebrates. Glycine, proline, alanine, aspartic acid and the sulfonic acid taurine are all involved in osmoregulation (Gilles and Schoffeniels, 1969). The uptake of these amino acids from sea water must be considered in the context that any reverse flow out of the organism functions to maintain osmoregulation. Stephens looked at the influence of salinity on the uptake of glycine by *Clymenella torquata* and showed that at low NaCl concentrations the uptake was virtually zero. At these salt concentrations this animal would be actively lowering its internal pool of amino acids to compensate for decreased ionic concentration in the medium.

The ability to remove dissolved amino acids and carbohydrates at isoionic sea water concentrations, however, is real and their net movement

into the organism may be important for a broad spectrum of organisms in which such pathways are not the main nutritional mechanism.

Lipids and Free Fatty Acids in the Marine Food Chain

The organic molecules which have been most exhaustively examined to date have been the amino acids and glucose; but there is a large and equally important class, the lipids, which are present in sea water at metabolically significant concentrations. Table 2 is a compilation of data from several laboratories on the concentration of lipids, specifically free fatty acids. The variability of the data comes from the diverse methodologies used in sampling, storing, filtering, and extracting the specimens as well as to differences in source. The latest papers use filterability through a 0.45 μm filter to define dissolved matter and employ solvent extraction to separate the lipids.

It is certain that there are large amounts of hydrophobic lipoidal material dissolved in the oceans of the world, not just in isolated areas of phytoplankton slicks or polluted coastal waters. While Stephens and many others were conducting investigations on dissolved amino acids and carbohydrates, only two investigators were working on the uptake of dissolved free fatty acids. Southward and Southward (1971, 1972) described experiments with pogonophoran species, and Testerman (1972) published data on two nereid species. These experiments demonstrated uptake processes for fatty acids that were saturable and inhibitable by other fatty acids. Such uptake operated efficiently at the free fatty acid concentrations to which the organisms are exposed in their natural environment. The fatty acids, once removed, were incorporated into several complex lipid compounds. The loss of label from these organisms

Table 2. Fatty Acids in Marine Waters.

Compound	Method of Extraction	Concentration in mg/l	Investigator(s)
Fatty Acids	Liquid-Liquid// pH 3//ethyl acetate	0.1 - 0.8	Slowey *et al.*, 1959, 1962
Fatty Acids	Liquid-Liquid// $CCl_4 + CHCl_3$	0.01 - 0.12	Williams, 1961, 1965
Lipids	Liquid-Liquid// pH 2//petroleum ether + ethyl acetate	0.4 - 8.0	Jeffrey, 1962, 1966
Fatty Alcohols, Acids, Esters, and HC	Coprecipitation w/ $FeCl_3$//extract w/ $CHCl_3$	0.2 - 1.0	Garrett, 1967
Fatty Acids	Liquid-Liquid// pH 2.0-2.5// Extract w/$CHCl_3$	0.01 - 0.025	Stauffer & Macintyre 1970
Lipids/Fatty Acids	Liquid-Liquid// pH 2//$CHCl_3$ (saponfication)	0.11 - 0.13 / 0.06 - 0.05	Testerman, 1972

Source: Taken in part from Jeffrey (1970) and Testerman (1972).

into the medium, the so-called "leakage" rate, was only 5 percent, with the majority of the "leaked" radioactivity being in the form of $^{14}CO_2$ indicating the catabolism of the free fatty acid (Testerman, 1972).

The work on lipid uptake by marine animals does not suffer from some of the problems of amino acid experiments. The lipid material, due to its hydrophobic nature, is not as freely soluble as amino acids. The lipophilic compounds involved are not readily diffusible in nature and are not involved in osmoregulation processes as are the amino acids. After a lipid compound is transported into an experimental animal, the reverse diffusion rate back into the water is not expected to be as large as that for amino acids; hence, the major direction of the movement is into the animal. Therefore, this movement may be much more metabolically significant.

Increasing coastal pollution problems ascribed to oil spills and natural oil seepage from the sea floor have caused several laboratories to investigate the effect of petroleum hydrocarbons on lamellibranch molluscs (Lee *et al.*, 1972; Fossato and Siviero, 1974; Stegeman and Teal, 1973). These investigations showed that *Crassostrea virginica* and *Mytilus edulis* were able to remove significant quantities of sub-lethal concentrations of petro-lipid material, up to 50 μg/gram wet body weight. This lipid material was assimilated in the gill and mantle areas as well as in the gut, indicating a possible direct adsorption pathway (Lee *et al.*, 1972). The naturally occurring hydrocarbons in the lipid pools of the organisms were not as saturated nor as aromatic in nature as the exogenous petro-hydrocarbons and were not affected by the large concentrations of the foreign compounds. Stegeman and Teal (1973) found that the fat content of the animal was proportional to the maximum ability to store the foreign hydrocarbon

material. This would seem to indicate that, once removed, the material mixes with the lipid pools of the organism.

The Oyster as a Possible Experimental Subject for Lipid Uptake

Studies on the feeding behavior of the American oyster, *Crassostrea virginica*, have been designed to determine the type and approximate size of particles filtered, and the nature of the filtering process. Because of the economic importance of the species, much of this work is reported in Wildlife Fisheries bulletins and other governmental publications, and deals with growth rates almost exclusively (Collier *et al.*, 1953; Galstoff, 1964; Korringa, 1952). The work that has been done concerns the filtration system of oysters and its ability to remove the several size classes of organic material which make up its diet (Haven and Morales-Alamo, 1970). The results indicate that oysters filter several different classes of material: (1) dissolved organic material 0.8 - 1.5 μm, (2) nano- and ultra-plankton 5.0 μm, (3) marine bacteria 1.0 - 2.5 μm, and (4) macroparticulate organic matter 1 - 10 μm and larger. Data from Ward and Aiello (1973) on the mussel *Mytilus edulis*, a lamellibranch like *Crassostrea*, imply that the gill is a dual purpose organ serving both as a surface of oxygen exchange and as an ultra-structure for ciliary-mucoid filtration. The controversy surrounding the importance of the mucus strand in entrapment of particles smaller than the interfilamental ostia of the gill has not been resolved, but it now appears likely that the structure of the gill lamellae can filter particles down to 1 μm in size without mucus (Haven and Morales-Alamo, 1970).

The first *in vitro* work on uptake by lamellibranchs showed that the gill tissue is the most important site in the animal for free amino acid

and sugar uptake (Bamford and Gingles, 1974; Bamford and McCrea, 1975). By excising gill tissue from *Cerastoderma edule*, the common cockle, and measuring the uptake of ^{14}C labeled amino acids, these workers demonstrated that the uptake mechanism is saturable, has a diffusion component, and that there is inhibition by other amino acids. Their work with the Japanese oyster, *Crassostrea gigas*, involved the uptake of labeled glucose and the inhibition of such uptake by glucose analogs. The impetus for this work came from a series of autoradiographic studies by Péquignat (1973) on the uptake of amino acids and glucose by *Mytilus edulis*. In these whole animal experiments, labeled amino acids, removed from sea water concentrated in tissues of the mantle, foot, and gills, i.e., those soft tissues exposed to organics in the water as it passed through the shell. It is obvious that the gill is vitally important in the feeding process both for large macromolecular aggregates and detritus in filter-feeding and for direct assimilation of dissolved material.

The metabolic importance of lamellibranch filtration of sea water can be expressed in the following energetic calculation derived from Nicol (1970). The oyster can filter sea water at a rate of 3 liters/hr during which time it consumes 0.20 ml of O_2; this rate of filtration may then be expressed as 15 liters H_2O/1 ml O_2. If 1 ml of O_2 will oxidize 0.8 mg of organic matter, and if the basal metabolism represents approximately one-third of the total oxygen consumption, the amount of organic matter that must be removed from the sea water is

$$\frac{0.8}{15} \times 3 = 0.16 \text{ mg/liter.}$$

Nicol suggests that the particulate diet of oysters, detritus and phytoplankton, can provide 0.14 - 2.8 mg of organic matter/liter. Since the results of *in vitro* and *in vivo* uptake experiments with lamellibranch

molluscs (Bamford and Gingles, 1974; Bamford and McCrea, 1975; Péquignat, 1973; Stephens, 1963), show that dissolved material, present in concentrations up to 10 mg/ml, can be removed from sea water, dissolved organic matter should be considered as a possible source of metabolic energy.

Research Objectives

The purpose of this research was to study the uptake and incorporation of dissolved free fatty acids by a marine filter-feeding mollusc, the American oyster, *Crassostrea virginica*. To formulate and organize the objectives, the following questions were asked:

(1) What are the ambient concentrations of free fatty acids in the water in the Cedar Key estuary and what is the free fatty acid distribution?

(2) Can the oyster remove free fatty acids from sea water at those concentrations found naturally and are the free fatty acids, once removed, incorporated into the lipid pools of the organisms?

(3) Is this uptake a saturable process? If so, what are the initial rates and concentration dependence of the process or processes?

(4) How does the uptake of dissolved material (i.e., smaller than 0.45 μm in diameter) compare with the uptake of particulate material 50 μm in size?

(5) Is there any temperature dependence of the uptake?

(6) Do different fatty acids have the same kinetic parameters of accumulation and assimilation? Is there competition between fatty acids for the uptake mechanism?

MATERIALS AND METHODS

Materials

Animals

Oysters of the genus and species *Crassostrea virginica* were collected from an estuary on the west coast of Florida north of Cedar Key known as Shell Mound. An area of collection was chosen which was accessible without a boat and at mean low tide was covered with two-three inches of water. The experimental plot was sheltered from heavy boat traffic and was exposed to a minimum of pollution due to the unpopulated area around it. Animals of 7 - 10 cm shell length (2.5 - 3.5 grams soft tissue weight) were selected at low tide and only during stretches of good weather so that there would be no effects due to large fresh water influx and salinity change. The normal salinity for the area ranged from 22 - 29 parts per thousand salt depending upon the tide. The animals were brought back to the laboratory in plastic buckets covered with wet canvas and were placed in a 20 gallon glass holding aquarium equipped with two Dynaflow circulating filters and an undergravel filtration apparatus. They were not fed in the holding tank and were used within 72 hours after collection. Animals were used from July through May because those collected during the early summer were small and gravid, frequently releasing eggs into the holding tanks or during the uptake experiments.

Chemicals

[1-^{14}C]palmitic acid, [3-^{14}C]stearic acid, [16-^{14}C]palmitic acid, and [7,8-^{3}H]oleic acid were purchased from Schwarz-Mann. All non-radioactive fatty acids were reagent grade and were recrystalized before use. Standard samples of phospholipid and neutral lipids for thin layer chromatography (TLC) and fatty acid methyl ester standards for gas liquid chromatography (GLC) were purchased from Applied Science, Supelco or Sigma Chemical.

Petroleum ether for extraction, column chromatography and TLC was purchased from Eastman Chemical or City Chemical of New York, and glass distilled two times over potassium permanganate. It was separated into 30 - 60°C and 60 - 78°C boiling fractions and was stored in dark bottles.

Aquasol was purchased from New England Nuclear and spectral grade toluene PPO-POPOP was made with reagents purchased from Sigma Chemical.

Chloroform and methanol for extractions were purchased from Eastman Chemical as analytical reagent grade solvents and were not redistilled prior to use. Anhydrous diethyl ether was purchased from Mallinckrodt and was not redistilled prior to use.

All other organic and inorganic chemicals were analytical or reagent grade.

Silanization of glassware

All glassware for uptake experiments, extraction, transporting and storage of lipid material in aqueous or organic solvents was treated with an aqueous silanizing reagent, "Siliclad," purchased from Clay Adams, Inc.

Column packings for GLC

EGSS-X and Apiezon-L column packings for the gas chromatography of fatty acid methyl esters were purchased from Applied Science Labs.

Thin layer plates

Thin layer plates of Silica Gel 60 of 250 μm thickness on 20 x 20 cm glass were obtained from E. Merck. Silica Gel G with no binder was obtained from Applied Science and was spread on glass plates.

Column chromatography

Specially prepared 400 mesh silicic acid for lipid column chromatography was purchased from Bio-Rad. Hi-Flosil, a silicic acid derivative for rapid separation of lipid classes, was purchased from Applied Science.

Sea water collection and filtration

Sea water used in the uptake experiments was collected from the Cedar Key estuary along with the oysters and transported to the laboratory in 12-liter glass carboys or 5-gallon vinyl plastic containers. Before use, the water was first filtered through a Whatman #1 paper under vacuum to remove large particles and then filtered through a Whatman GF/A glass fiber filter of 0.45 μm porosity. The filtered sea water was stored in glass at $4^{o}C$ until used as an uptake medium. Sea water to be extracted for background free fatty acid levels was sampled as soon as the filtration steps were completed.

Methods

Uptake Experiments

Closed shell experiments

The oysters to be used were removed from the holding tank and cleaned of all epiphytic and epizoic material with an oyster knife and a heavy bristle brush. They were then rinsed clean of all sand and left until the shells were dry.

The labeled fatty acid was added to a small glass petri dish and the carrier solvent, usually benzene, was removed with a stream of N_2 gas. A Teflon stirring bar was placed in the petri dish and the dish was placed in a six-liter glass vessel. Four liters of bacteriologically filtered sea water containing 200 mg/liter of streptomycin sulfate was added with stirring. The sea water was sampled by removing 1 ml aliquots and counted in 10 ml of Aquasol. After the extracts reached a constant specific activity, the animals were placed in the sea water, then removed at various times and extracted.

In early experiments, extraction was carried out by a modified Bloor method using a perchloric acid precipitation step followed by an ethanol-ether (3:1) extraction (Bloor, 1928). This procedure is outlined in Figure 3. In later experiments, a modified Bligh and Dyer (1959) extraction was used. This involved homogenization of the whole animal tissue in chloroform-methanol (2:2) followed by isolation of the chloroform fraction (Figure 4). In either extraction method, an aliquot of 1 ml of the ethanol-ether or 200 µl of the chloroform extract was added to Aquasol and counted.

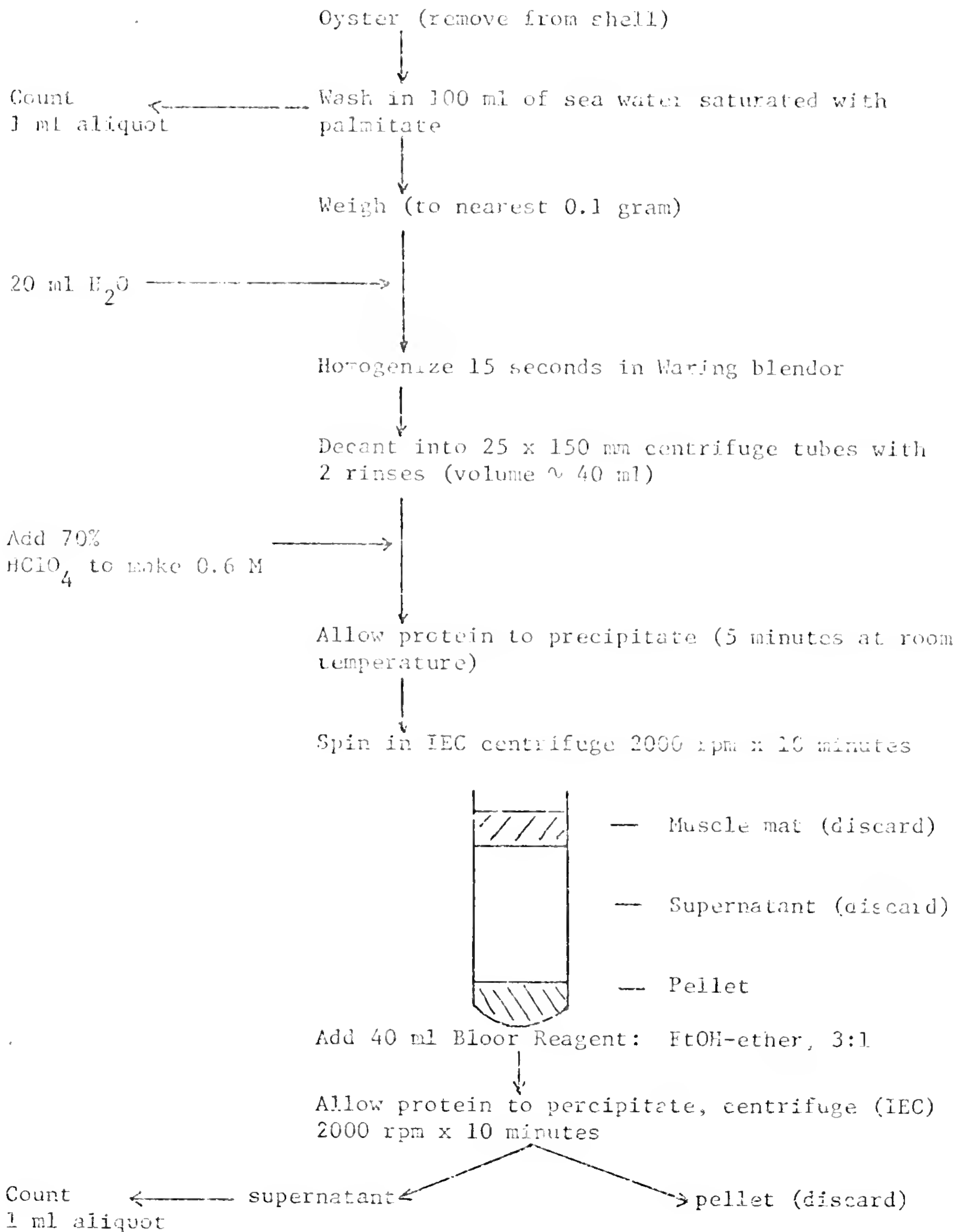


Figure 3. Extraction with Adapted Bloor Method.

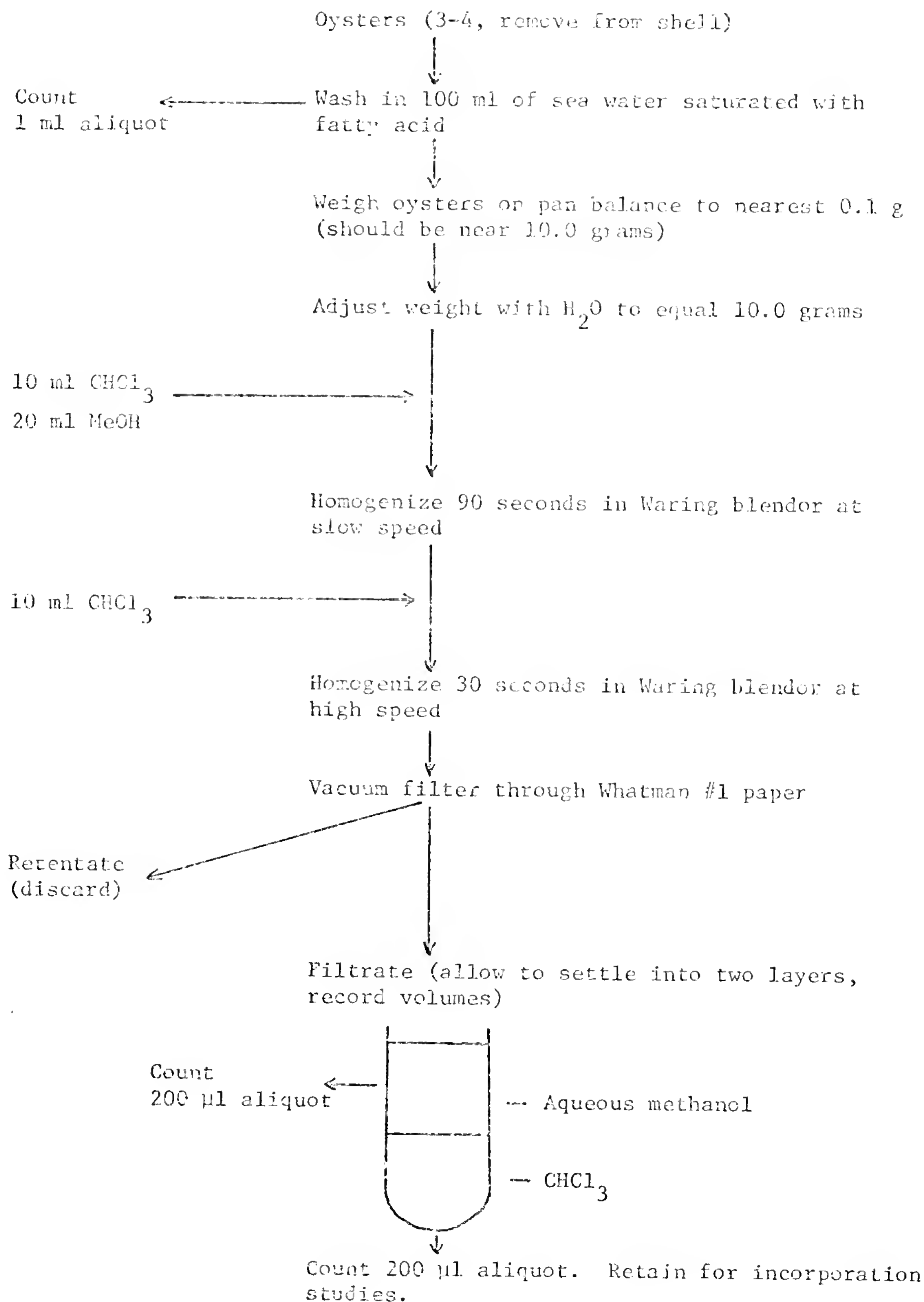


Figure 4. Extraction with Adapted Bligh and Dyer Method.

Open shell experiments

For those uptake experiments in which concentration dependence and competition were investigated, a modified procedure was used in order to eliminate variations in the data caused by any periodicity of valve opening and closing by the experimental animals. The upper valve was removed by wedging the hinge and then carefully separating the adductor muscle from its upper shell insertion. Only those animals in which no traumatic tissue damage was evident were used in these experiments. Continuation of regular heart beat and a non-ruptured pericardial cavity were used as a test of viability and successful removal of the upper shell. The animals were rinsed in sea water and then placed in the vessel containing 4 liters of filtered sea water. At zero time the labeled fatty acid and any competing fatty acid dissolved in ethanol were added to 4 liters of sea water below the surface of the vortex created by a stirring bar, ensuring that the ethanol and the fatty acid were dispersed rapidly throughout the medium. In these experiments, the carrier ethanol concentration never exceeded 5 parts per thousand and no effects of the solvent were ever seen. The sea water was sampled by removing 1 ml aliquots at various times and counting in Aquasol. Samples were also taken and filtered through 0.45 μm filter and counted in Aquasol to determine whether the labeled fatty acid aggregated or was adsorbed on aggregated material.

Celite uptake experiment

The uptake of fatty acids adsorbed on celite was studied using Johns-Mansville celite sieved to approximately 50 μm size particles. The fatty acid in appropriate concentration in ether solution was added to

the dried celite and the solvent removed under vacuum with a Rinco evaporator. This process of solvent addition and removal was repeated 3 times and the celite dried under N_2 to remove traces of remaining solvents. The celite bound fatty acid was then added with continuous stirring to the sea water containing the experimental animals and after suitable time periods, samples were taken and the procedure outlined in Figure 3 or 4 followed. The sea water was sampled both unfiltered and after 0.45 μm filtration to determine the concentration of free fatty acids, and therefore, the degree of dissociation of the fatty acid from the celite particles.

Temperature dependent uptake experiments

The temperature of the sea water solution was maintained using a copper-coil cooled/heated water reservoir around the 6 liter glass vessel The cooling or heating water in the coil was circulated from a Forma Scientific water bath. The temperature of the sea water was thermostatically maintained with $\pm$ 1°C of the desired temperature. The studies of uptake were the same as described previously.

Turnover experiment

The animals were prepared as for the uptake experiments but the shells were not removed. The oysters were placed in a glass vessel with 4 liters of filtered sea water. Seven mg of sodium [^{3}H]acetate (5 mCi) was added to the sea water. After 18 hours, the animals were removed, washed in sea water, and placed in a glass vessel with 4 liters of non-radioactive sea water. Groups of 3 oysters were removed at 0.5, 1.0, 2.0, 3.0, and 5.0 hours and extracted by the chloroform-methanol method.

Aliquots of the extract were separated on TLC plates and counted and quantitated.

Resolution of Lipids

Thin layer chromatography

The neutral lipid classes were resolved by thin layer chromatography techniques and identified by comparison with standard compounds. The 250 μm silica gel plates were divided into 2 cm channels and activated by heating for 30 minutes at 120°C. Lipid extracts (100 or 200 μl) were applied with an Oxford pipetter 1.5 cm from the bottom of the plate and the solvent evaporated with a stream of hot air. The plates were developed in a PE/EE/HOAc (petroleum ether (30 - 60°C)/diethyl ether/acetic acid) solvent (90/10/1) for approximately 1 hour. The solvent was removed with a stream of air and the material on the plate was visualized with either iodine vapor or by charring with sulfuric acid (Mangold, 1960). This TLC solvent system completely resolved the neutral lipid classes of *Crassostrea* and the sea water extracts into sterols, triglycerides, alkyl diglycerides, wax esters and sterol esters.

The phospholipid classes were resolved on silica gel plates activated for 30 minutes at 80°C, and developed in a chloroform/methanol/water solvent (65/25/4) for approximately 80 - 100 minutes (Wagner, 1961). The plates were channeled and the extracts spotted in the same manner as the neutral lipids.

A better separation of the phospholipids could be achieved when the neutral lipids were first removed from the extract by column chromatography over a 1 x 10 cm Hi-Flosil column. The extract, in chloroform, was applied at the top and all the neutral lipids eluted with 2 column

volumes of $CHCl_3$. The phospholipids were then stripped from the column bed with methanol. After removing the methanol in flash evaporator, the extract was taken up into chloroform, spotted on a TLC plate, and run in the polar lipid TLC system described previously.

For complete resolution of phospholipids, a two-dimensional method was used in which the plate was developed in chloroform/methanol/water/ 28% aqueous ammonia (130/70/8/0.5) in one direction and chloroform/ acetone/methanol/acetic acid/water (50/20/10/10/5) in the 90° direction (Parsons and Patton, 1967).

Table 3 lists the visualization reagents which were employed in the identification of the neutral and phospholipid compounds.

These reagents, together with a saponification step for esterified compounds (Stahl, 1969), permitted the identification of the lipids found in the oysters.

Quantitation of lipid material

The lipids following separation by thin layer chromatography were quantitated using the method of Amenta (1964). The lipid on the TLC plate was visualized with I_2 vapor and scraped into glass tubes; 1 or 2 ml of a 8.5 μM solution of potassium dichromate in concentrated sulfuric acid were added. The tube was stoppered and heated at 90 - $100^{\circ}C$ for 45 minutes in a water bath with constant agitation. The tubes were removed, allowed to cool, and centrifuged in a clinical centrifuge to pellet the silicic acid. A 0.5 ml aliquot of the supernatant was removed, diluted with 10 ml of distilled water, and stirred to mix thoroughly. The absorbance of this solution was determined at 350 nm, comparing against a water blank. The difference in absorbance between a

Table 3. Visualization Reagents for TLC.

Reagents	Function	Reference
I_2 Vapor	General Screen	Lettschart and Fluck, 1956
Chromic Acid-Sulfuric Acid	General Screen	Bertetti, 1954
Rhodamine B	General Screen	Kaufmann and Budwig, 1951
Ninhydrin-Butanol	Amino-Phospholipid and Glycolipids Containing Glucosamine	Fahmy *et al.*, 1961
Chromic Acid-Glacial Acetic Acid (1:1)	Cholesterol Cholesterol Esters	Michalec, 1956
Ammonium Molybdate	Phospholipids	Hanes and Isherwood, 1949
Hydroxyl Amine-Ferric Chloride	Esterified Carboxylic Acids	Whittaker and Wijesundera, 1952

standard tube and a sample tube was compared to curves for cholesterol, tripalmitin, cholesteryl stearate, dimyristyl phosphatidyl choline, and palmitic acid.

Scintillation counting

The aqueous samples of sea water, wash, and filtered sea water from the uptake experiments were counted in Aquasol (1 ml aqueous sample added to 10 ml scintillant). The chloroform and methanol layers of the extracted material were counted in Aquasol at 200 ul/10 ml to reduce quenching of the organic solvents. All samples were counted in a sub-ambient Packard Tricarb at $0^{\circ}C$ and compared to suitable standards. The double label experiments were counted in a refrigerated Nuclear Chicago counter in the double label mode.

The radioactive lipids, once separated on TLC plates, were either counted directly in a Packard TLC radioscanner or the lipids were scraped off the plates directly into 5 ml of Toluene POPOP and counted in a refrigerated Packard Tricarb. The efficiency of this method is much less than reported by others (Kritchevsky and Malhotra, 1970) but it is much simpler than a solvent extraction-Aquasol counting procedure.

All scintillation counting work was corrected for background and counting efficiency by coincidence counting with [^{14}C]toluene, [^{14}C]benzoate, and [^{3}H]water standards purchased from Packard Instruments and diluted as required.

Fatty Acid Methylation--GC Separation

Preparation of methyl esters

The fatty acids were methylated according to the method of Stoffel

et al. (1959). To the fatty acid samples separated by thin layer chromatography were added 4 ml of 0.24 N HCl in methanol and 0.5 ml of dry benzene. The solution was refluxed at 80 - 100°C for 2 hours in a ground glass apparatus fitted with a $CaCl_2$ drying tube. The reaction mixture was cooled to room temperature and 9 ml of H_2O were added to quench the reaction. The aqueous solution was extracted 3 times with petroleum ether (30 - 60°C) and this extract was dried over Na_2SO_4 and $NaHCO_3$. The petroleum ether was added to a sublimation apparatus (a side arm test tube fitted with a cold finger) and evaporated. Then the fatty acid methyl esters were sublimed in 200 μm vacuum and at 60 ± 2°C. The methyl esters were rinsed with hexane from the cold finger into a small vial and were injected into the GC.

A second procedure (Hoshi *et al.*, 1973) was employed for methylation at room temperature. It required 0.2 ml of the sample fatty acid in chloroform, 0.2 ml of 20 mM cupric acetate in methanol and 1.0 ml of 0.5 N HCl in methanol. The solution was allowed to react at room temperature for 30 minutes and then, after the addition of 0.4 ml H_2O, was extracted 3 times with 2 ml of petroleum ether (30 - 60°C). The extracts were pooled, washed with H_2O, evaporated to dryness, and redissolved in hexane before injection into the GC.

Saponification and methylation

The direct saponification and methylation of fatty acids in the lipid extracts were performed using a modification of the procedure of Christopherson and Glass (1971). The lipid extracts were added to Teflon-capped tubes and the solvent evaporated to dryness with N_2 gas. Five ml of 2 M potassium hydroxide in methanol solution were added and heated at

40 - 50°C for 30 minutes. After the addition of 6 ml of water, the solution was extracted 2 times with 5 ml of petroleum ether (30 - 60°C). The ether solutions were pooled, evaporated to dryness, taken up in 200 - 500 µl of hexane, and stored in small 1 ml vials with Teflon-lined screw caps. Aliquots (5 - 10 µl) of this hexane solution were injected into the gas chromatograph.

Gas Chromatography

The fatty acid methyl esters were run on 2 different column systems in a Beckman GC-65 gas chromatograph with N_2 as the carrier gas and dual hydrogen flame detector. An organo-silicone polymer, EGSS-X, at a 10 percent loading on 100/120 Gas Chrom P-Support in 2 m x 4 mm glass column was run isothermally at 180°C. This column resolved the 16-C and 18-C series of fatty acid esters, but even at its maximum temperature the higher boiling poly-unsaturated acid esters were not eluted. Therefore, initial experiments were run on dual Apiezon-L columns at a 2.25 percent loading on 100/120 Gas Chrom G in 1.3 m x 4 mm glass columns. The gas chromatograph was programmed from 170 - 275°C at 1.5°C/minute at which temperature the higher boiling esters were eluted.

The later determinations were done on an EGSS-X column run isothermally at 174°C with a 45 ml/minute flow rate and at 190°C with a 60 ml/minute flow rate. At 174°C EGSS-X columns resolve lower boiling fatty acids and the 18 series; at 190°C the long chain unsaturated acids are eluted. This column does not suffer from large bleed rates that Apiezon columns show at higher temperatures; therefore, almost all acids reported in 10-C - 22-C range can be resolved without difficulty (Applied Science, 1973).

DATA AND DISCUSSION

Lipids and Free Fatty Acids in Sea Water

The sea water of the Shell Mound estuary was sampled in 8 liter quantities for determination of total lipids, compound lipids, and specific free fatty acids during the spring, summer, and fall. The water was extracted as described in the section on methods and fractionated by thin layer chromatography. The results of the neutral and phospholipid chromatography of the June 21, 1974, October 31, 1974, and the March 31, 1975 samples appear in Figures 5 and 6. The absence of phospholipids from the June 21 and March 31 extracts and their presence in the chloroform extract of the October 31 sample can be attributed to the use of petroleum ether (30 - 60°C) for their extraction. Jeffrey has shown that a complete polar lipid extraction can be achieved only with chloroform (Jeffrey, 1970). However, because we were interested primarily in the uptake of free fatty acids, the use of petroleum ether was justified. Preliminary experiments with ^{14}C labeled fatty acid revealed that better than 90 percent extraction of the label could be effected with 1 extraction step with petroleum ether (30 - 60°C) and 3 subsequent washes of the extract with 2 N HCl.

By comparison of the lipid extracts with known standards, those lipid classes which are separated by TLC can be identified and quantitated by the methods previously described. The results appear in Table 4. For the June 21 extract the majority of the lipid appears to be in the free fatty

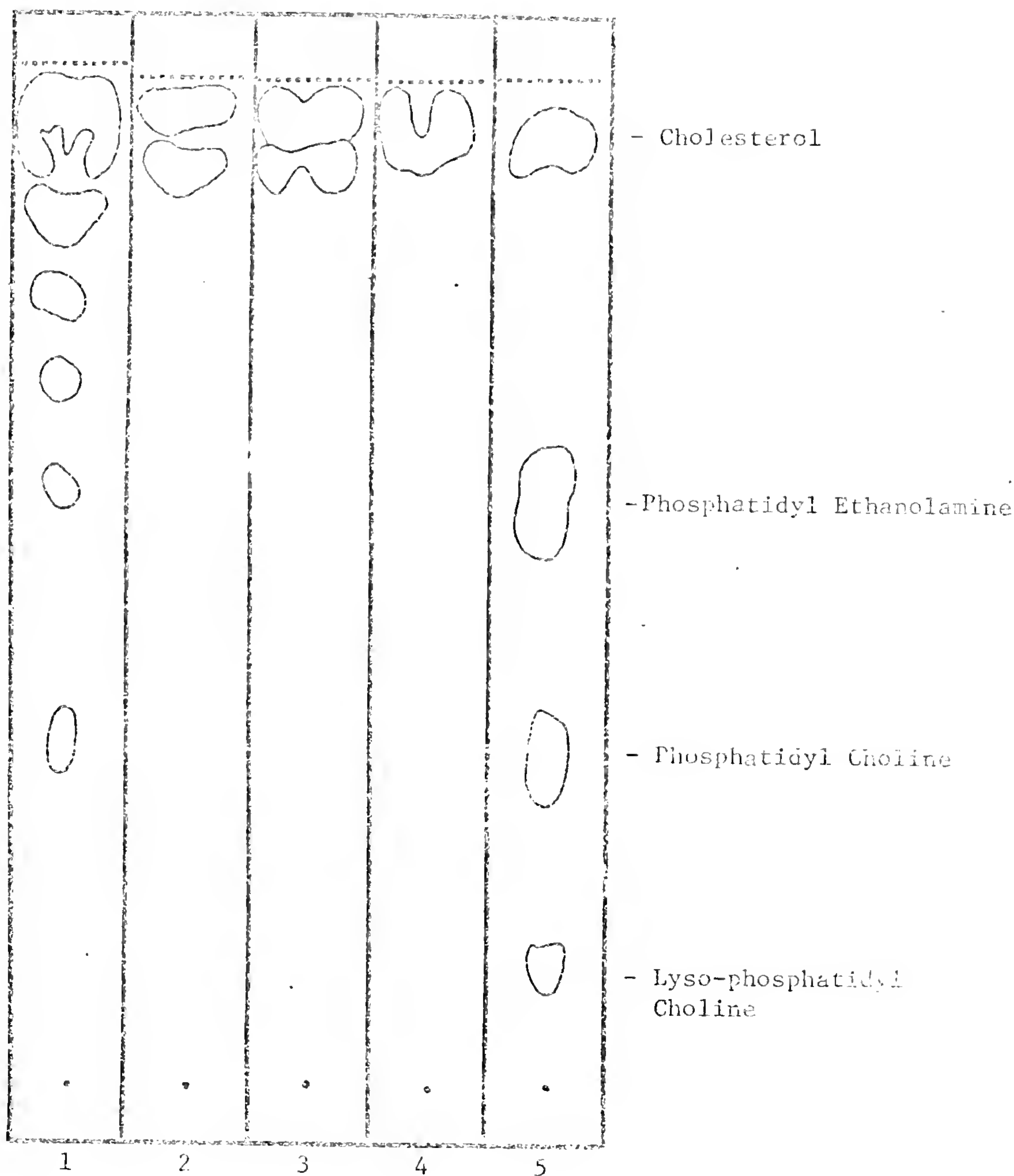


Figure 5. Separation of Polar Lipids in Sea Water Extracts.

Sea water was extracted with petroleum ether (June 21) or chloroform (October 31) and 200 µl aliquots run on the polar lipid TLC system. 1, BFSW (bacterially filtered sea water) from Oct. 31; 2, NBFSW (non-bacterially filtered sea water) from June 21; 3, BFSW June 21; 4, cholesterol standard; 5, phospholipid standard with standards listed on the right margin. Dotted line at the top: solvent front.

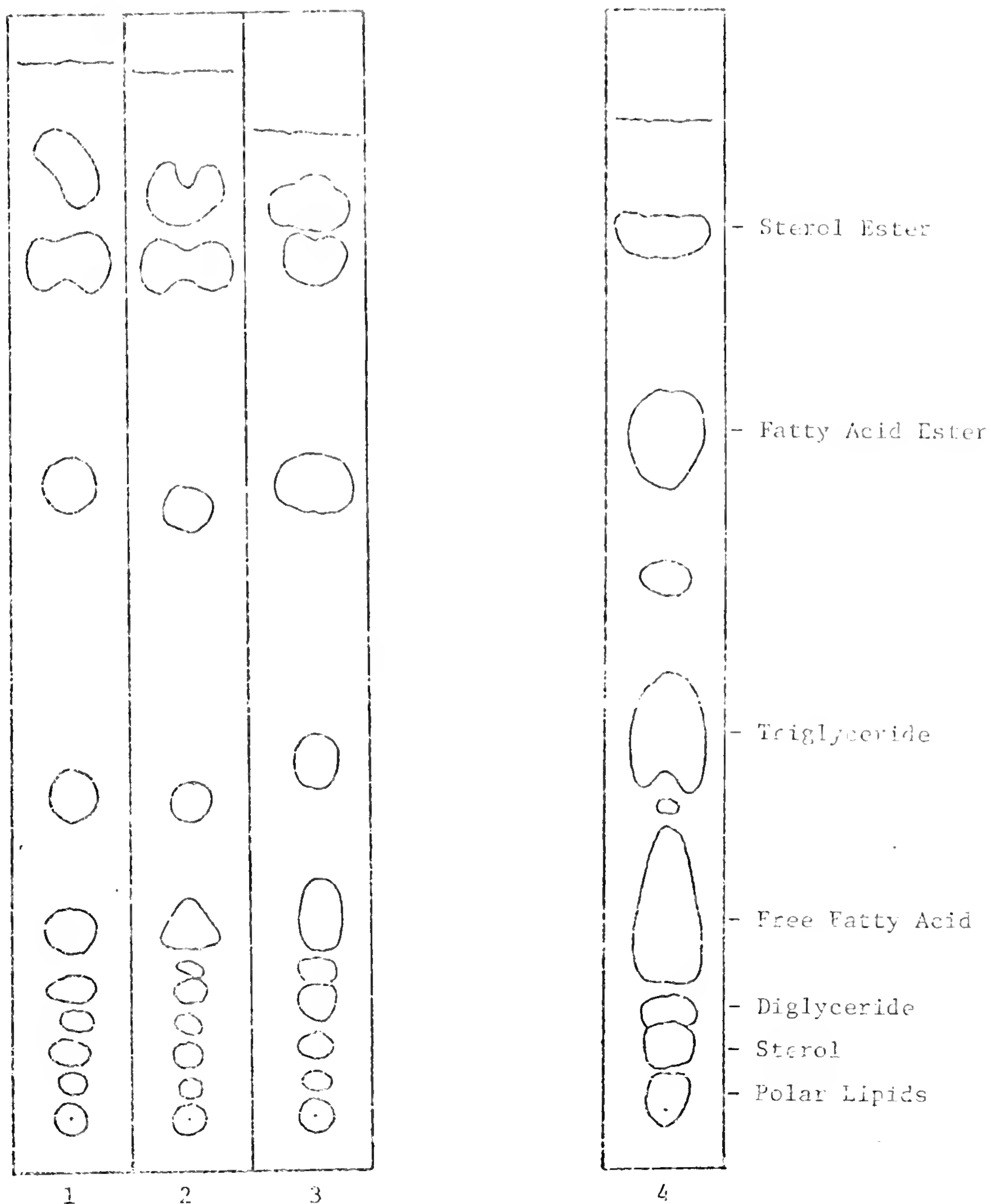


Figure 6. Separation of Neutral Lipids in Sea Water Extracts.

Sea water was extracted with petroleum ether and 200 µl aliquots run on the neutral lipid TLC system. 1, NBFSW June 21; 2, BFSW from June 21; 3, BFSW from March 31; 4, standard neutral lipid mixture with components listed in the right margin. Solid line at the top was the solvent front.

Table 4. Concentrations of Extractable Specific Lipids in the Sea Water Collected on June 21, 1974 (Extract A) and March 31, 1975 (Extract B).

Rf[a]	Lipid Class	Concentrations in Sea Water in μg/l	
		Extract A	Extract B
0.04	Monoglyceride	Trace	Trace
0.06	Sterol	14	32
0.12	Diglyceride	Trace	Trace
0.21	Free Fatty Acid	77	56
0.36	Triglyceride	31	47
0.65	Alkyl Diglyceride	36	38
0.88	Sterol Esters	13	62
0.94	Hydrocarbons	104	53
	Total	275	288

[a]Relative migration of lipid class on a neutral lipid chromatographic system relative to the solvent front migration.

acid and hydrocarbon fractions: together they comprise greater than 50 percent of the total lipid. The concentration of the free fatty acid, 77 μg/liter, compares favorably with previous determinations reported in the introduction. For the June 21 extract, the free fatty acids were eluted from the silica gel and methylated. The methyl esters were run on the gas chromatograph with the results shown in Figure 7. The fatty acid distribution is similar to that obtained by Testerman (1972).

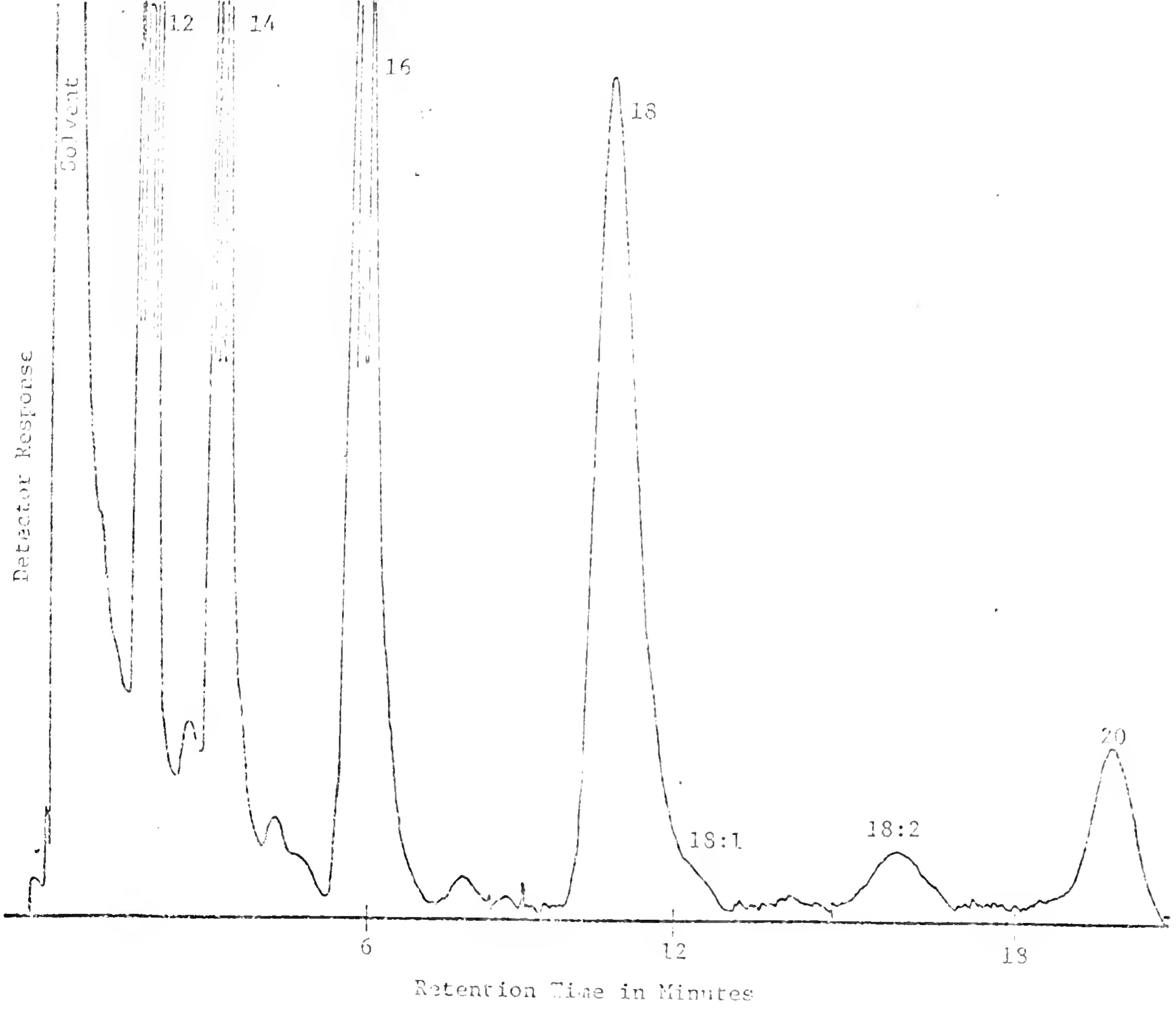
The percentage of each fatty acid present, corrected for differences in detector sensitivity, appears in Table 5. From these data, the predominant fatty acid in the sea water at Shell Mound appears to be palmitic acid. The notable absence in our work of those long chain unsaturated acids, 18:3, 18:4, 20:1, 20:2 found by others (Jeffrey, 1970), can be attributed to the complete removal of all algae and bacteria prior to extraction, for these acids are characteristic of such organisms.

In the sea water extracts from Shell Mound, the fatty acids which are characterized are free by definition of the experimental methods used. The saponification step, used by others, has been intentionally eliminated from the extraction-separation-methylation steps so that only those fatty acids which are free in solution are extracted. The inclusion of a saponification step before methylation by Testerman, Jeffrey, and others was intended to break up any lipid organic aggregates in the sea water so that complete extraction might be effected.

The data in Table 4 indicate that large amounts of free fatty acids are present in the sea water at Shell Mound and that these might be expected to be readily available for removal by any animal possessing an uptake system which functions at these naturally occurring concentrations.

Figure 7. Gas Liquid Chromatograph of Fatty Acid Methyl Esters Prepared from Sea Water Extract of June 21.

The fatty acid esters were labeled as identified from comparison to standard acid esters. The sudden shift in detector response in the 12-, 14-, and the 16-carbon peaks represented a 4 times decrease in detector sensitivity. The esters were run on an EGSS-X column at 174°C and a nitrogen carrier gas flow of 45 ml/min.



Solvent
12
14
16
18
18:1
18:2
20
Detector Response
6
12
18
Retention Time in Minutes

Table 5. The Free Fatty Acids in the June 21 Sea Water Extraction.

The retention time and percent composition of the fatty acid methyl esters are from GC run Figure 7 and corrected for detector response.

Carbon Number	Retention Time in Minutes	Percent Composition
C-12	2.2	27.6
C-14	3.5	8.7
C-16	6.1	35.5
C-18	10.8	22.1
C-18:1	12.1	2.8
C-18:2	16.0	1.0
C-20:0	20.2	2.3

Uptake of Palmitic Acid

The ability of oysters to remove palmitic acid from natural sea water solutions was investigated with [1-^{14}C]palmitate at a concentration of 2.8×10^{-7} M. The background concentrations of total lipids and free fatty acids were determined and the specific activity of the palmitate was computed from the amount of isotope and carrier used. In each set of experiments sea water from the same sample was used throughout to minimize any differences in salinity which might have affected the uptake. Stephens has shown that the salinity of the sea water drastically affects the uptake of amino acids by coelenterates (Stephens, 1963). Natural sea water was chosen so that any trace elements or dissolved organics which are not present in artificial mixtures, but which may affect uptake processes, would be present. With artificial salts, in the quantities needed to make a 28 parts per thousand salt solution, organic contaminants will be present in large concentrations compared to 10^{-7} M fatty acids. Even reagent grade salts could contain significant quantities of non-extractable lipid and hydrocarbon impurities.

In the experiments on lipid uptake by oysters, the lipid label might be expected to adhere to the mucus and the soft tissues of the animals. A satisfactory method of removing this adventitiously adsorbed material had to be developed. In the early work with hydrocarbon uptake by Lee *et al.* (1972), a methanol wash was employed, but we found this severely dehydrated the animals and could possibly cause the removal of more than just adsorbed material. A wash procedure in sea water saturated with the experimental fatty acid was found to exchange effectively any simply adsorbed material (see Figure 8). The loss of label could then be monitored by sampling the wash solution at 30 to 60 minutes. In all

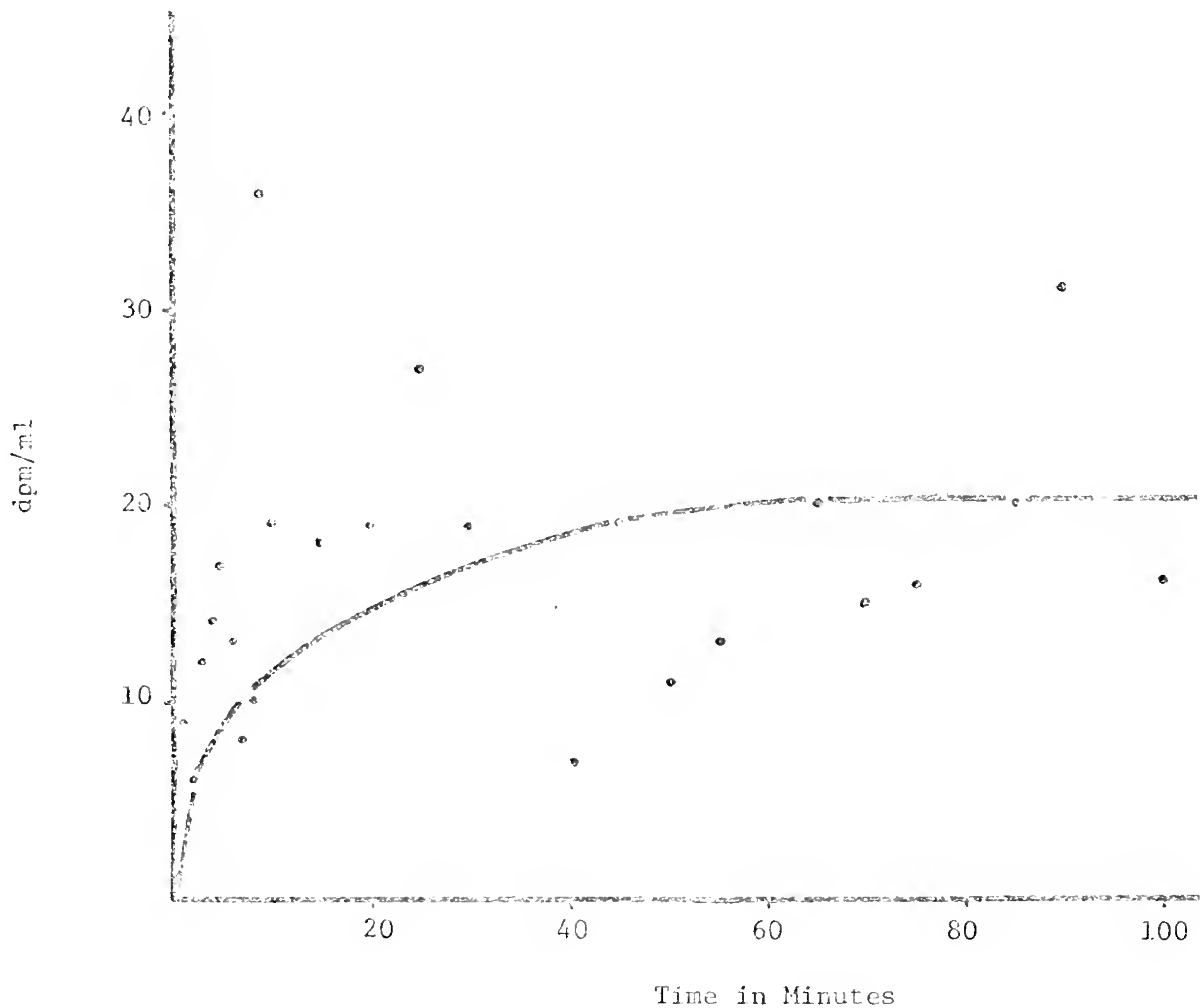


Figure 8. Diffusion of Adsorbed Labeled Fatty Acid into a Sea Water Wash Saturated with Unlabeled Palmitate.

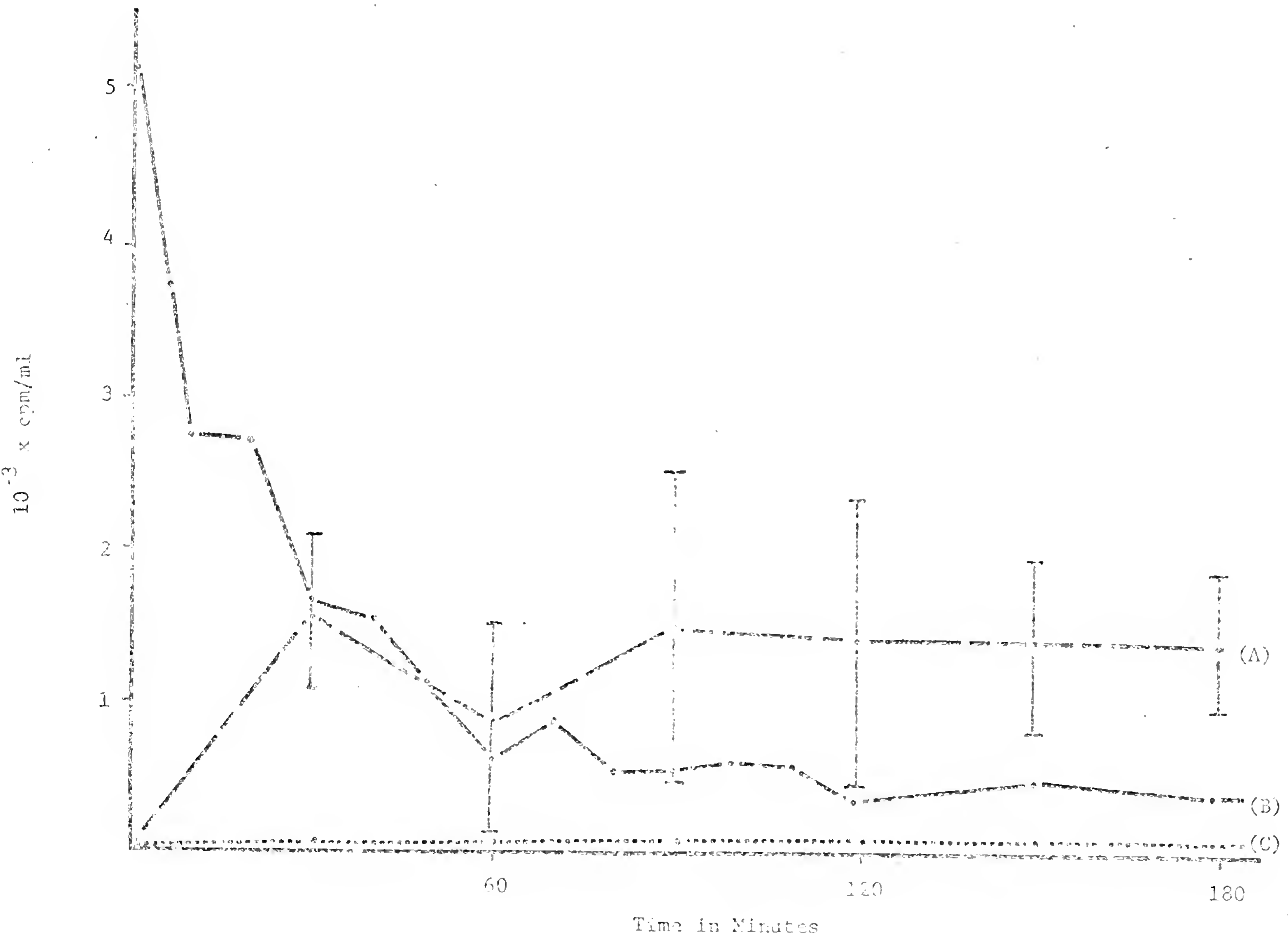
Animals labeled with palmitate for 240 minutes were placed in 100 ml of filtered sea water containing a saturating amount of palmitate. The sea water was sampled in 1 ml aliquots and counted in 10 ml of Aquasol. Animals were labeled with 10 µCi ^{14}C palmitate at a concentration of 2.8×10^{-7} M.

uptake experiments such a wash step was employed and found to be satisfactory.

In order to monitor fatty acid uptake by the oyster, procedures involving lipid extraction were used. Experiments with tissue solubilizers proved unsatisfactory with animals as large as oysters, since their weight (3 grams average in experimental animals) is above the upper limits of the tissue sample weight for such alkaline solubilizers. Although the work with nereid and pogonophoran species utilized such a digestion step to sample single animals or groups of animals, the oysters had to be extracted. Preliminary experiments with petroleum ether (30 - 60°C) extraction techniques on aqueous homogenates proved unsuccessful due to the stable emulsion formed at the organic-water interface. After using a step involving perchloric acid, the precipitated protein could be pelleted along with included lipid material. This pellet could then be isolated and extracted with ethanol-ether (3:1). The lipids were solubilized and the protein remained as a precipitate. Using tracer techniques of labeled fatty acids, this method of Bloor (1928) was shown to be 75 percent effective in extracting lipids from the oyster aqueous homogenate. The results of an uptake experiment at a palmitate concentration of 2.8×10^{-7} M using the saturated wash step and the Bloor extraction method appear in Figure 9. The major loss of label from the sea water occurs in the first 60 minutes and is coincident with the appearance of the label in the lipid extract. The loss of labeled material from sea water was shown to be a function of the living animals and was not due to adsorption onto the shells or the walls of the glass vessel by carrying out a blank experiment with a similar weight of oyster shells cleaned and washed according to the methods for whole animals

Figure 9. The Uptake of Palmitic Acid Measured Using the Bloor Extraction Technique.

The radioactivity appearing in the lipid extract (A) was plotted as the average incorporation of 3 or 4 animals plus or minus 1 standard error of the mean. The radioactivity in 1 ml aliquots of the sea water was plotted as (B). The label that was removed in the wash step was plotted as (C). Concentration of palmitate was 2.8×10^{-7} M with 10 μCi of ^{14}C.


10^{-3} x cpm/ml
5
4
3
2
1
(A)
(B)
(C)
60
120
180
Time in Minutes

(see Figure 10). In this control experiment less than 10 percent of the label was removed from the water.

The effect of 200 mM sodium cyanide on the uptake of 2.8 x 10^{-7} M palmitate was investigated. As seen in the data in Figure 11, the radioactivity in the lipid extract remained very low and the label in sea water remained constant, indicating that the background adsorption of lipid onto the animals in the absence of uptake was indeed small. The animals were not killed by the cyanide for at least 2 hours but their respiration was severely inhibited. A large concentration of cyanide was used because of the oyster's known ability to carry on anaerobic metabolism (Hammen, 1969).

The Bloor method of extraction did not permit the quantitation of the lipid classes because of the hydrolysis and esterification that occurred in the acidic ethanol/ether extraction step. A chloroform-methanol extraction (Bligh and Dyer, 1959) as described in the methods section was therefore utilized for all further uptake investigations.

The variability of the amount of radioactivity in the sea water at time zero in Figures 9 and 10 was ascribable to an artifact in the addition of the labeled acid to the sea water. At first the labeled fatty acid, dissolved in ether or benzene, was added to a glass petri dish. The solvent was removed with nitrogen, and the petri dish was placed into the reaction vessel. The amount of label that dissolved in the sea water was dependent upon the temperature, the solubility of the fatty acid, and the degree of agitation of the solution. Of these variables, the agitation was least reliable, so a method involving direct addition of the labeled fatty acid dissolved in ethanol was devised. This was shown in preliminary experiments to be a simple and most reliable method of

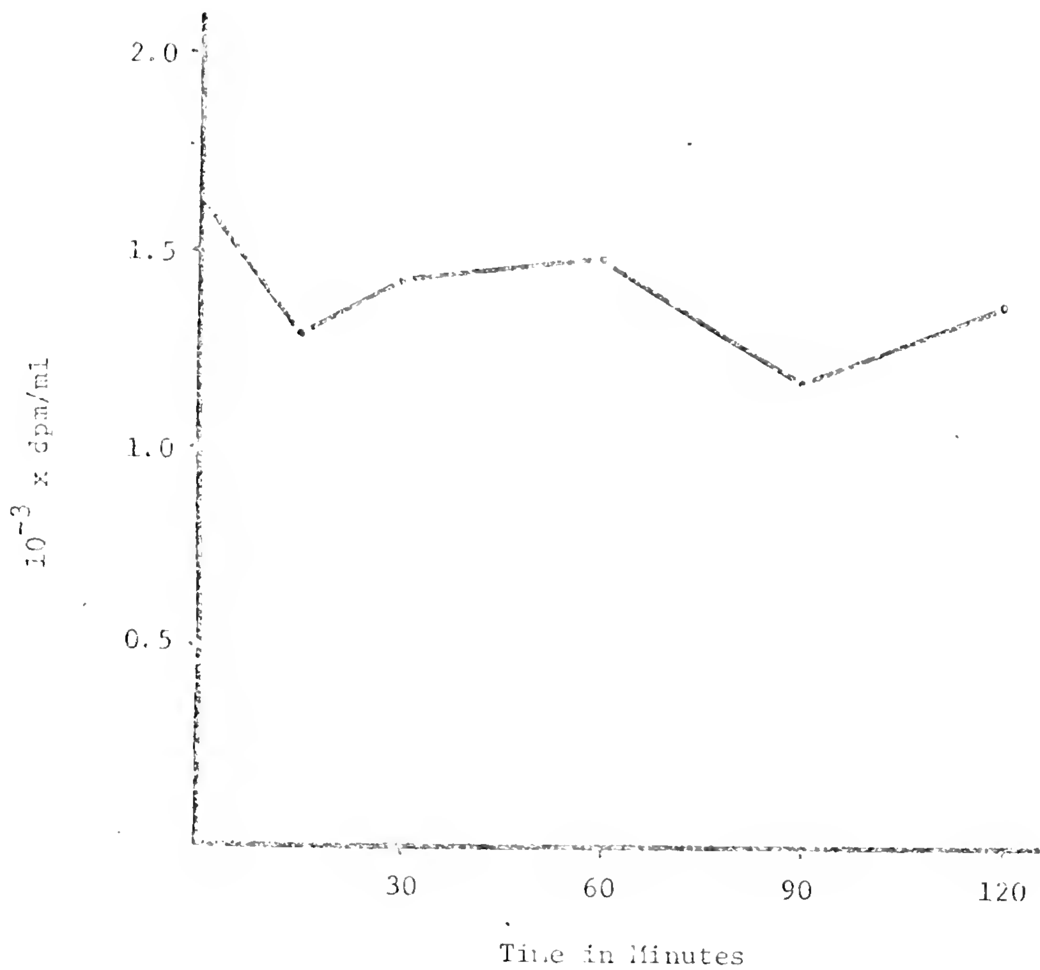
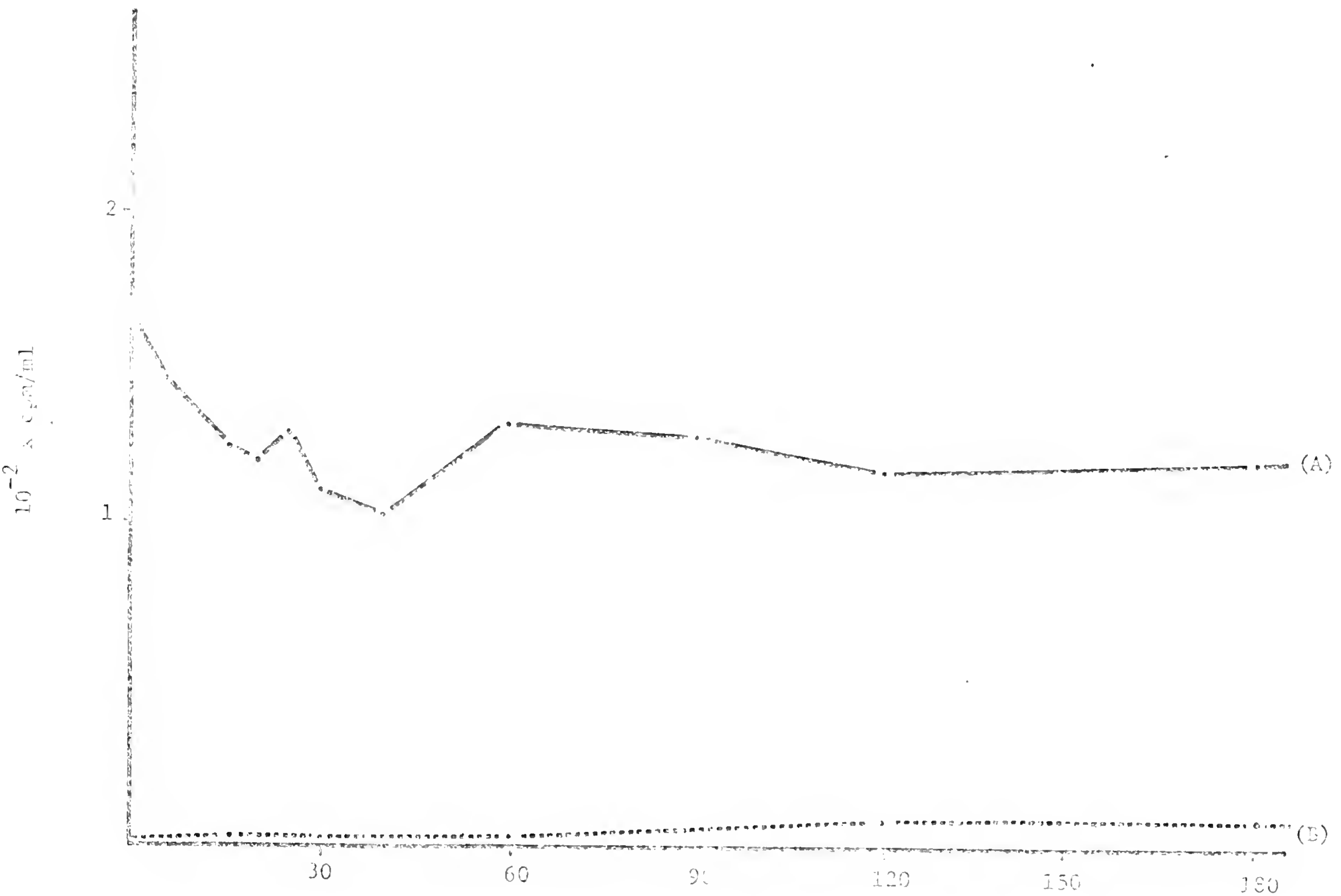


Figure 10. Removal of ^{14}C Fatty Acid by Background Absorption onto Shells and Glass Surfaces.

The loss of labeled fatty acid from the sea water in a vessel containing 2.8×10^{-7} M palmitate with 10 μCi ^{14}C isotope and the shells of the same number of animals as normally used in the uptake experiments was plotted against time. The shells were washed according to the methods used for whole live animals.

Figure 11. The Uptake of Palmitic Acid in the Presence of 200 mM Sodium Cyanide.

The radioactivity in the sea water (A) and the lipid extract of the animals (I) were plotted against time. The sodium cyanide and the labeled fatty acid were added at time zero. Palmitic acid concentration was 2.8×10^{-7} M with 10 μCi ^{14}C isotope. The lipids were extracted from the animals using the Bloor method.


(A)
(B)
2
1
30
60
90
120
150
180
Time in Minutes

dispersing the acid. The problems of variability of initial label concentration in sea water were reduced significantly without any side effects of the ethanol on the animals.

From the early series of experiments involving long-term uptake of up to 3 to 6 hours, it was apparent that the uptake maximum occurred at about 1 hour with a subsequent leveling off of the radioactivity in the sea water and lipid extract pools. That this leveling off was due to the removal of most of the free fatty acids by the animals was shown in a repeated pulse experiment in which the labeled fatty acid, dissolved in ethanol, at a concentration of 2.8×10^{-7} M (palmitate) was added at time zero and at 180 minutes. The results, shown in Figure 12, indicate that the labeled fatty acid concentration in the sea water decreases rapidly in the first 3 hours, coincident with the appearance of label in the lipid extracts of the animals. After the second pulse at 180 minutes, the fatty acid level in the sea water again decreases with a concomitant increase of labeled acid in the lipid extracts. The regular differences in the counts in the lipid extracts were caused by the periodicity of valve opening and closing in the animal's normal feeding cycle, but the data show that during the first 2 hours almost all the label is removed.

The presence of $^{14}CO_2$ in the sea water, shown in Figure 12, indicates that the animals were metabolizing some, at least, of the fatty acid removed. The gradual increase in slope after the second addition of labeled fatty acid may indicate that the breakdown of free fatty acid is proportional to the amount of the fatty acid removed.

To avoid irregular valve opening, a method of synchronization was employed. The best method took advantage of the normal behavior of animals exposed to air during the tidal cycle. When experimental

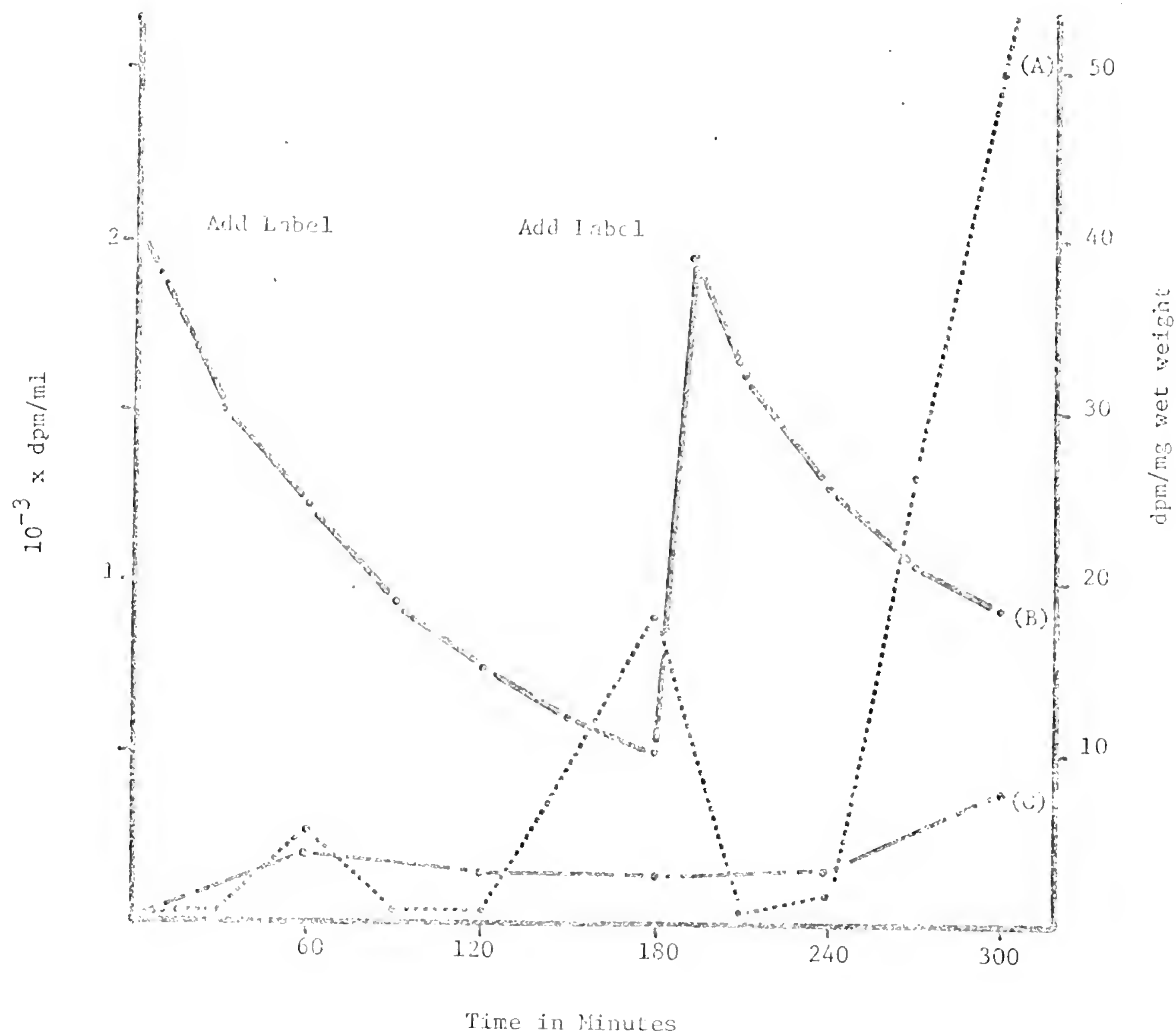


Figure 12. The Uptake of Palmitate, Double Addition of Label.

The radioactivity in aliquots of the sea water (B), the lipid extract (A), and CO_2 in an aliquot of sea water (C) were plotted against time. The concentration of palmitate in the sea water was 2.8×10^{-7} M after the first addition at time zero and [illegible].8 x 10^{-7} M after the second addition. A total of 20 μCi of ^{14}C isotope was used; 10 μCi at each addition. The label was added in ethanol. $^{14}CO_2$ was counted after trapping in hyamine hydroxide and adding to Aquasol. The lipid was extracted by the chloroform/methanol method and plotted as the dpm/mg oyster tissue in each sample.

animals were removed from the holding tank, cleaned as usual, and left to dry in the air for 3 hours, then placed in the radioactive free fatty acid containing sea water, the shells opened almost immediately. Opening in the first few minutes is essential to the determination of initial rates of uptake necessary for kinetic determinations. The variability of the data even after such a synchronization attempt by an out-of-water phase necessitated experiments in which the top shells were removed.

When the upper shell was removed carefully and the muscle, gill, mantle, and pericardial tissue were not traumatized, the uptake of label into the animal was more reproducible (see Figure 13). The maximum labeling of the lipid pools was linear with time and occurred during the first 90 - 120 minutes.

The temperature dependence of the uptake process was investigated using the experimental apparatus described in the methods section. The temperature dependence of palmitate uptake at a 2.8×10^{-7} M concentration was investigated at temperatures of 20, 25, 30, and 35°C. The results appear in Figure 14 as the average uptake for two experiments at each temperature. The inverse dependence of the uptake on temperature which is seen in the experiments is similar to what has been reported before in uptake experiments on other marine animals (Snick, 1975). The uptake of fatty acid at 20°C was virtually zero with the response of the animals being a decreased shell opening cycle. The temperature dependent uptake therefore may represent a physiological response of the animals to temperature and not a response of the uptake machinery to temperature.

<u>Celite Uptake Experiments</u>

The major assimilatory pathway utilized by the oyster is filter feeding via the ciliary apparatus of the gills. While uptake of free

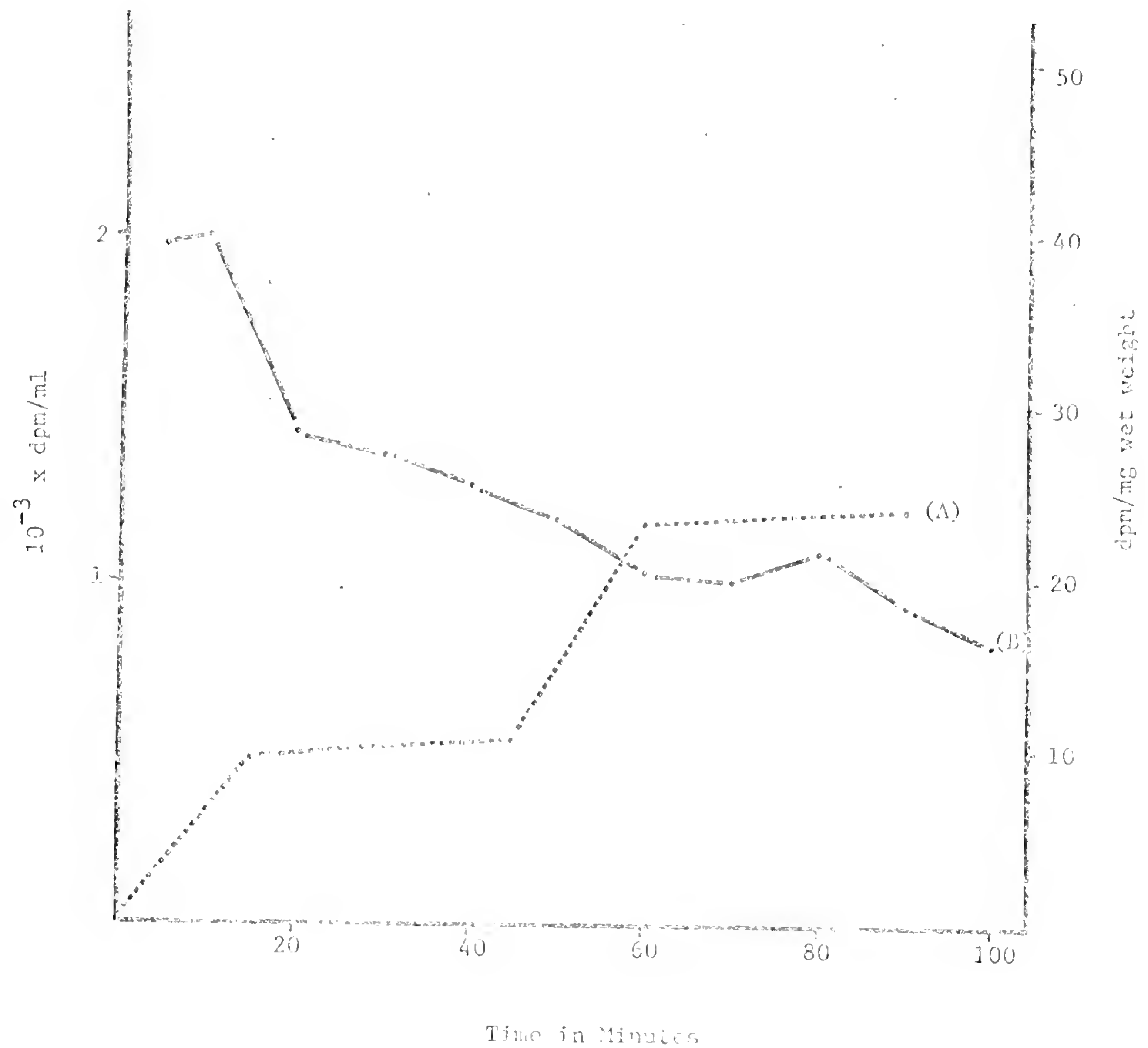


Figure 13. The Uptake of Palmitic Acid by Open Shell Animals.

The loss of labeled fatty acid from the sea water (B) and the appearance of label in the lipid extract (A) was plotted against time of exposure. Animals with the upper valve removed were placed in 4 liters of sea water with a palmitate concentration of 2.8 x 10^{-7} M and containing 10 µCi total ^{14}C isotope. The lipids were extracted by the chloroform/methanol method and the dpm/mg wet weight of the oysters plotted.

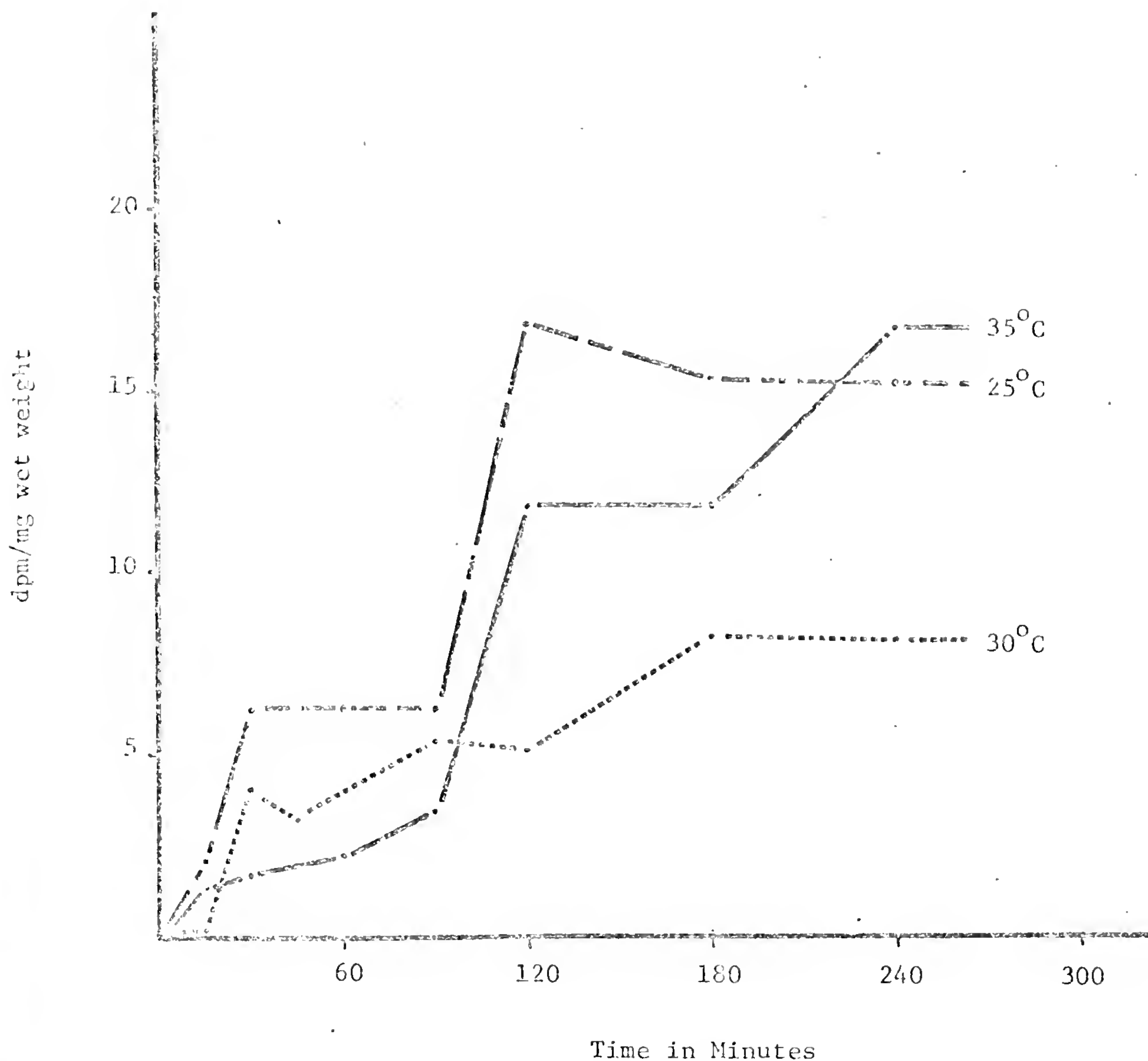


Figure 14. Temperature Dependent Uptake of Palmitate.

The radioactivity in the lipid extract of the animals was plotted for three different temperatures. The concentration of palmitate was 2.8×10^{-7} M with 10 µCi total ^{14}C isotope in all experiments. Each point was the average of two experiments at each temperature. The results for 20°C were negative to 300 minutes. The lipids were extracted with chloroform/methanol.

fatty acids can be established, it may represent merely the removal of fatty acid particles through prior adsorption on a mucus thread followed by the ciliary transport of this thread through the digestive apparatus.

In the autoradiographic work by Péquignat (1972), the labeled amino acids which were taken up from the sea water by *Mytilus edulis* were first found in the gill, the mantle, and the foot. Only after a much longer period of time were silver grains on the photographic emulsions found in positions corresponding to the digestive tract and to the mucus secretions on the gills. In order to establish the time sequence of particulate filtration in oysters, a preliminary experiment with celite of 50 µm particulate size was used. An aniline dye, oil red O, in ether solution was adsorbed onto the celite particles by successive washes with the ether solution followed by evaporation of the solvent. Fifty mg of dyed particles were added to 4 liters of sea water and the extent of uptake determined by visual inspection before and after dissection of the animals. The presence of red particles was noted on the external surfaces and in the digestive tract. Aliquots (5 ml) of the sea water in which the particles were suspended were extracted with petroleum ether and the absorbance at 525 nm (the maximum for oil red O) determined. The results, shown in Table 6, indicate that particles are adsorbed onto the mucus thread within the first 30 minutes and into the digestive tract after 90 minutes. Because oil red O is not digested by the animal, it is sorted and appears in the feces after 90 - 120 minutes.

Knowing that celite particles are removed from sea water by oysters, the uptake of celite-adsorbed [^{14}C]palmitate was investigated. Ten µCi of ^{14}C labeled fatty acid was adsorbed onto 50 mg of 50 µm celite particles with successive ethyl ether evaporations as described in the

Table 6. Localization of Oil Red O Celite Particles Removed from Sea Water by Experimental Animals.

Localization of Celite Particles	Time of First Appearance (Minutes)	Absorbance (525 nm[a]) of Pet Ether Extract of Sea Water
Sea Water	0	0.299
Mucus Thread	30	0.232
Oral Cavity	60	0.291
Digestive Tract	90	0.218
Anus, Feces	90-120	0.147

[a]Five ml sea water extracted with petroleum ether 30 - 60°C and read into a visible spectrophotometer.

methods section. The uptake of this labeled celite was investigated with whole animals. Figure 15 shows that the total radioactivity in the sea water decreases as the incorporation into the lipid extract increases, with the exception that the appearance of the label is delayed by some 45 minutes when compared with the uptake of similar concentrations of freely soluble palmitate at 2.8×10^{-7} M. This delay has been seen in every celite particle uptake experiment run with oysters. It represents a delay in the incorporation of labeled acid particles into the animal by the filter feeding apparatus when compared to the uptake of non-particulate fatty acid. These results are, therefore, similar to Péquignat's findings on the uptake of amino acids by *Mytilus edulis*, the label appearing in the gut much later than that which appears in the soft tissues.

The concentration of free acids in the sea water was determined by the dpm/ml in a 0.45 µm GF/A filtered aliquot. From Figure 15 there appears to be a constant amount of radioactivity in the filtrate indicating only minor dissociation of the particle-bound fatty acid into free acid.

The uptake of celite-adsorbed palmitate at 2.8×10^{-7} M was also investigated using the open shell animals (Figure 16). There is a difference between their accumulation of label and that in the whole animal experiment. The organism can remove the label very efficiently and at a linear rate up to 90 minutes. If this celite uptake is compared to the uptake of 2.8×10^{-7} M palmitate for open shell animals (Figure 17), the rates (slopes) of uptake are different. The use of a concentration factor (Taylor, 1969) allows comparison of the two different sea water concentrations as dpm/ml of sea water//dpm/mg of animal tissue in the

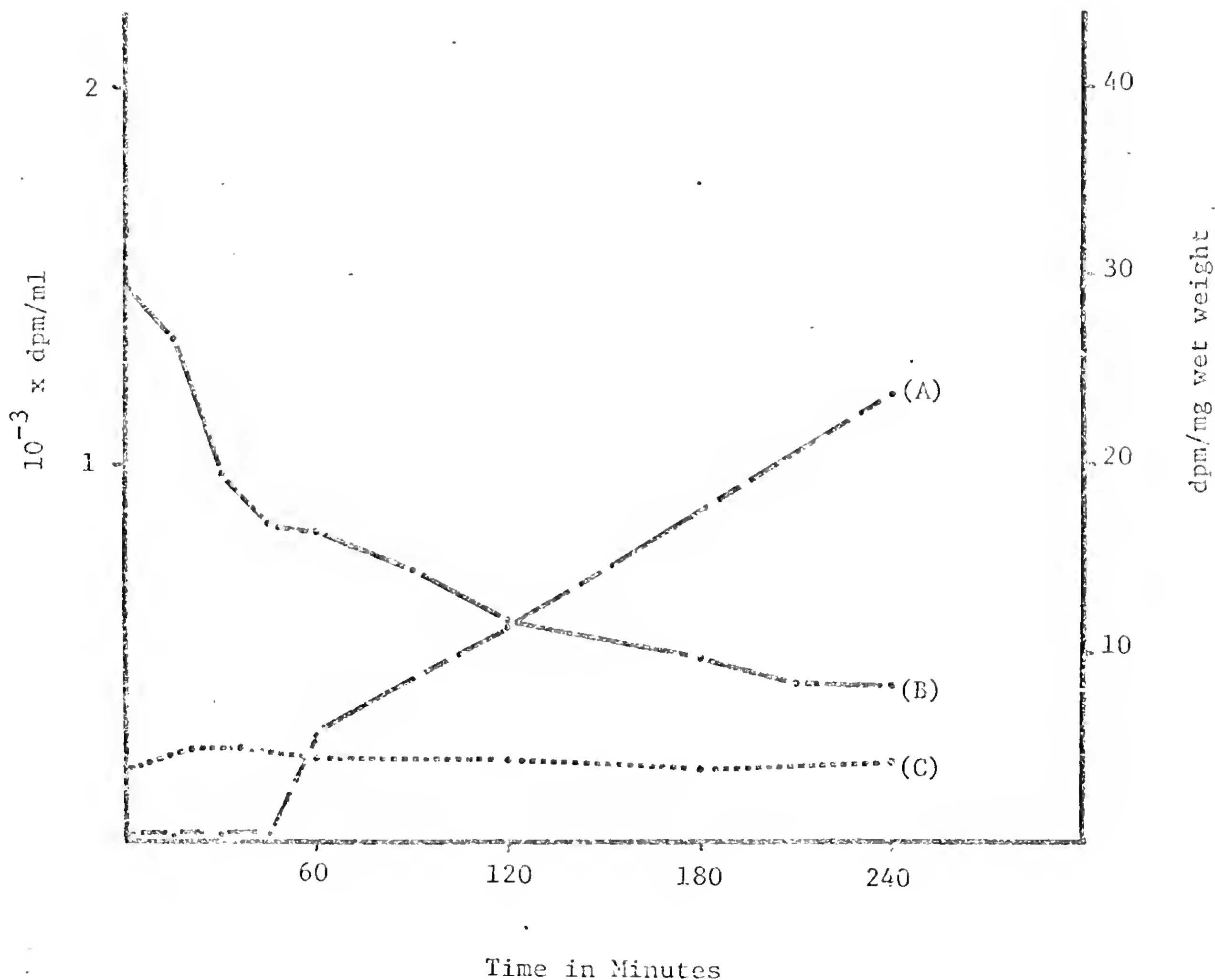


Figure 15. The Uptake of Celite-adsorbed Palmitate.

The total radioactivity in a 1 ml aliquot of the sea water (B), a 1 ml aliquot of 0.45 µm filtered sea water (C), and 200 µl of the chloroform extract of the animals (A) was plotted against time. The concentration of palmitate used to prepare the 50 µm celite was 2.8×10^{-7} M. Three animals were extracted at each point.

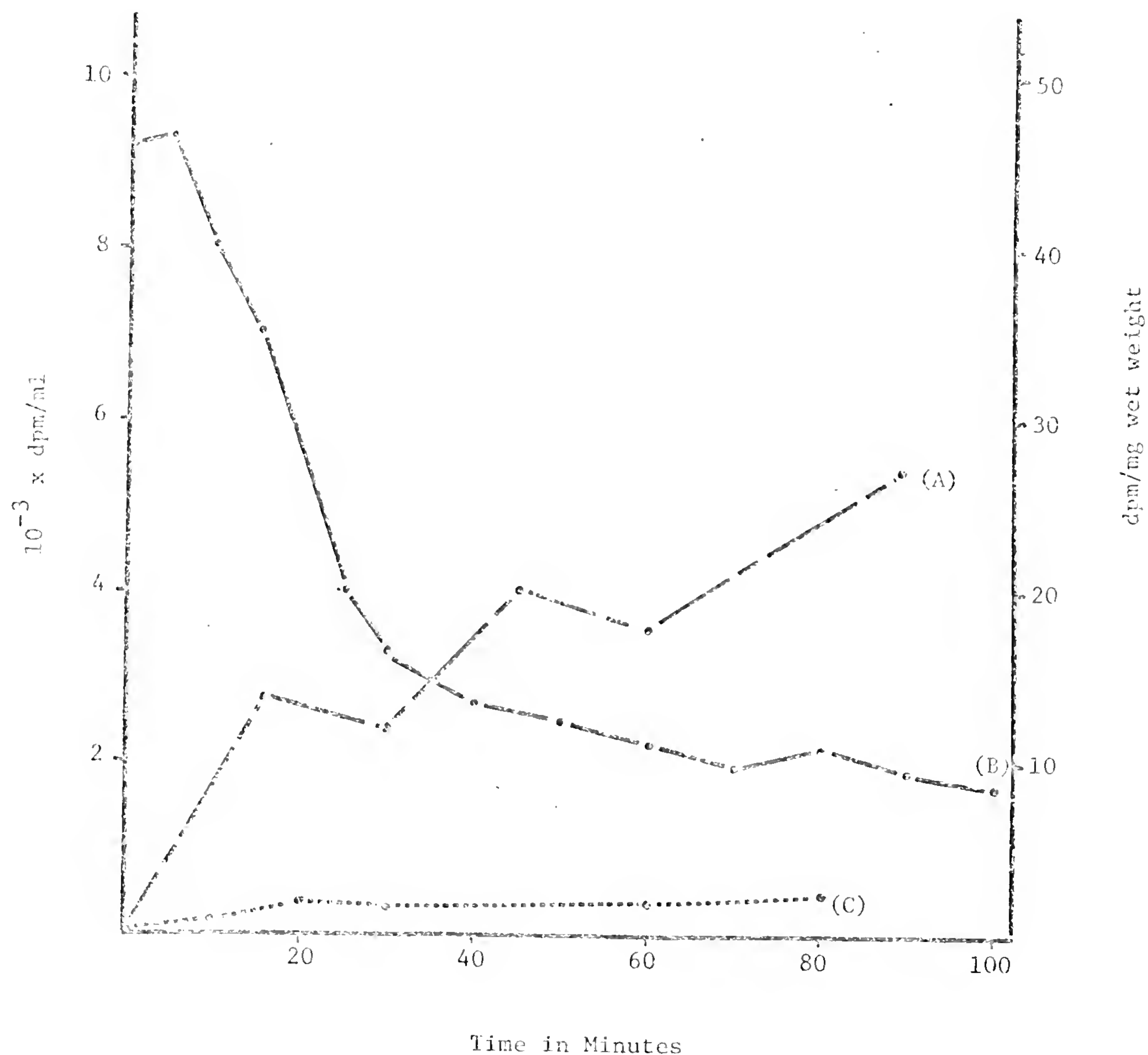
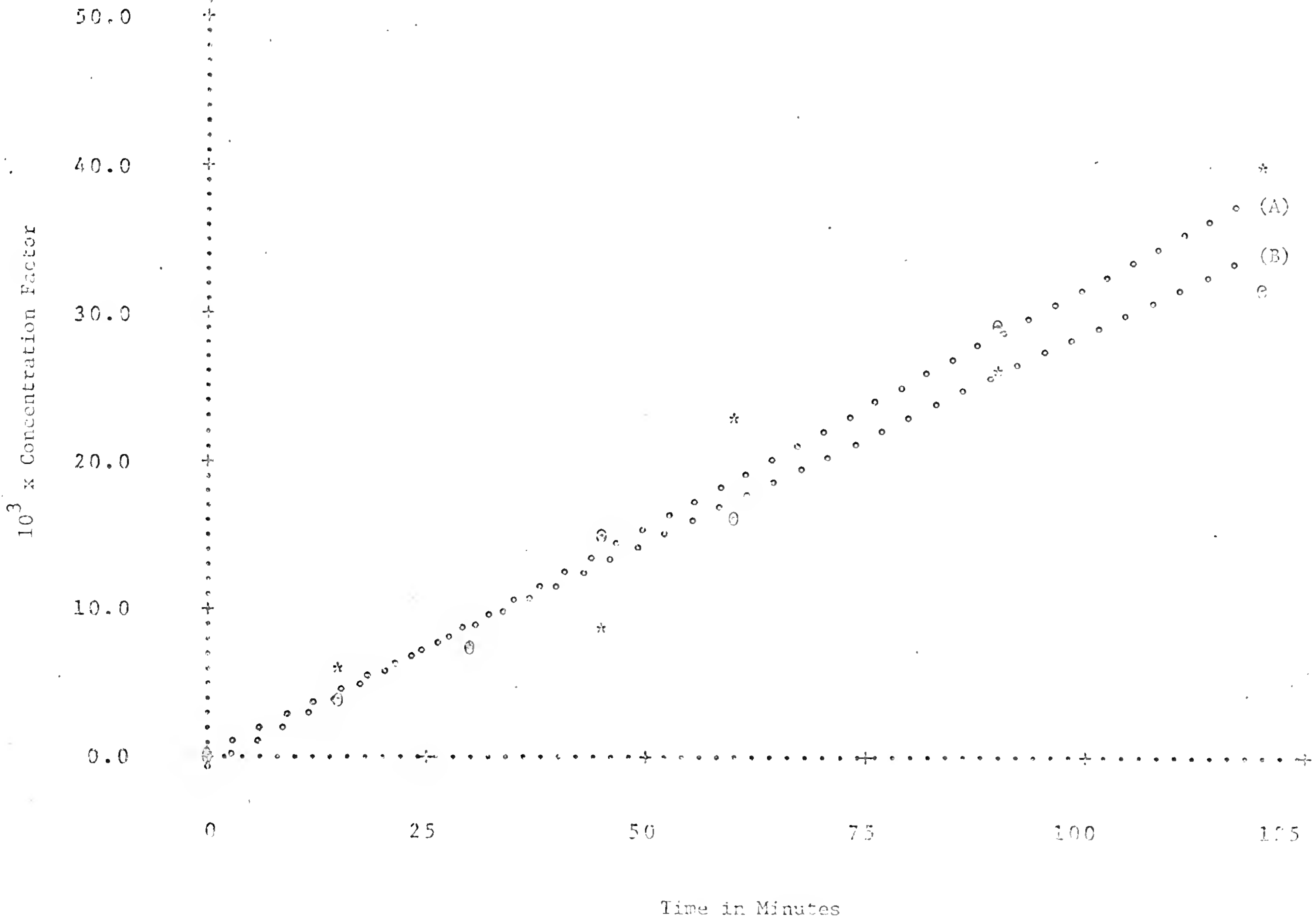


Figure 16. The Uptake of Celite-adsorbed Palmitate, Open Shell Animals.

The radioactivity in 1 ml aliquots of the sea water (B), 1 ml 0.45 μm filtered aliquots of the sea water (C), and aliquots of the chloroform extract (A) were plotted against time. The concentration of palmitate used to prepare the celite was 2.8×10^{-7} M. The animals were added after the removal of the upper valve. Three animals were sampled at each point.

Figure 17. The Uptake of 2.8×10^{-7} M Palmitate, Celite-adsorbed and Free.

The plot of a concentration factor; as dpm/ml sea water//dpm/mg oyster tissue was made against time for open shell animals. The data is computer plotted with the small open circles designating the least squares line through the data points. (A) and * indicate celite adsorbed label, and (B) and ⊙ indicate free palmitate.



50.0
40.0
30.0
20.0
10.0
0.0
10^3 x Concentration Factor
(A)
(B)
0
25
50
75
100
125
Time in Minutes

chloroform extract. From Figure 17, the celite uptake for open shell animals occurred at a faster rate than the uptake for whole animals. The uptake by open shelled oysters is facilitated by the celite particles dropping out of circulation in the glass beaker and onto the animals. The fatty acids on the celite could then be exchanged from particle to animal either in a mucus thread or across the water-tissue surface. This process would not and does not occur in whole animals where the movement (by ciliary currents) of celite containing sea water through the shell would bring the particles into contact with the filtering apparatus of the gill.

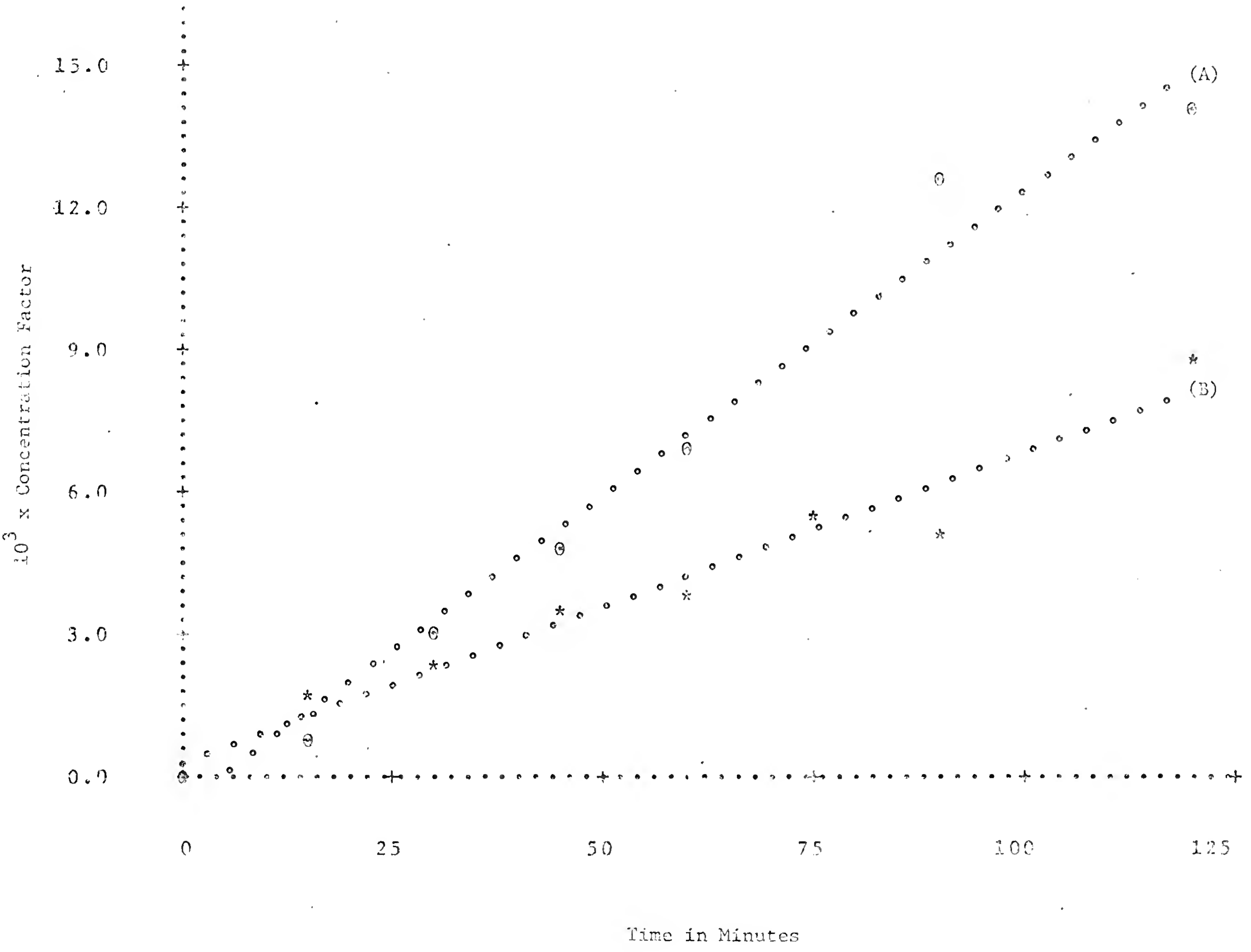
The comparison of the uptake of free stearic acid and celite-bound stearate by open shell animals is shown in Figure 18. The rates of uptake are much lower than those for palmitate, but the celite-adsorbed label is removed at a faster rate than free stearate. The explanation of these results would parallel that for palmitate; the rate of uptake is enhanced due to particulate aggregates settling out of solution onto the animals.

Concentration Dependent Uptake--
Kinetic-Parameters of Uptake

The concentration dependent uptake process was investigated with open-shell animals and ^{14}C labelled palmitic, stearic, and oleic acids. The incorporation of [^{14}C]palmitate and [^{14}C]stearate into the lipid extracts are plotted in dpm/min/mg wet weight as a function of the time after uptake. The lines were computer plotted by least squares. See Figures 19 and 20. The slopes of the plots of the initial rate of uptake is plotted versus concentration. Figures 21 and 22, the saturation plots

Figure 18. The Uptake of 2.8 x 10^{-7} M Stearate, Celite-adsorbed and Free.

The plot was the same as described in Figure 17. (A) and ⊙ indicated celite adsorbed stearate, and (B) and * indicated free stearate.



(A)
(B)
15.0
12.0
9.0
6.0
3.0
0.0
0
25
50
75
100
125
Time in Minutes
10^3 x Concentration Factor

Figure 19. Concentration Dependent Uptake of Palmitate.

The appearance of radioactivity in the chloroform extracts of the animals was plotted against time for 5 different concentrations of palmitate. The plots were made by computer with the least squares lines plotted as the open circle lines. The animals were used after the removal of the upper shell. Three animals were sampled for each point. Palmitate concentrations were (A) and * - 0.07 µM, (B) and Θ - 0.14 µM. (C) and O - 0.28 µM, (D) and X - 0.43 µM, and (E) and Θ - 0.56 µM. The specific activity for all experiments was 12.8 µCi/ mole.

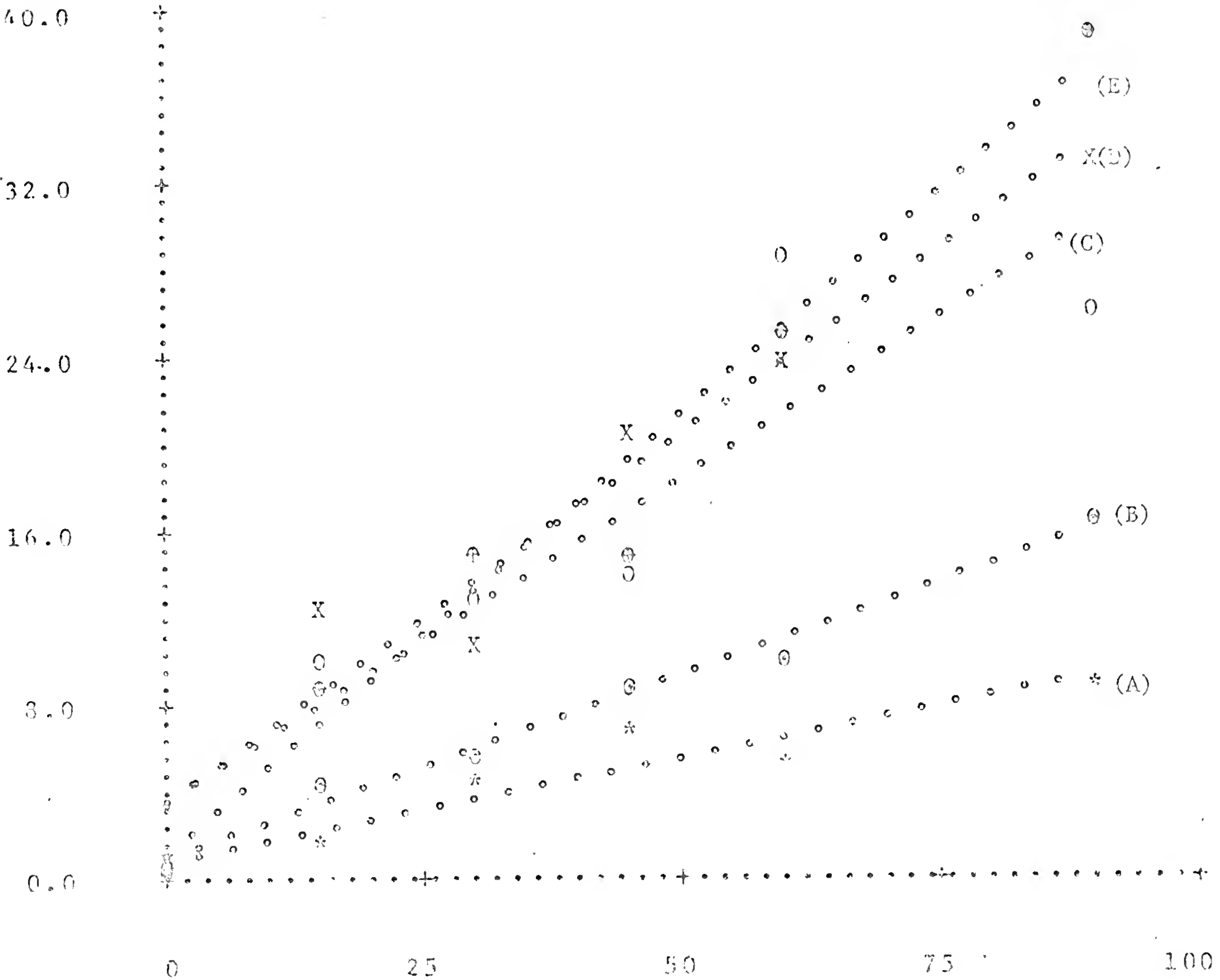
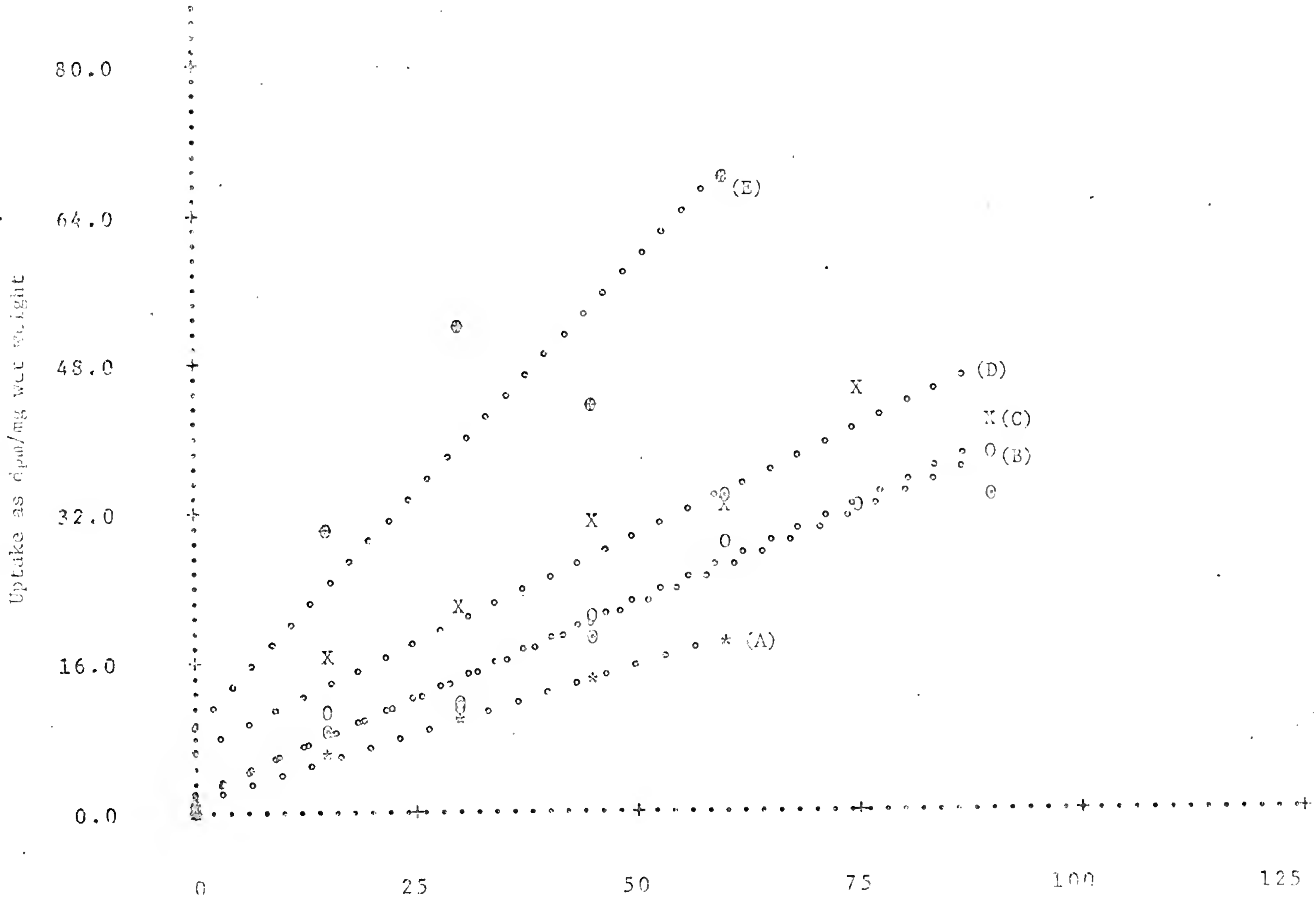

40.0
32.0
24.0
16.0
8.0
0.0
Uptake as dpm/mg wet weight
(E)
X(D)
(C)
(B)
(A)
0
25
50
75
100
Time in Minutes

Figure 20. Concentration Dependent Uptake of Stearate.

The appearance of radioactivity in the chloroform extracts of the animals was plotted against time for 5 different concentrations of stearate. The plots were made the same as described in Figure 19. The concentrations of stearate were (A) and * - 0.057 μM, (B) and ⊖ - 0.084 μM, (C) and O - 0.14 μM, (D) and X - 0.28 μM, and (E) and ⊕ - 0.42 μM. The specific activity for all experiments was 14.0 μCi ^{14}C/μmole.


80.0
64.0
48.0
32.0
16.0
0.0
Uptake as dpm/mg wet weight
0
25
50
75
100
125
Time in Minutes
(E)
(D)
X (C)
O (B)
* (A)

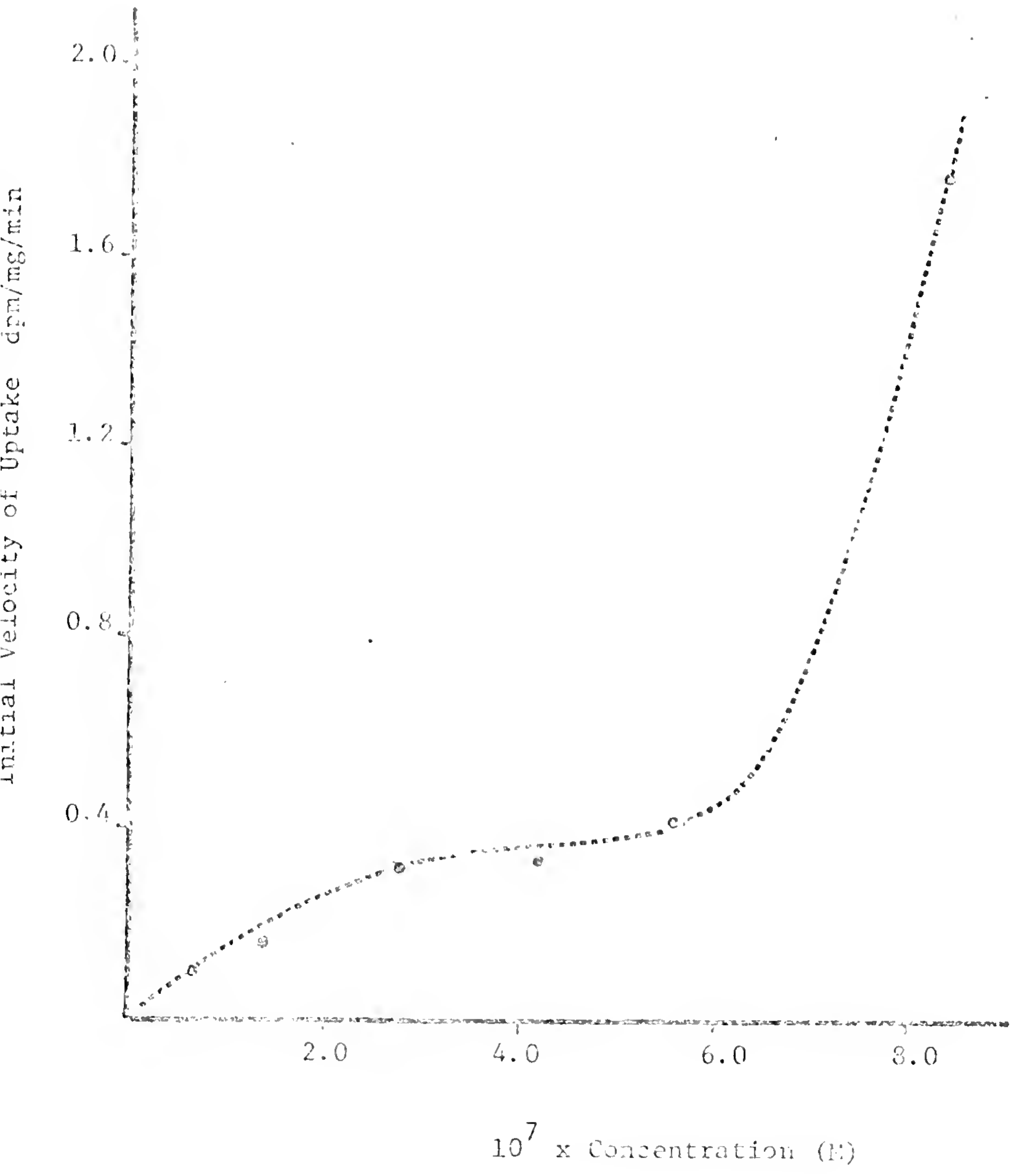


Figure 21. The Concentration Dependent Rate of Uptake of Palmitate.

The initial rate of uptake determined from the slopes of Figure 19 were plotted against the concentration of palmitate in the experiments. The animals had the upper shell removed prior to addition to the sea water.

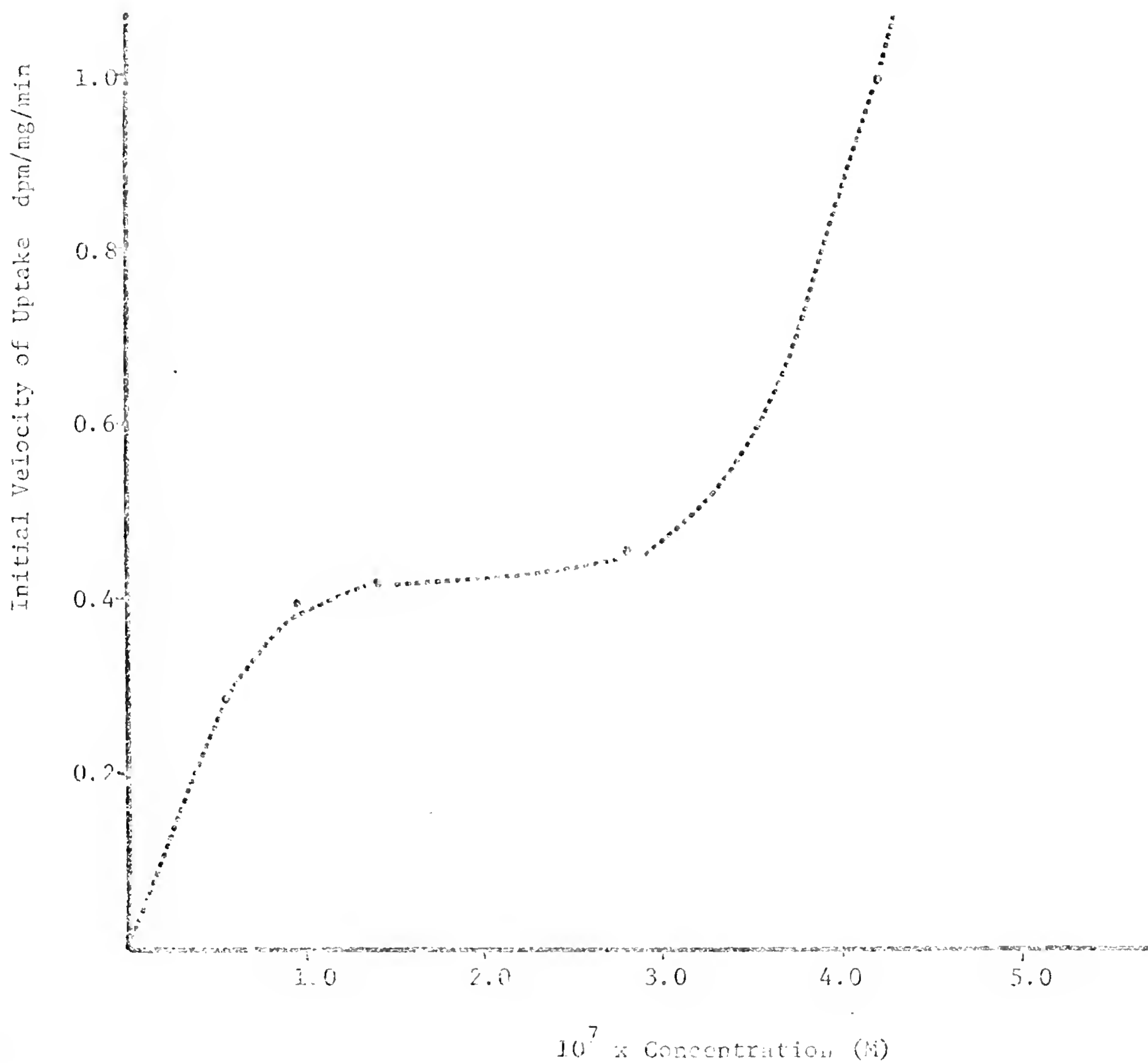


Figure 22. The Concentration Dependent Rate of Uptake of Stearate.

The initial rates of uptake determined from the slopes of Figure 20 were plotted against the concentration of stearate in 5 experiments. The animals had the upper shell removed prior to placement in the sea water.

for palmitate and stearate, show similar saturations at low concentrations, then a sudden burst in the uptake rate appears at 3.0 or 6.0×10^{-7} M. This is probably due to self-aggregation of the fatty acids at the elevated concentrations promoting either an enhanced rate due to large particle effects or due to generation of particles large enough to permit the animals to filter them. The increased uptake rate is seen in conjunction with increased turbidity of the sea water solution. The same concentration effect was seen by Testerman (1972) in his experiments with fatty acid uptake. From his experimental work with artificial sea water as a medium, he found the micellar concentration of palmitate to be about 5×10^{-6} M. In the experiments with natural sea water reported here the micellar concentration is about 7.0×10^{-7} M. The difference in the two figures emphasizes the importance of considering the contribution of other fatty acids in sea water when investigating uptake rates.

The plots of the velocity-concentration data for palmitate and stearate treated by the Lineweaver-Burk reciprocal method yield straight lines. Figure 23, the palmitate plot for all data points below 6.0×10^{-7} M, i.e., below the aggregation concentrations, has a y intercept, Km of 5.0×10^{-7} M, and a maximal velocity of 0.78 dpm/mg/min. If this rate is converted to the actual concentration of palmitate removed, the rate becomes 2.3 pmoles/gram/hr. For stearate (Figure 24) the Km is 0.59×10^{-7} and the maximal rate of uptake is 0.63 dpm/mg/min. The rate of uptake of stearate expressed in molar terms becomes 1.9 pmole/gram/hr. These figures for the Km relate to the sea water concentrations of the acids in natural coastal waters. From the data at Shell Mound, the ambient concentrations of the acids in sea water are 1.1×10^{-7} M

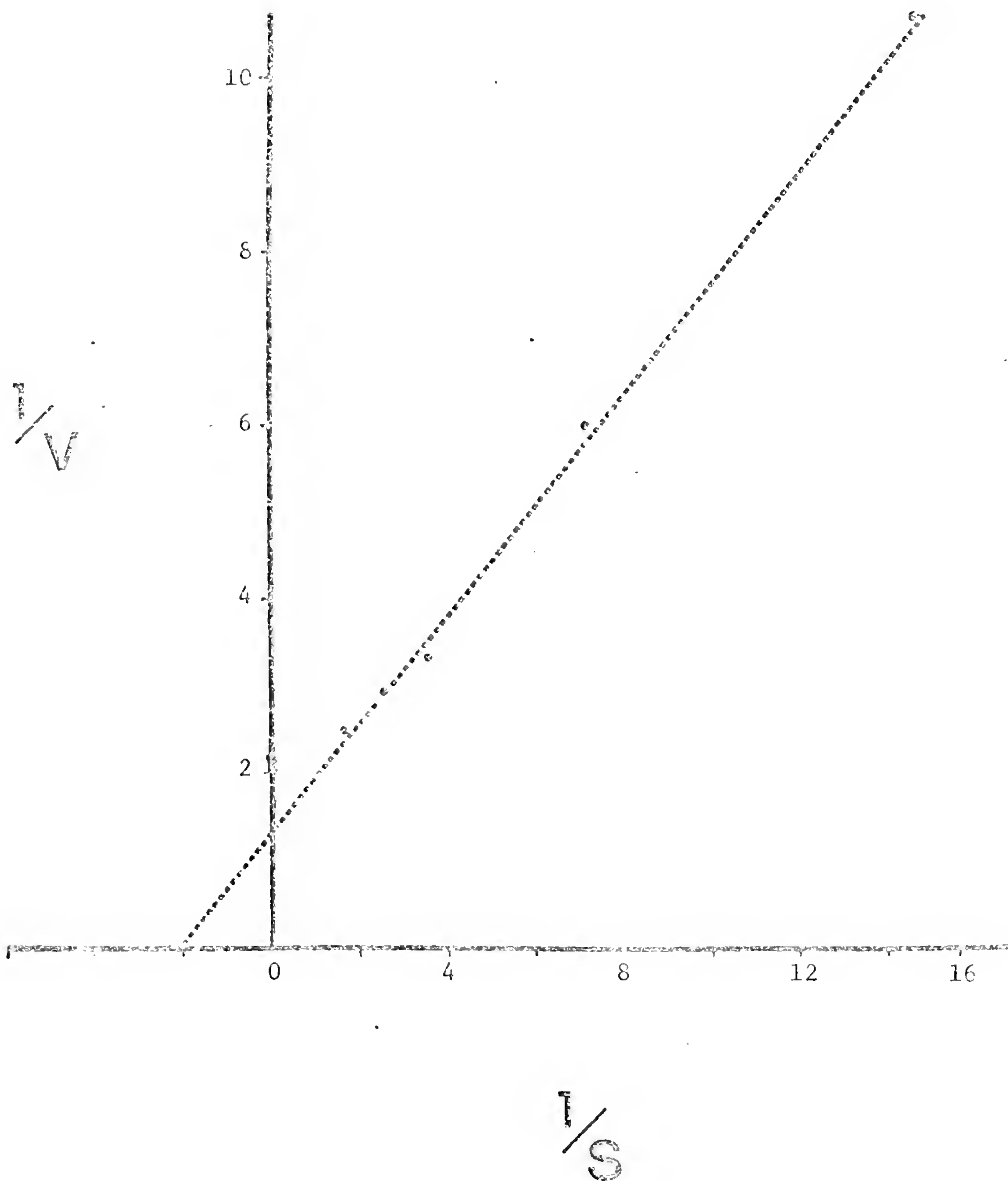


Figure 23. Lineweaver-Burk Transformation of Palmitate Uptake Data.

The initial rates of uptake for 5 concentrations of palmitate were plotted by the double reciprocal method. The maximum velocity was determined from the y-intercept and the Km for the uptake process from the slope (V = dpm/mg wet weight/min) ($S = 10^{-7}$ M omitting the point at 8×10^{-7} M).

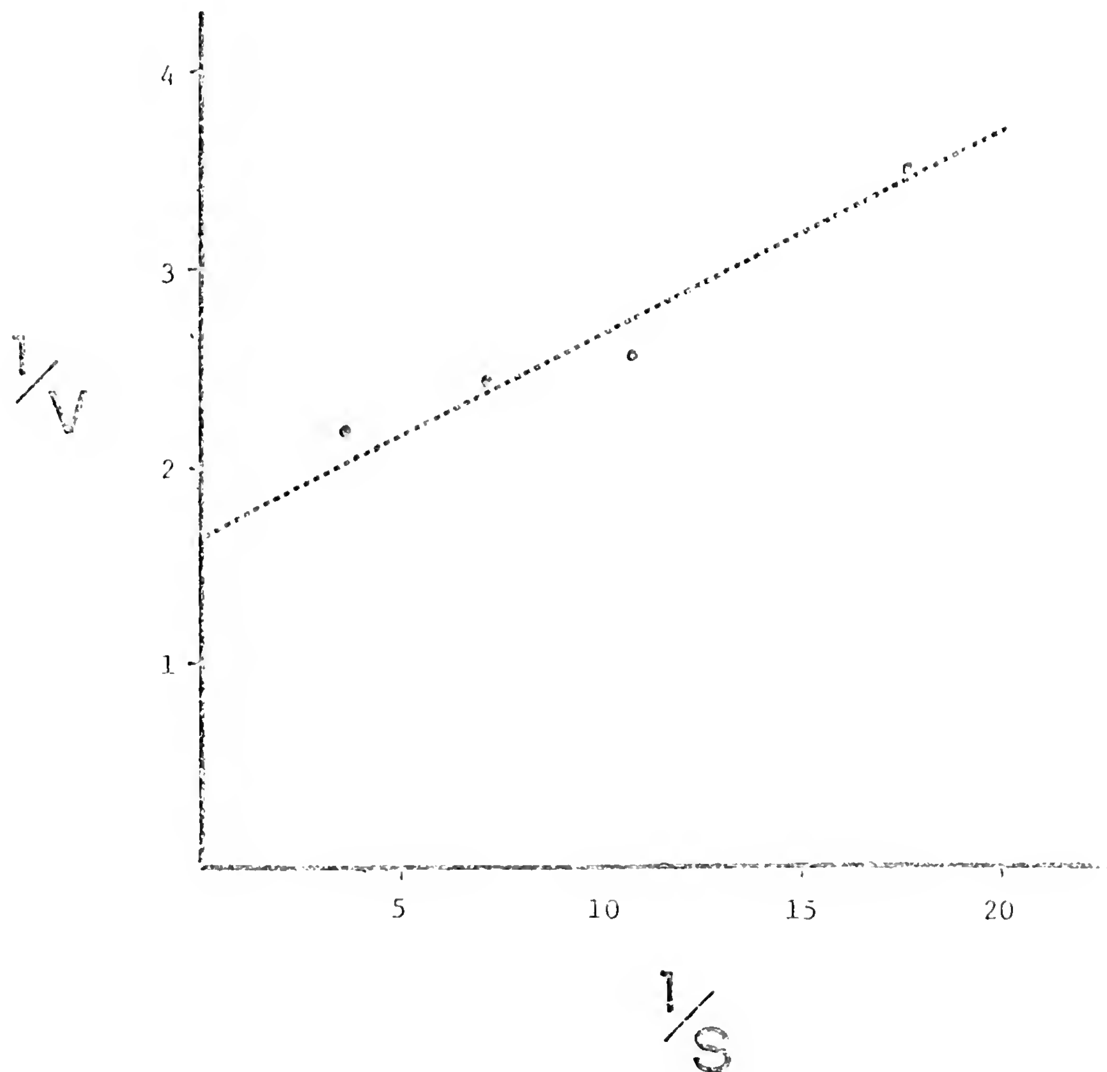


Figure 24. Lineweaver-Burk Transformation of Stearate Uptake Data.

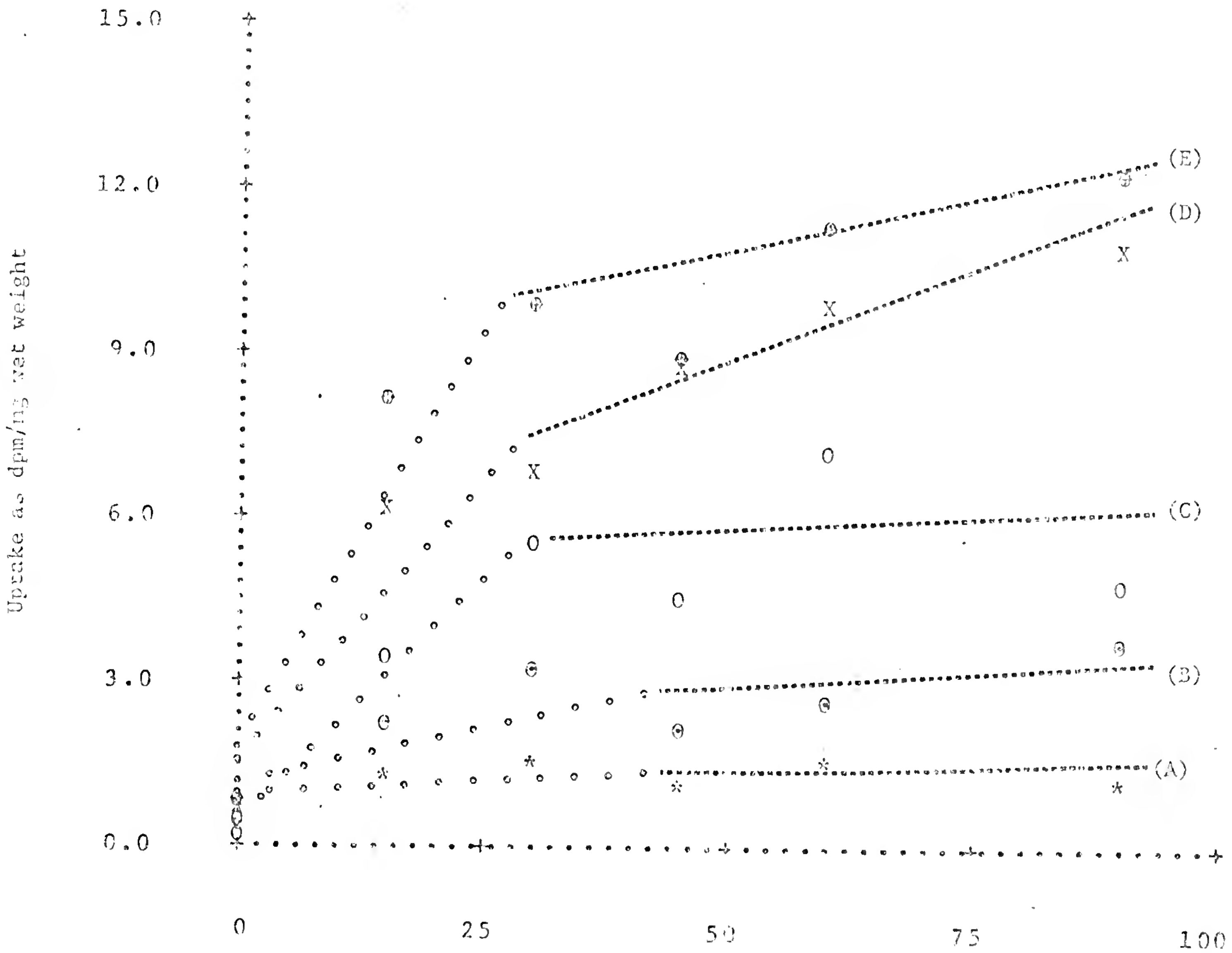
The initial rates of uptake for 4 concentrations of stearate were plotted by the double reciprocal method. Values for velocities and concentrations are the same as for Figure 23. The rate for $S = 4.2 \times 10^{-7}$ M was omitted.

for palmitate and 0.60 x 10^{-7} for stearate. At naturally occurring concentrations the oysters are able to remove both palmitate and stearate from the water because palmitate is below the half-saturating concentration and stearate is about equal to the half-saturating concentration. Other data on the fatty acid distribution indicate that the levels of palmitate may represent a greater percentage of the total free fatty acid and stearate a lower percentage for other areas and methods of determination (Jeffrey, 1970). Our evidence then indicated that the animals had a system which is saturated at 10^{-7} M which enables them to remove palmitate and stearate at naturally occurring concentrations.

Uptake measurements were made with oleic acid at a range of concentrations from 1.25 - 15.0 x 10^{-7} M. The initial rates of the uptake are shown in the computer plot of least squares velocities in Figure 25. The velocities are only linear for the first 30 to 45 minutes and show a saturation at longer times. When the initial rates of uptake are plotted, a linear relationship is found with no saturation even at a 1.5 x 10^{-6} M concentration. (See Figure 26.) The ambient concentration of oleate in the sea water at Shell Mound was determined to be 0.7 x 10^{-9} M. At this concentration, much less than those used in the uptake experiments, the rate of uptake is essentially zero. From these data the uptake of oleate from naturally occurring concentrations is not significant and represents a very small contribution to the total fatty acid removed from sea water.

Figure 25. Concentration Dependent Uptake of Oleate.

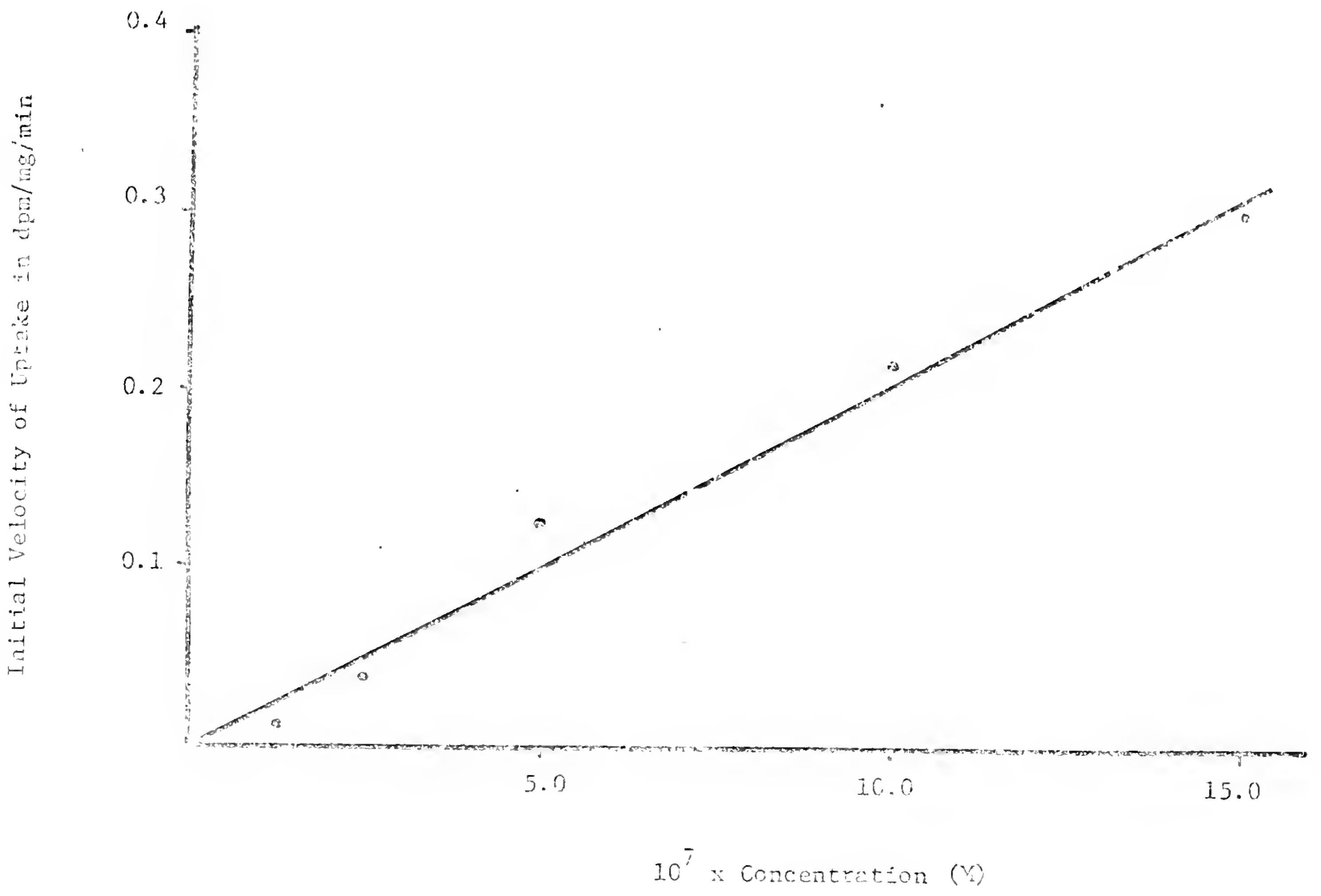
The appearance of radioactivity in the chloroform extracts of the animals was plotted against time for 5 different concentrations of oleate. The plots were made as described in the legend of Figure 19, but only the initial rate for the first 30 minutes was used. After the first 30 minutes, the uptake was no longer linear with time. The concentrations of oleate used were (A) and * - 0.125 µM, (B) and ⊙ - 0.25 µM, (C) and O - 0.50 µM, (D) and X - 1.0 µM, and (E) and ⊖ - 1.5 µM. The specific activity for all experiments was 200 µCi ^3H/µmole.



15.0
12.0
9.0
6.0
3.0
0.0
Uptake as dpm/mg wet weight
0
25
50
75
100
Time in Minutes
(E)
(D)
(C)
(B)
(A)

Figure 26. The Concentration Dependent Rate of Uptake of Oleate.

The initial rate of uptake determined from the slopes of Figure 25 was plotted against the concentration of oleate in 5 experiments. The animals had the upper shell removed prior to placement in the sea water.


0.4
0.3
0.2
0.1
5.0
10.0
15.0
10^7 x Concentration (M)
Initial Velocity of Uptake in dpm/mg/min

Lipids of *Crassostrea* and the Incorporation of Labeled Fatty Acids

The neutral lipids of *Crassostrea virginica* have been characterized by column chromatography and thin layer chromatography (Watanabe and Ackman, 1972). We found 5 major classes of neutral lipids as can be seen from a TLC of the lipid extracts from a [^{14}C]palmitate incorporation experiment in Figure 27. The classes listed in order of increasing Rf are sterols, triglycerides, alkyl diglycerides, wax esters, and cholesterol esters. The polar lipids, which remain at the origin in a neutral lipid TLC system, can be separated in a polar solvent system as described in the methods section. In the lipid extract of oysters there are 4 or 5 major polar lipid classes as can be seen from a TLC from a palmitate uptake experiment in Figure 28. The 2 major compounds are those with relative mobilities of 0.3 and 0.63, phosphatidyl choline and phosphatidyl ethanolamine, respectively.

The genus *Crassostrea*, unlike the genus *Ostrea*, contains no free fatty acid pools in the lipid extracts (Watanabe and Ackman, 1972). This fact is most important in evaluation of uptake experiments since any free fatty acid that is assimilated is either incorporated into an esterified lipid or catabolized for energy. Also, there is no problem of back diffusion of a labeled acid once it is incorporated into a large intra-cellular pool, as is seen in amino acid uptake (Johannes *et al.*, 196[illegible]) By determining the incorporation into specific lipids, the actual uptake and incorporation rates can be measured and quantitated.

The radiochromatographic scans of the neutral and polar lipid separated by TLC following a 2.8 x 10^{-7} M palmitate uptake experiment are shown in Figures 29 and 30. Superimposed on the scans are the traces of

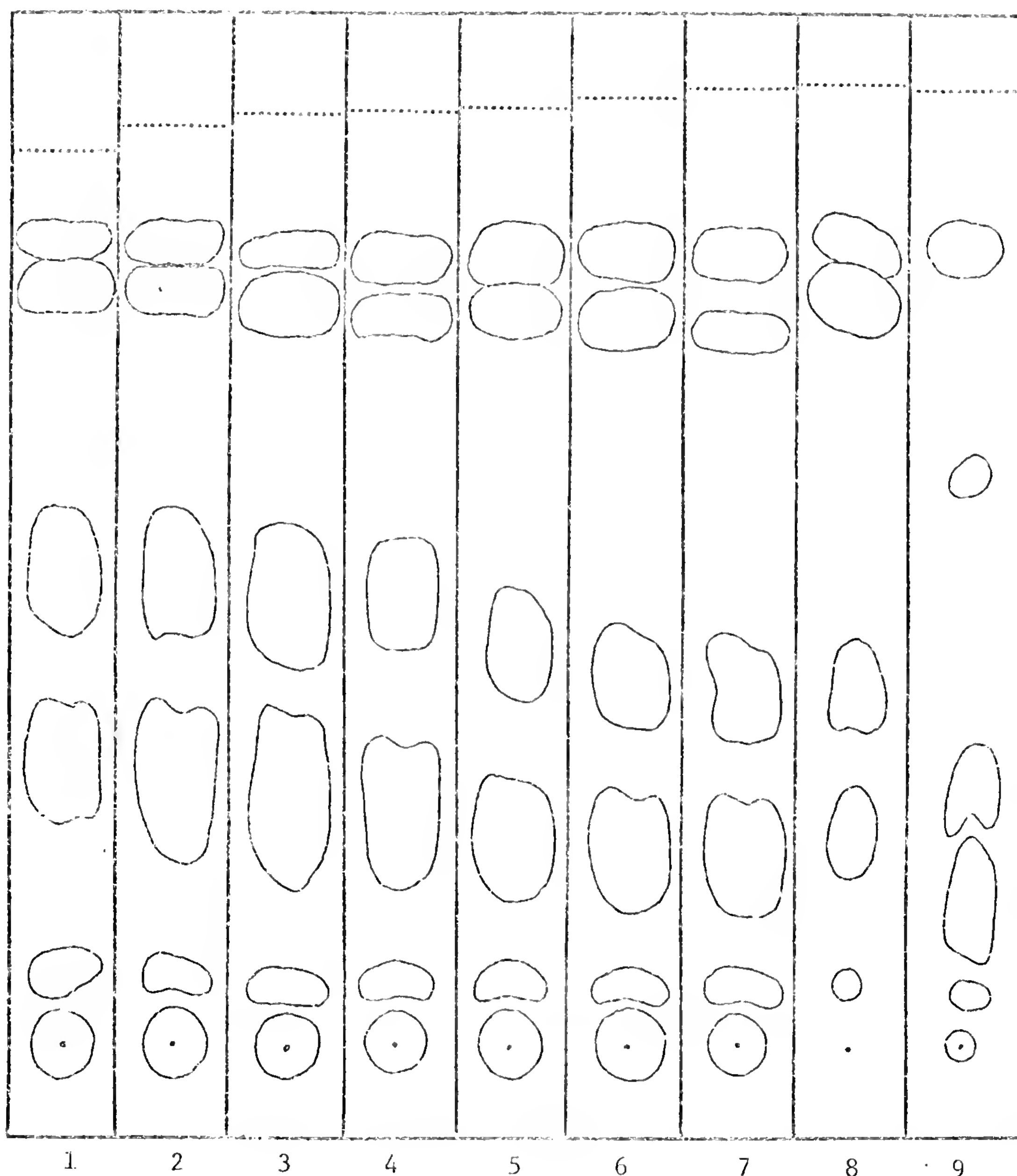


Figure 27. The Thin Layer Chromatographic Separation of Oyster Neutral Lipids.

The lipid extracts from a 2.8×10^{-7} M palmitate incorporation experiments were run on the neutral lipid system parallel with standard mixtures. The lipids were visualized with iodine. (1 - 7): 200 µl of the lipid extracts for 0, 15, 30, 45, 60, 90, and 120 minute samples. (8): standard mixture containing in order of increasing Rf: cholesterol, tripalmitin, 1 - alkyl 2, 3 dipalmitoyl diglyceride, hexadecyl palmitate, and cholesteryl palmitate. (9): standard mixture containing in order of increasing Rf: polar lipids, cholesterol, free fatty acid, triolein, methyl palmitate, and cholesterol oleate. The dotted line at the top of the plate was the solvent front.

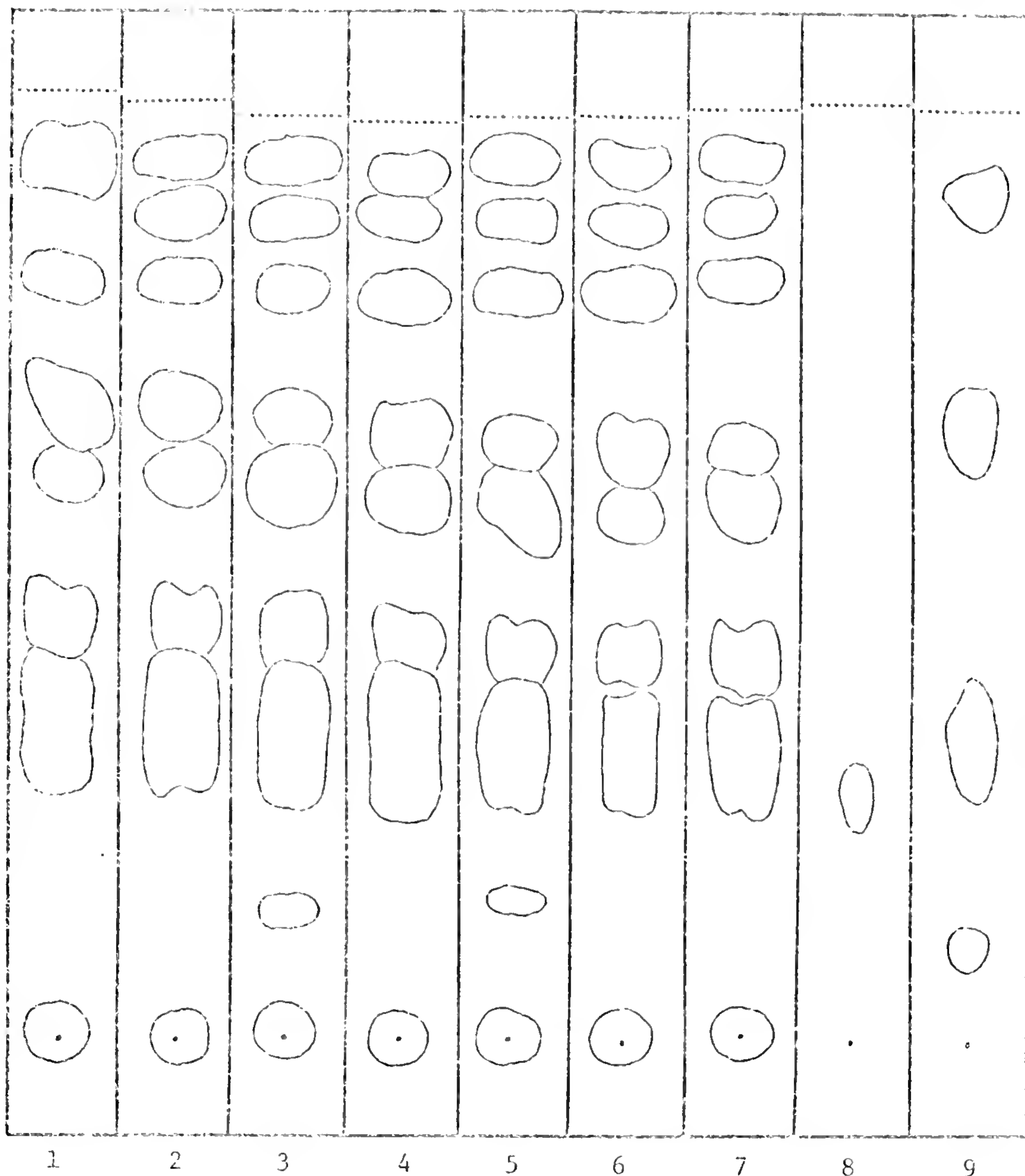


Figure 28. The Thin Layer Chromatographic Separation of Oyster Polar Lipids.

The lipid extracts from a 2.8×10^{-7} palmitate incorporation experiment were run on the polar lipid TLC system parallel with standard mixtures. The lipids were visualized with iodine. (1 - 7): 200 µl of the lipid extracts for 0, 15, 30, 45, 60, 90, and 120 minute samples. (8): standard of di-myristyl phosphatidyl choline. (9): standard mixture containing in order of increasing Rf: lyso-phosphatidyl choline, phospatidyl choline, phosphatidyl ethanolamine, and cholesterol. The dotted line at the top was the solvent front.

Figure 29. Radiochromatographic Scan of the Neutral Lipid TLC Separation.

The 60 minute extract from a 2.8×10^{-7} palmitate uptake experiment was spotted (200 μl) on a TLC plate and devaloped in the neutral lipid solvent system. The plate was scanned and the iodine stainable material overlaid on the scan. Scan speed was 1 cm/min. Maximum scale was 100 cpm/min. The lipids represented were (A) polar lipid, (B) cholesterol, (C) triglyceride, (D) alkyl diglyceride, (E) wax ester, and (F) cholesterol ester. (G) was the solvent front and (H) was the ^{14}C standard used to align the scans and the plates.

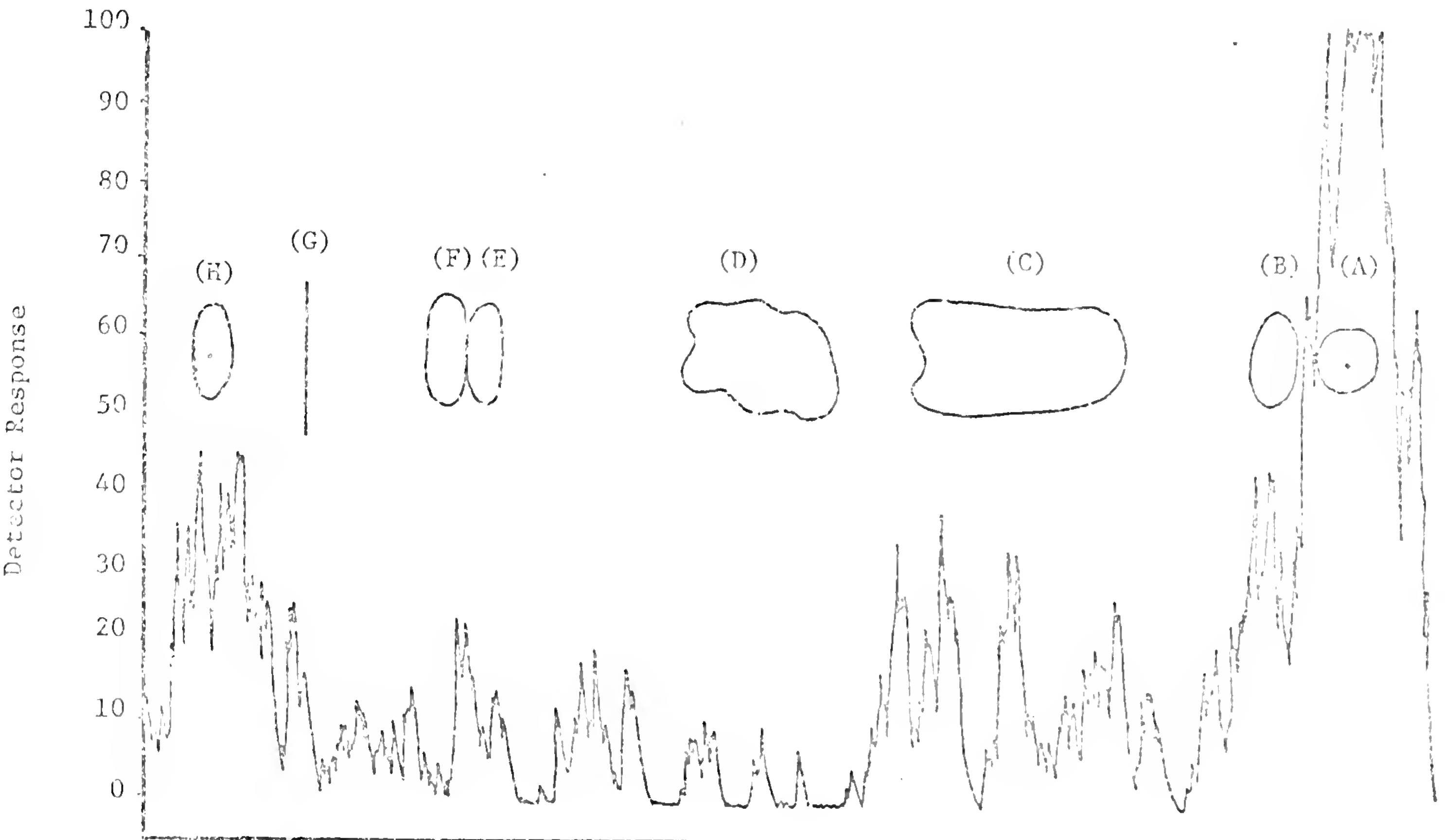
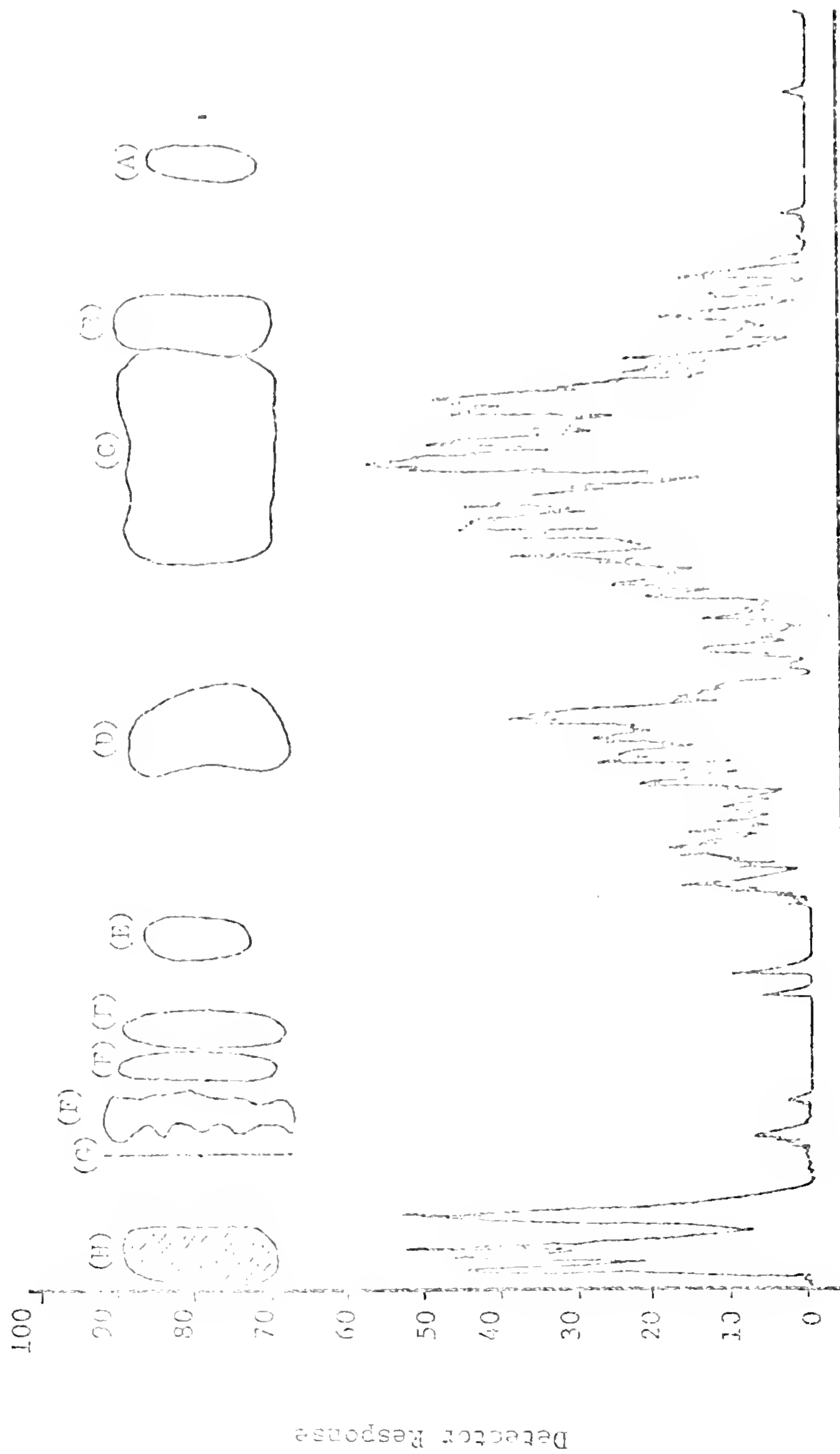

Detector Response
100
90
80
70
60
50
40
30
20
10
0
(H)
(G)
(F) (E)
(D)
(C)
(B)
(A)
Relative Migration

Figure 30. Radiochromatographic Scan of the Polar Lipid TLC Separation.

The 60 minute extract from a 2.8×10^{-7} M palmitate uptake experiment was spotted (200 µl) on a TLC plate and developed in the polar lipid system. The plate was scanned and the iodine stainable material overlaid on the scan. Scan speed was 1 cm/min. Maximum scale of the machine was 100 cpm/min. The lipids represented were (A) glycolipid, (B) sphingomyelin, (C) phosphatidyl choline, (D) phosphatidyl ethanolamine, (E) unknown, and (F) plant pigments. (G) and (H) were the same as in Figure 29.


Detector Response
Relative Migration
100
90
80
70
60
50
40
30
20
10
0
(H)
(G)
(F)
(F)
(F)
(E)
(D)
(C)
(A)

the lipids visualized by iodine vapor. In the neutral plate a large amount of ^{14}C activity was seen at the origin, representing incorporation into the phospholipid material. Incorporation was seen into triglycerides and cholesterol. The label incorporated into cholesterol was shown to be cholesterol and not phospholipid material by chromatography in a more polar solvent system in which the sterols and the phospholipids were more completely resolved. Very little incorporation was seen in the alkyl diglycerides and the cholesterol and wax esters.

The phospholipids were scraped from the origin of the neutral lipid plate and run in the polar solvent system and scanned. The scan showed 2 major areas of incorporation at the positions corresponding to phosphatidyl choline (Rf = 0.3) and phosphatidyl ethanolamine (Rf = 0.63). If a two-dimensional plate was run in the solvents described in the methods section, and all spots were removed and counted, only 2 areas had any significant radioactivity: the areas corresponding to phosphatidyl choline and phosphatidyl ethanolamine (see Figure 31).

The fatty acid distribution in the esterified lipids was determined for the total lipid extract and for the isolated triglycerides (Figures 32 and 33). The distribution indicated that palmitate was a major component of the esterified lipids in both the triglycerides and total lipid.

When the lipids were separated by TLC, and the individual compounds which showed activity in the radiochromatographic scans were counted and quantitated, the typical pattern seen is shown in Figure 34. The major lipids labeled were the phospholipids followed by the triglycerides and cholesterol. Further characterization of the phospholipid in all experiments indicated that over 90 percent of the activity was located in the phosphatidyl choline with the remainder found in phosphatidyl ethanolamine.

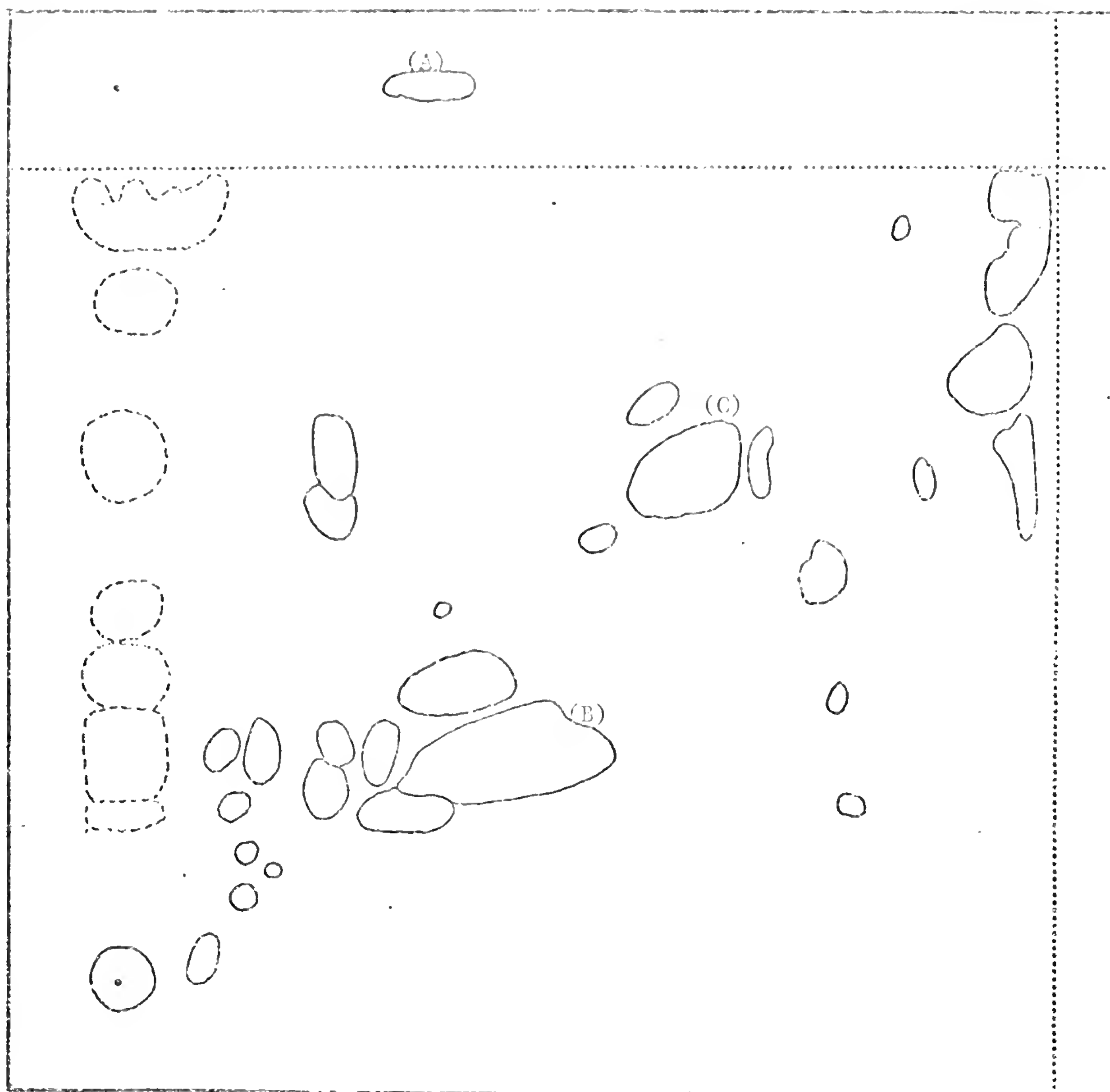


Figure 31. The Two-dimensional TLC Separation of Oyster Phospholipids.

The 120 minute extract of a 2.8×10^{-7} M palmitate uptake experiment was run in the two dimensional solvent system described in the methods. The separation achieved in the first solvent system was shown by the dotted outlines on the left. The labeled materials were (A) standard phosphatidyl choline run in the second solvent system, (B) phosphatidyl choline in the oyster extract, and (C) phosphatidyl ethanolamine in the extract. The origin was spotted with 200 µl of the chloroform extract. The solvent fronts were shown by the dotted line.

Figure 32. Gas Liquid Chromatograph of Fatty Acid Methyl Esters Prepared from Esterified Fatty Acids of Isolated Oyster Triglycerides.

Oyster triglycerides were isolated from chloroform extracts by preparative TLC in the neutral lipid solvent system. The triglyceride fraction was scraped from the plate and saponified and methylated as described in the methods. The esters were run on an EGSS-X column at 174°C and a nitrogen carrier gas flow of 45 ml/min. The fatty acid esters were identified from comparison to standard mixtures.

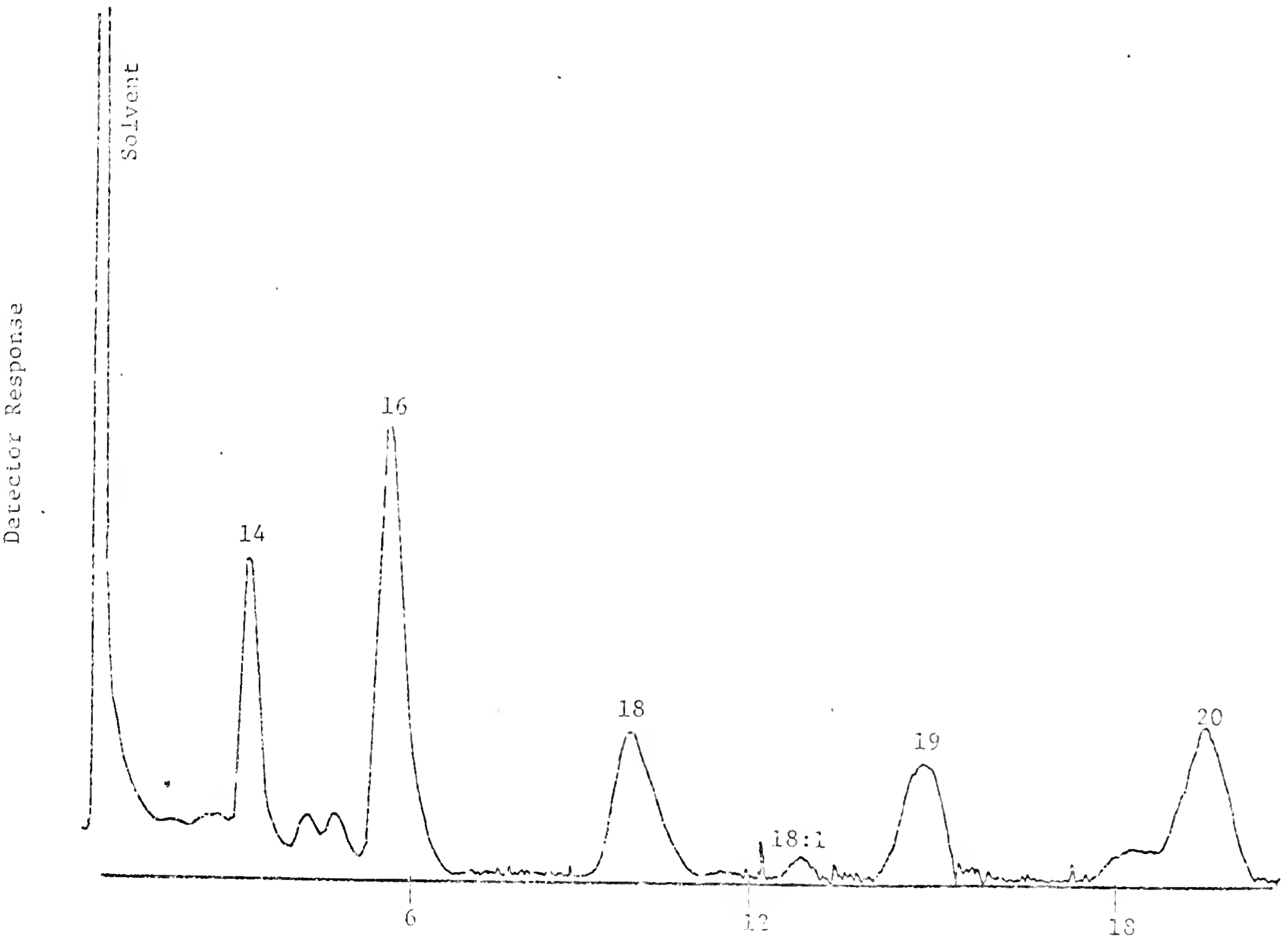

Solvent
Detector Response
14
16
18
18:1
19
20
6
12
18
Retention Time in Minutes

Figure 33. Gas Liquid Chromatograph of Fatty Acid Methyl Esters Prepared from Esterified Fatty Acids of Oyster Total Lipid Extracts.

The chloroform extract of oysters was saponified and methylated by the 1 step method previously described. The methyl esters were run on an EGSS-X column at 174°C and a nitrogen carrier gas flow of 45 ml/min. The fatty acid esters were identified from comparison to standard mixtures. The sudden shift in detector response seen in 16 carbon peak represented a 2 time decrease in sensitivity.

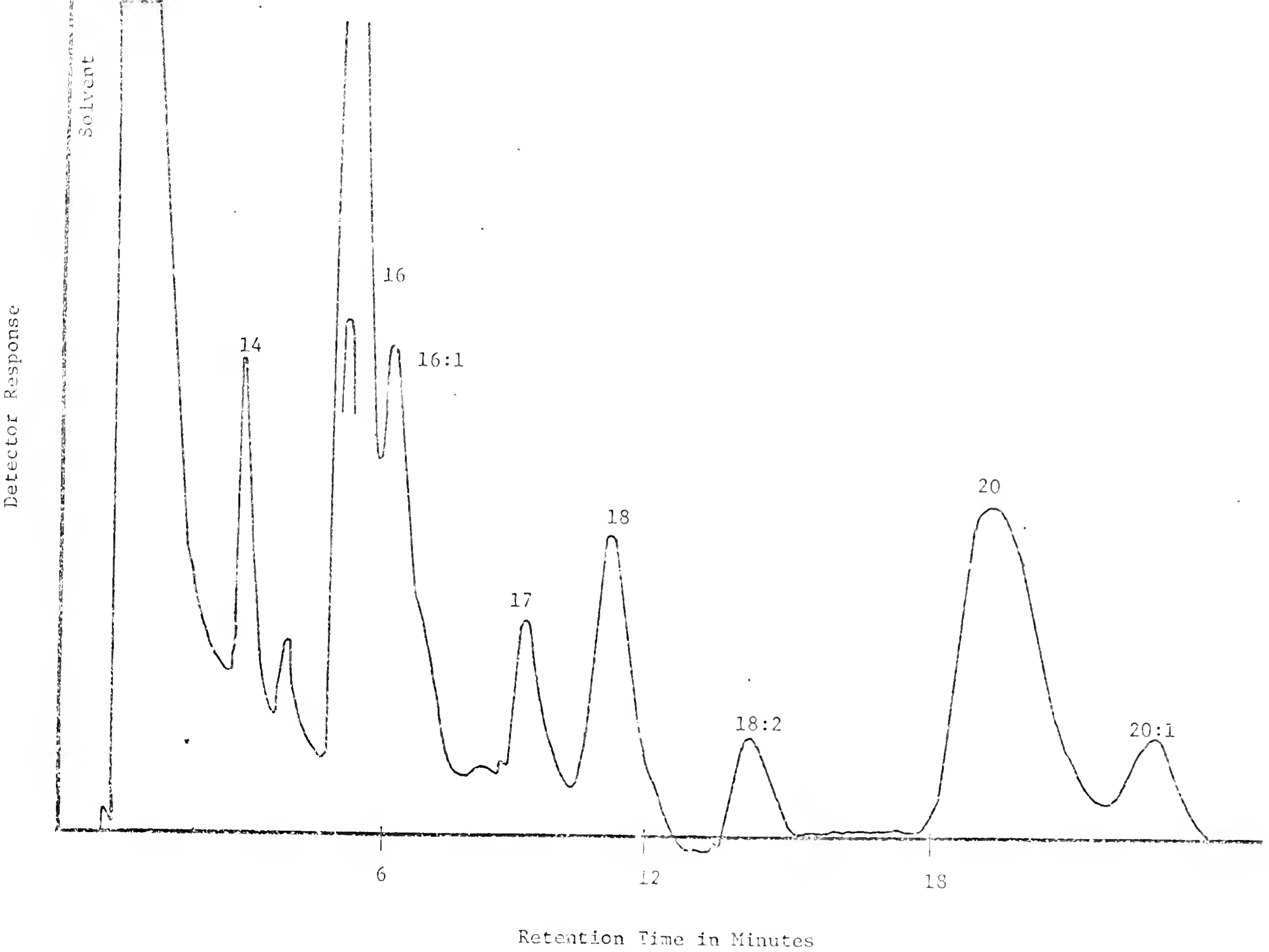
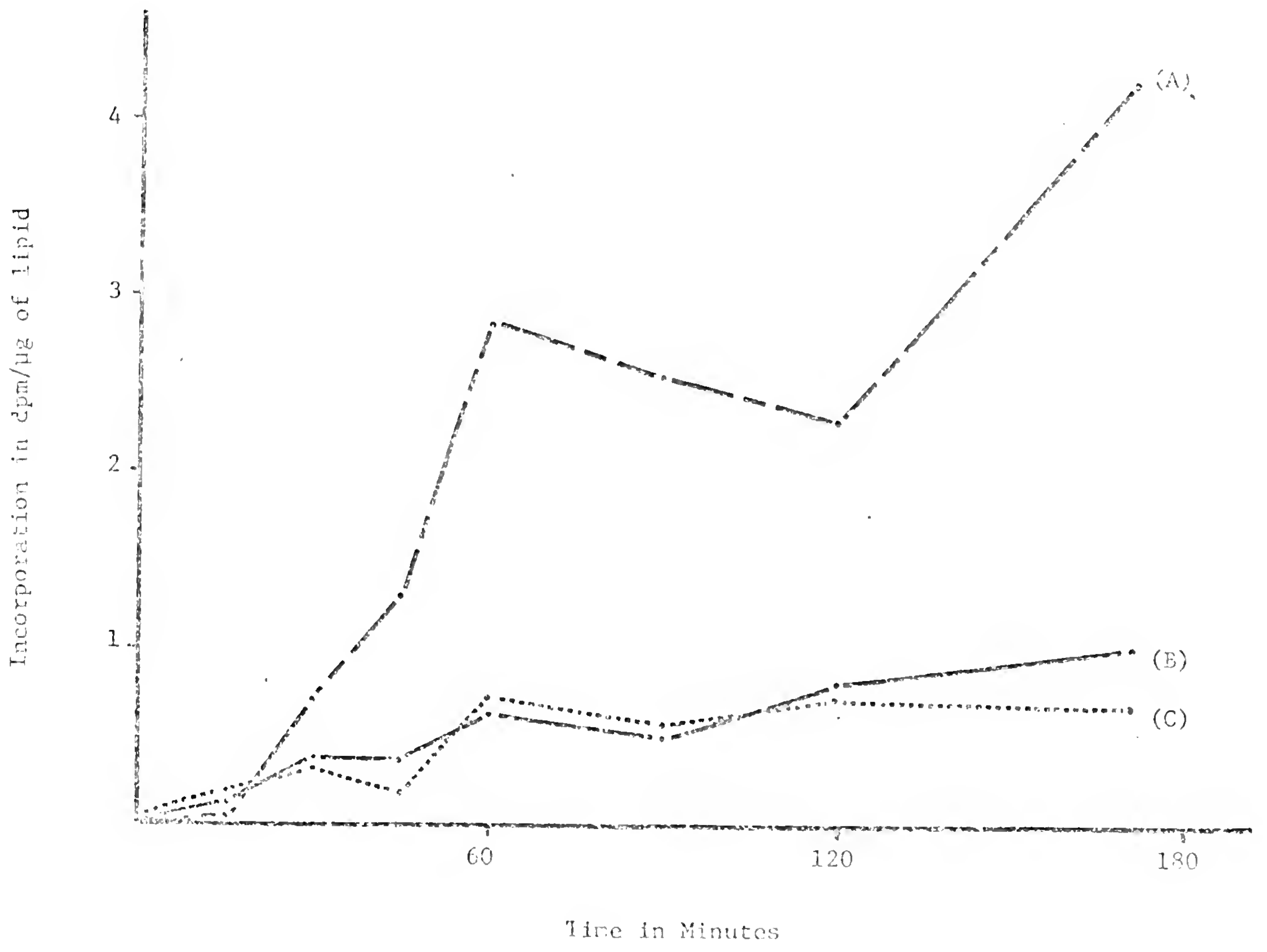

Solvent
14
16
16:1
17
18
18:2
20
20:1
6
12
18
Detector Response
Retention Time in Minutes

Figure 34. Incorporation of ^{14}C Labeled Palmitate into Isolated Lipid Classes.

The chloroform extracts from a 2.8×10^{-7} M palmitate uptake experiment at 30°C were separated on the neutral lipid solvent system. The isolated lipids were counted and quantitated as described in the methods. Three animals with shells intact were extracted at each point. Each point represents the average of two separate uptake experiments. The isolated lipids were (A) total phospholipids, (B) triglycerides, and (C) cholesterol.


(A)
(B)
(C)
4
3
2
1
60
120
180
Incorporation in dpm/μg of lipid
Time in Minutes

Other neutral lipid classes were found to contain some ^{14}C label in the palmitate and stearate experiments, but the incorporation was not significantly above the experimental background for counting and quantitating techniques.

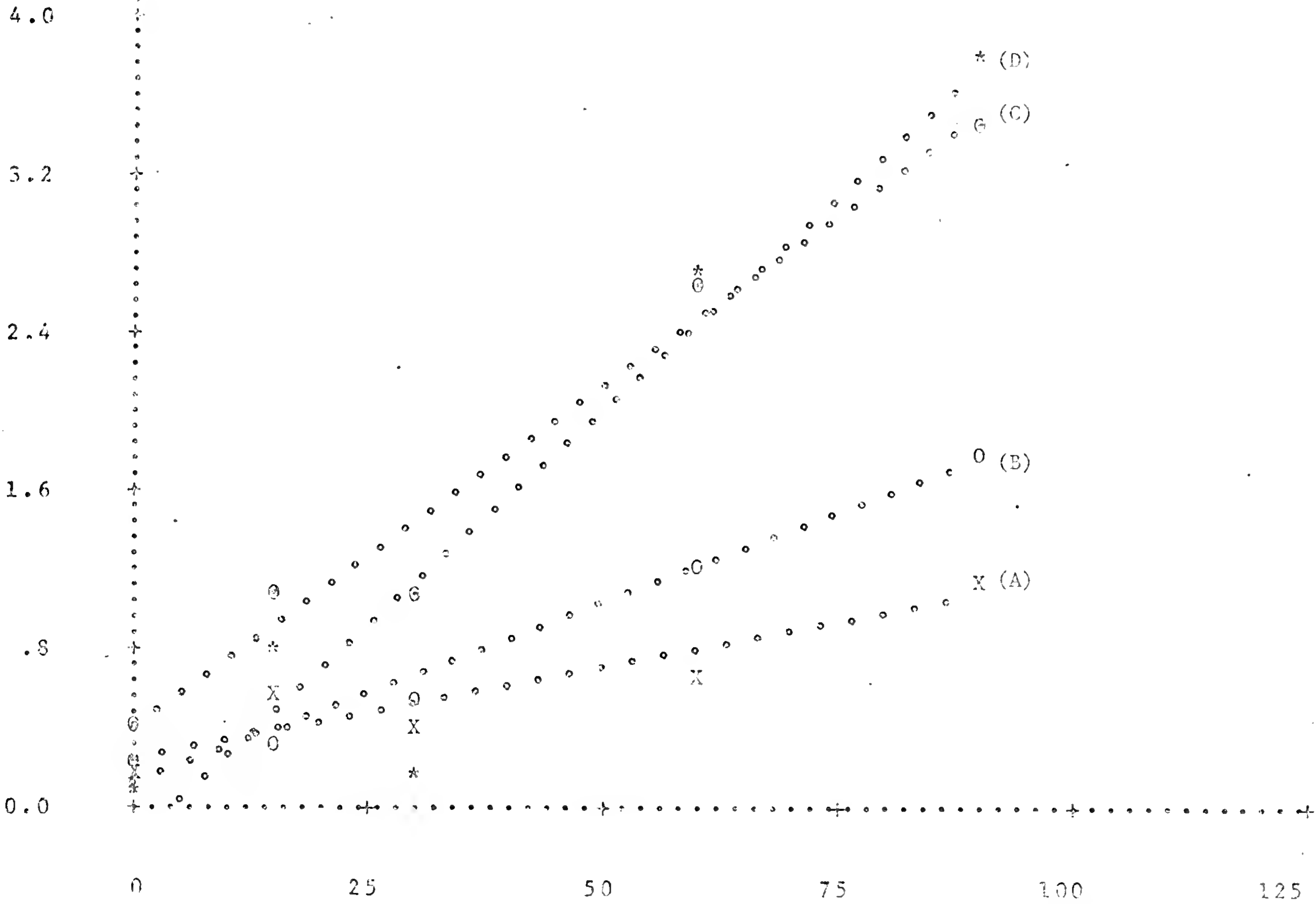
If the data for the concentration dependent incorporation into phosphatidyl choline are plotted for the series of palmitate experiments, the initial velocities can be determined by the least squares computer plot (see Figure 35). If the slopes are now plotted as dpm/μg incorporated/min, a saturation plot is obtained (see Figure 36). The Lineweaver-Burk treatment of these uptake-incorporation data, shown in Figure 37, indicates that the maximal rate of incorporation into the phosphatidyl choline pool is 0.4 dpm/μg/min.

The uptake expressed in molar terms is 15 μmoles of fatty acid incorporated into 1 mole of phosphatidyl choline per minute. The Km for the incorporation, measured from the slope of the reciprocal plot is 3.3×10^{-6} M.

The value for the Km represents the combination of processes to which it corresponds; it involves both an uptake event and an incorporation event which are quite distinct biochemically. The Km for the incorporation of ^{14}C label into total lipid, as measured before in Figure 23, was 5.0×10^{-7} M. The difference between the values may be ascribed to the multiple biosynthetic events necessary to incorporate a newly assimilated fatty acid into a phosphatidyl choline molecule. No statement can be made concerning the absolute nature of these events, but by using the data for incorporation of the fatty acid into phospholipid as a measure of uptake, the contribution of any back diffusion to the uptake process becomes moot.

Figure 35. Concentration Dependent Incorporation of Palmitate into Phosphatidyl Choline.

The increase in the specific activity of isolated phosphatidyl choline from chloroform extracts was plotted against time for 4 concentrations of palmitate. The plots were made by computer with the least squares lines plotted as the open circle lines. The animals were used after the removal of the upper shell. Three animals were sampled at each point. Palmitate concentrations were (A) and X - 0.17 μM, (B) and O - 0.14 μM, (C) and Θ - 0.28 μM, and (D) and * - 0.42 μM. The specific activity of the isotope for all the experiments was 12.8 μCi/μmole.


Incorporation dpm/mg Phosphatidyl Choline
4.0
3.2
2.4
1.6
.8
0.0
0
25
50
75
100
125
Time in Minutes
* (D)
(C)
O (B)
X (A)

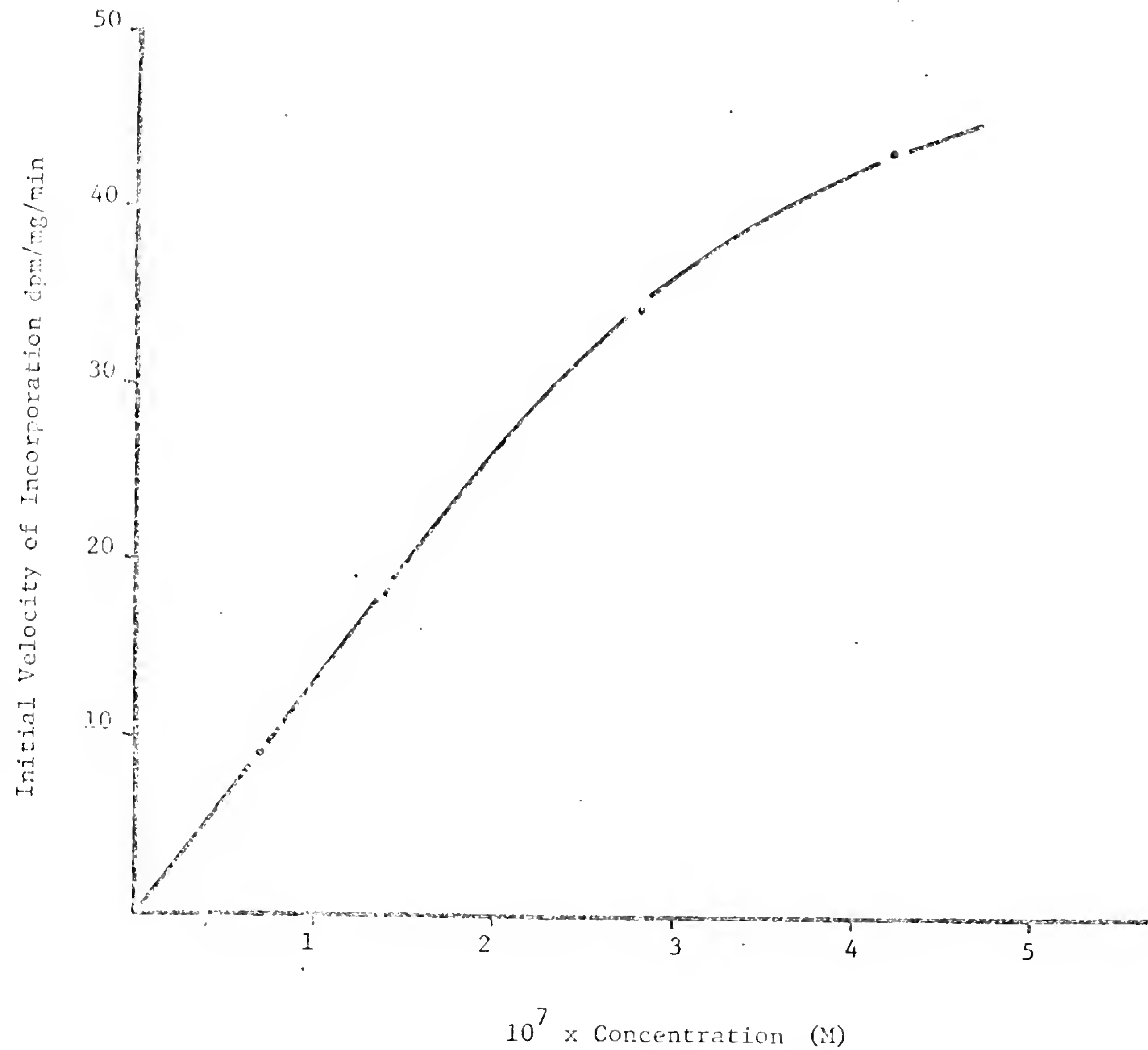


Figure 36. The Concentration Dependent Rate of Incorporation of Palmitate into Phosphatidyl Choline.

The initial rates of incorporation were determined from the slopes of Figure 35 and plotted against the concentrations of palmitate in the experiments.

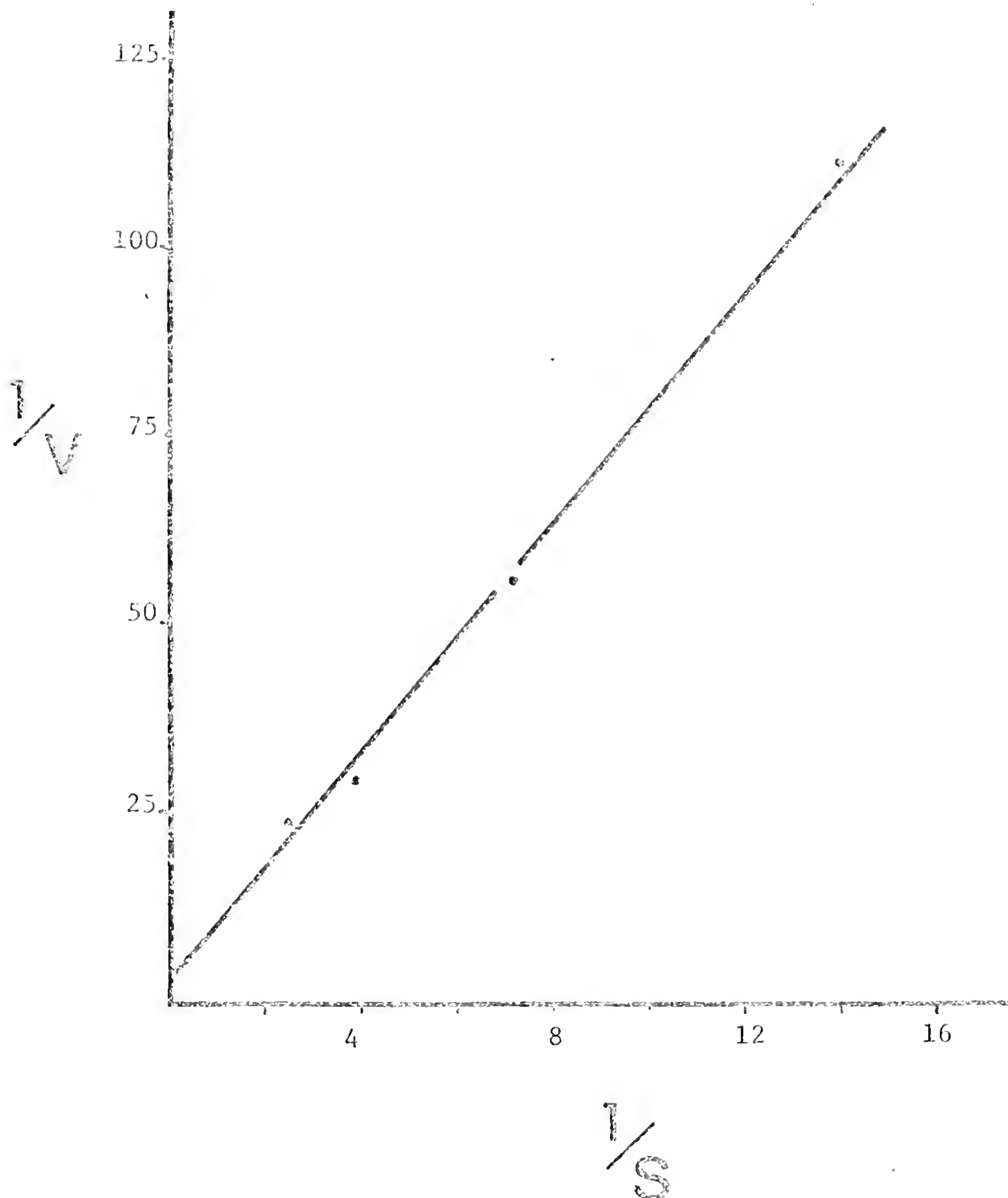


Figure 37. Lineweaver-Burk Transformation of Palmitate Incorporation Data.

The initial rates of incorporation into phosphatidyl choline for 4 concentrations of palmitate were plotted by the double reciprocal method. The maximum velocity was determined by the y-intercept and the Km for the process was determined from the slope. ($S = 10^{-7}$ M)(V = dpm/μg phosphatidyl choline/min).

The incorporation of stearate into total polar lipid pools is treated in the same manner as the palmitate data. The plots of the concentration dependence of uptake and the Lineweaver-Burk reciprocal plot appear in Figures 38, 39, and 40. The maximal rate of incorporation into total phospholipid is 3.4 μmoles stearate incorporated per 1 mole of polar lipid per minute. The Km measured from the slope of the reciprocal plot is 5.9×10^{-8} M. The Km for the total uptake determined from Figure 24 was 6.2×10^{-8} M. The Km for stearate incorporation into phospholipid is, as it is for palmitate, a misleading number for it represents both assimilation and incorporation.

Competitive Uptake

The investigations into amino acid and carbohydrate uptake by marine invertebrates demonstrated specific inhibitions of such uptake by groups of amino acids and metabolic analogs of carbohydrates. Testerman's (1972) work on fatty acids revealed competition of oleic acid uptake by linoleic, palmitic, and caproic acids. Our investigations on uptake by oysters revealed that palmitate and stearate uptake was much greater than that of oleate. The animals did not have a saturable uptake system for oleate; therefore, the effect of naturally occurring concentrations of oleate (10^{-9}) on the uptake of stearate was investigated. (See Table 7.)

Oleic acid in concentrations 10 times greater than that found in sea water was shown to inhibit the uptake of stearic acid. The assimilation of stearate or equimolar concentrations of oleate was completely inhibited. The variability of the data in these competition experiments using whole animals prevented determinations of the type of inhibition and the inhibitor constants, but the data indicate that stearate uptake can be inhibited by oleic acid.

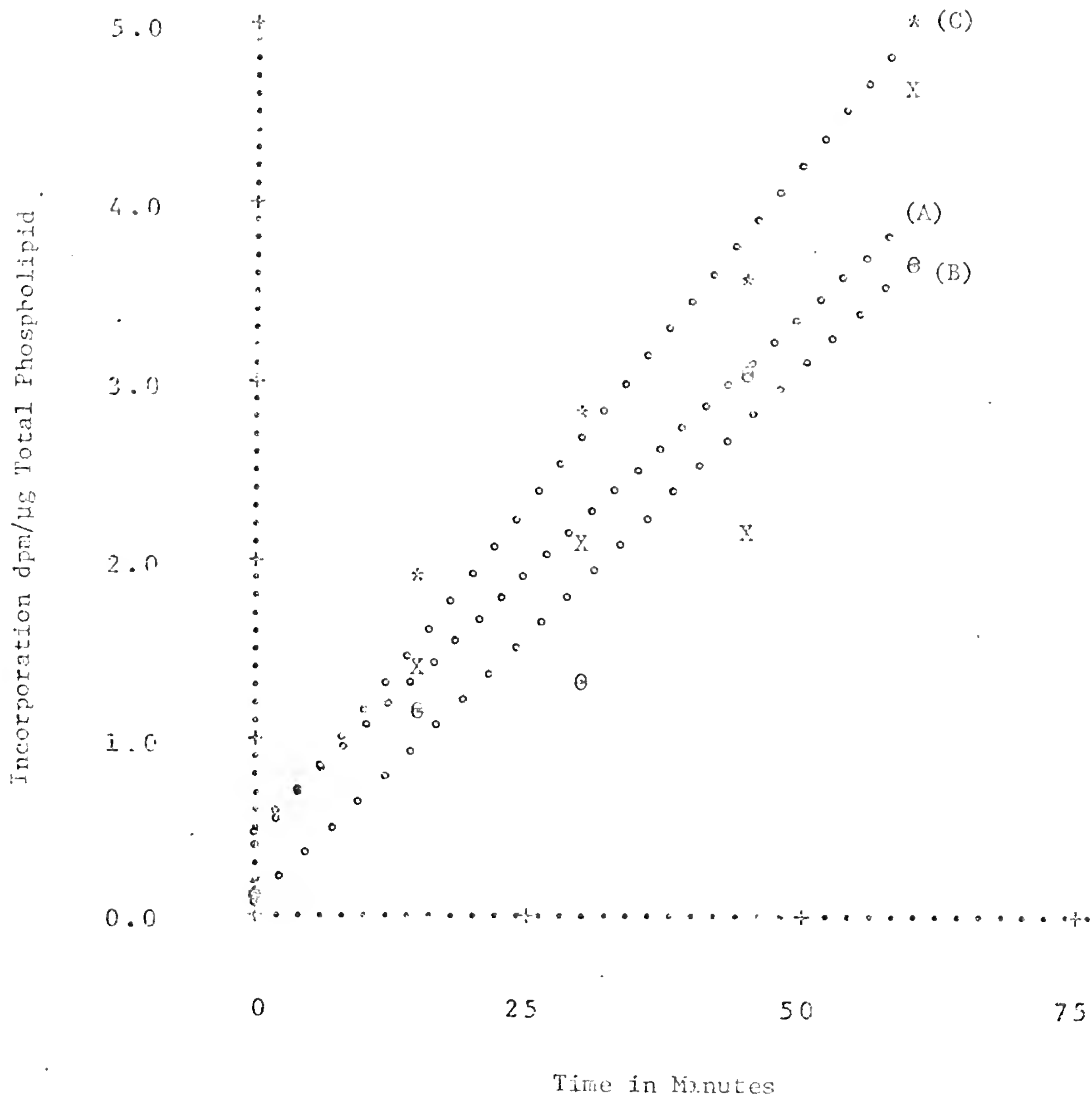


Figure 38. Concentration Dependent Incorporation of Stearate into Total Phospholipids.

The increase in the specific activity of the total phospholipid fraction from the chloroform extracts was plotted against time for 3 concentrations of stearate. The plots were the same as described as in Figure 35. Stearate concentrations were (A) and X - 0.093 µM, (B) and Θ - 0.14 µM, and (C) and * - 0.28 µM. The specific activity for the stearate isotope in all experiments was 14.0 µCi ^{14}C/µmole.

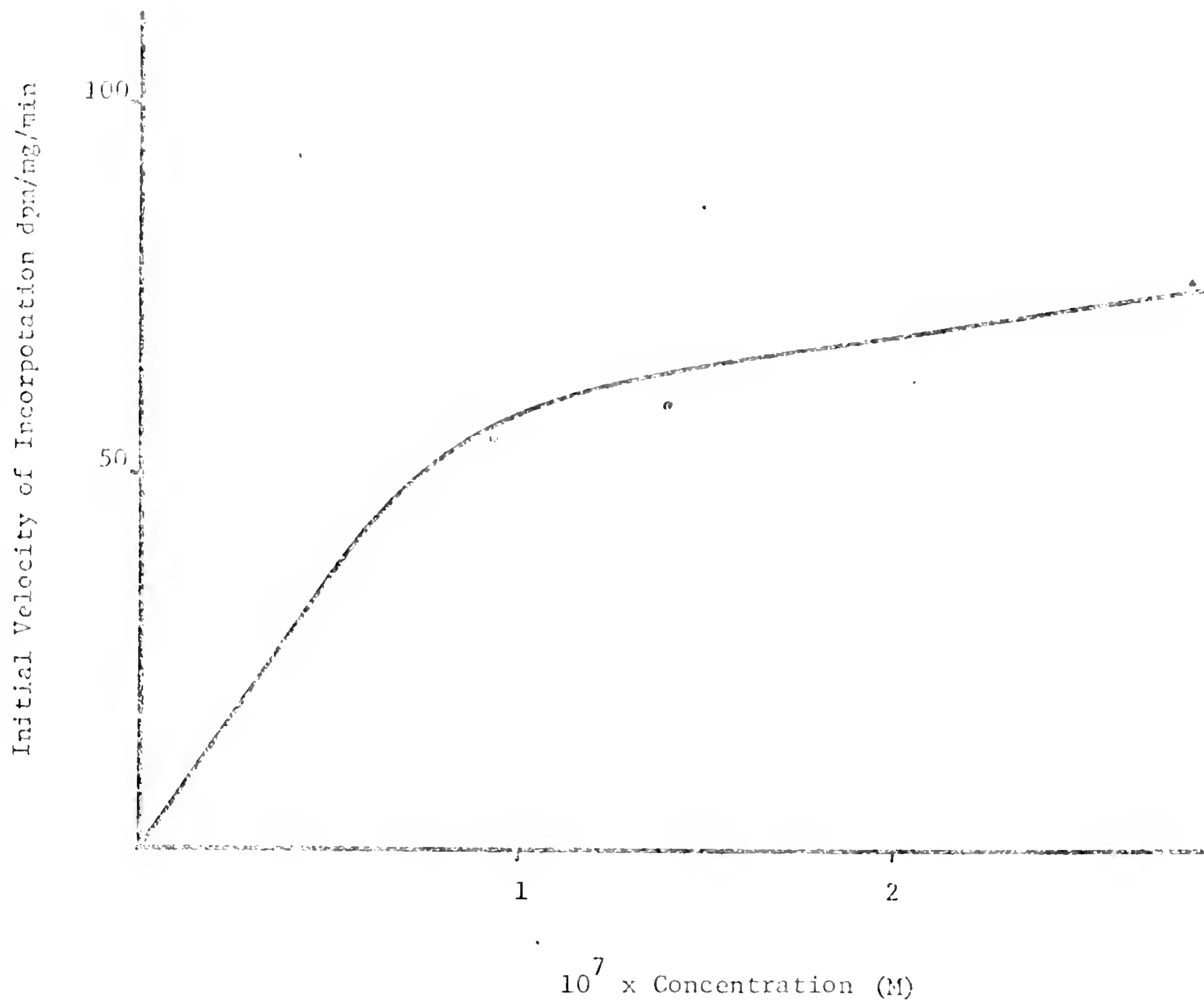


Figure 39. The Concentration Dependent Rate of Incorporation of Stearate into Total Phospholipid.

The initial rate of incorporation determined from the slopes of Figure 38 was plotted against the concentrations of stearate in the experiments.

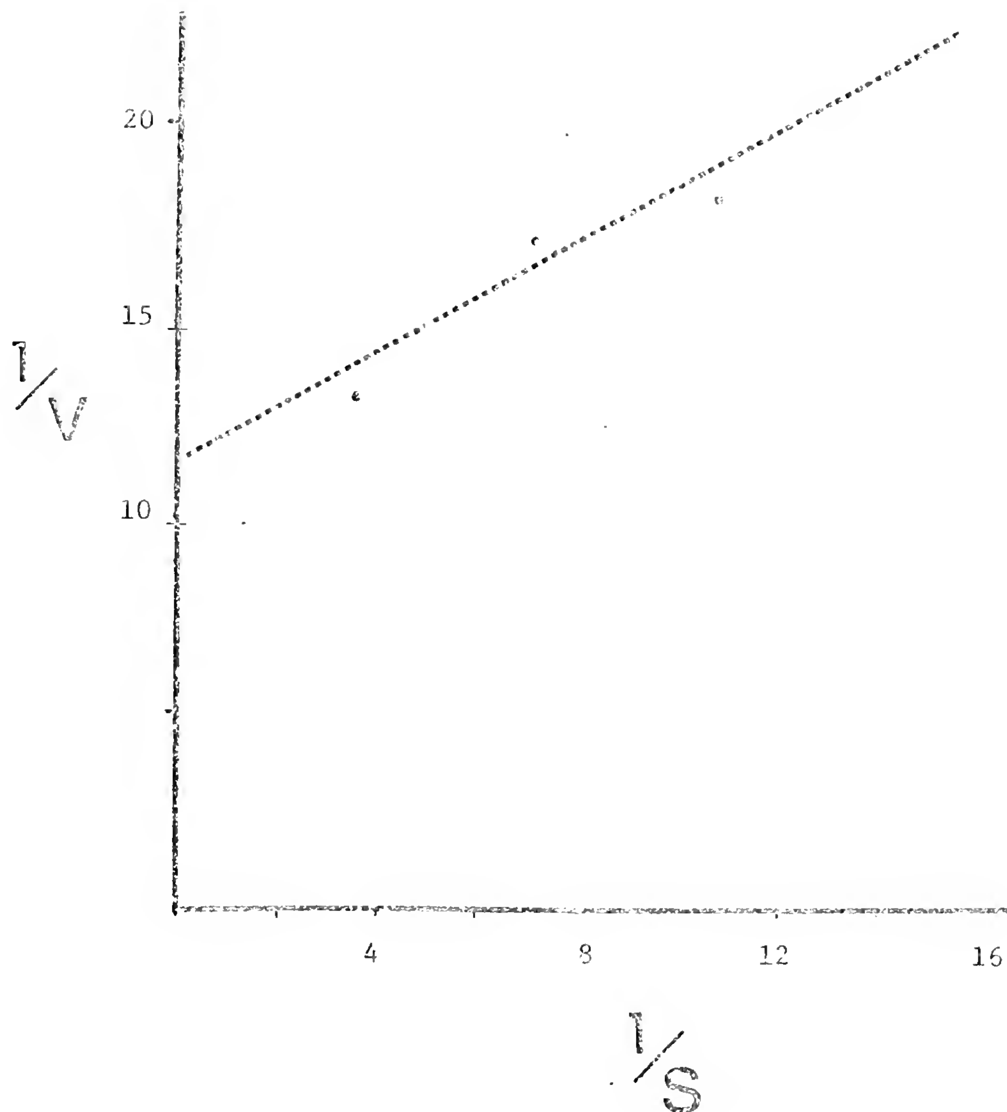


Figure 40. Lineweaver-Burk Transformation of Stearate Incorporation Data.

The initial rates of incorporation into phospholipid for 3 concentrations of palmitate were plotted by the double reciprocal method. The maximum velocity was determined from the y-intercept and the Km for the process was determined from the slope. (V = dpm/μg total phospholipid/min)(S = 10^{-7} M).

Table 7. The Effect of Oleic Acid on Stearic Acid Uptake.

Stearate Concentration	Oleate Concentration	Rate of Stearate Uptake[a] in pmole/gr/hr
2.8×10^{-7} M	0	0.91
2.8×10^{-7} M	8.8×10^{-9} M	0.68
2.8×10^{-7} M	3.5×10^{-8} M	0.40
2.8×10^{-7} M	1.8×10^{-7} M	0.00

[a]Determined from the least squares slope of initial velocity measurements.

The effect of oleate on palmitate uptake was investigated at up to 100 times the naturally occurring concentrations for oleate because no effect of oleate at naturally occurring concentrations could be demonstrated. The data in Table 8 indicate that a 1/1 molar ratio of oleate/palmitate has little effect on the rate of assimilation, but a 2/1 ratio increases the rate of uptake of palmitate. The total concentrations of the fatty acids in the last experiment (palmitate, oleate, and background fatty acids in the sea water), now exceed the micellar concentration for the solution and the mixed micellar aggregates are formed. The aggregation of these acids then promotes the uptake of the included palmitate as was seen in the data for the uptake of large concentrations of palmi- alone (Figure 21).

The concentration effects of added oleate in the palmitate uptake experiments indicated the need for further work into this concept of promoted uptake by particle generation. The effect of palmitate on the oleate uptake was investigated and the results appear in Table 9. The assimilation of oleate has been shown to be much less than palmitate and stearate and not saturable at 10^{-7} M concentrations (Figure 26). If palmitate is added to sea water containing 2.5×10^{-7} M oleate, the rate of uptake of oleate increases. If a similar concentration of palmitate is added to a 5.0×10^{-7} M oleate solution, there is little effect on the uptake. The results demonstrate that the addition of palmitate promotes micellar aggregation and an increase in uptake; but the results from the larger concentration may mean that there is a limit to this effect on acids like oleate which are not taken up to any appreciable extent by oysters.

In view of these data from the inhibition experiments the results must be interpreted very carefully. If a small inhibition is seen it

Table 8. The Effect of Oleic Acid on Palmitic Acid Uptake.

Palmitate Concentration	Oleate Concentration	Rate of Palmitate Uptake[a] in pmole/gr/hr
2.8×10^{-7} M	0	0.88
2.8×10^{-7} M	2.5×10^{-7} M	0.82
2.8×10^{-7} M	5.0×10^{-7} M	1.20

[a]Determined from the least squares slope of initial velocity measurements.

Table 9. The Effect of Palmitic Acid on Oleic Acid Uptake.

Oleate Concentration	Palmitate Concentration	Rate of Oleate Uptake[a] in nmole/gr/hr
2.5×10^{-7} M	0	3.6
2.5×10^{-7} M	2.8×10^{-7} M	16.8
5.0×10^{-7} M	0	13.5
5.0×10^{-7} M	2.8×10^{-7} M	15.9

[a]Rates measured in least squares slope of initial velocity plots.

may be entirely due to dilution effects. At large fatty acid concentrations, an inhibition effect may be masked by the promotion effects caused by particulate formation. The data suggest an obvious inhibition of stearate uptake by oleate, but no effect on palmitate uptake was seen with oleate.

Turnover of Lipid Classes

Data on the rate of fatty acid incorporation into various lipid classes have been obtained as described previously. In order to investigate the extent of this incorporation and its importance to the lipid metabolism of the oyster, determinations were made of the lipid turnover rate.

A method of lipid labeling with radioactive sodium acetate has previously been applied in order to determine the relative metabolic activities of various lipids in copepods (Farkas *et al.*, 1973). This method was applied to oysters by labeling for 18 hours with sodium [^{3}H]acetate (8mCi) in artificial sea water. In these experiments the assumptions are made that all lipid classes will be labeled within the period of exposure and that the label will be incorporated in sufficient amounts to make the specific activity determinations accurate. A preliminary experiment with labeled acetate indicated that the acetate could be removed from the sea water by the animals and that it was incorporated into all the lipids of the chloroform extract.

Figure 41 shows the results of the labeling experiment in the loss of label from the methanol and chloroform extracts of the animals. The incorporation is much greater in the non-lipid, methanol soluble material indicating that the acetate has entered several metabolic pathways not

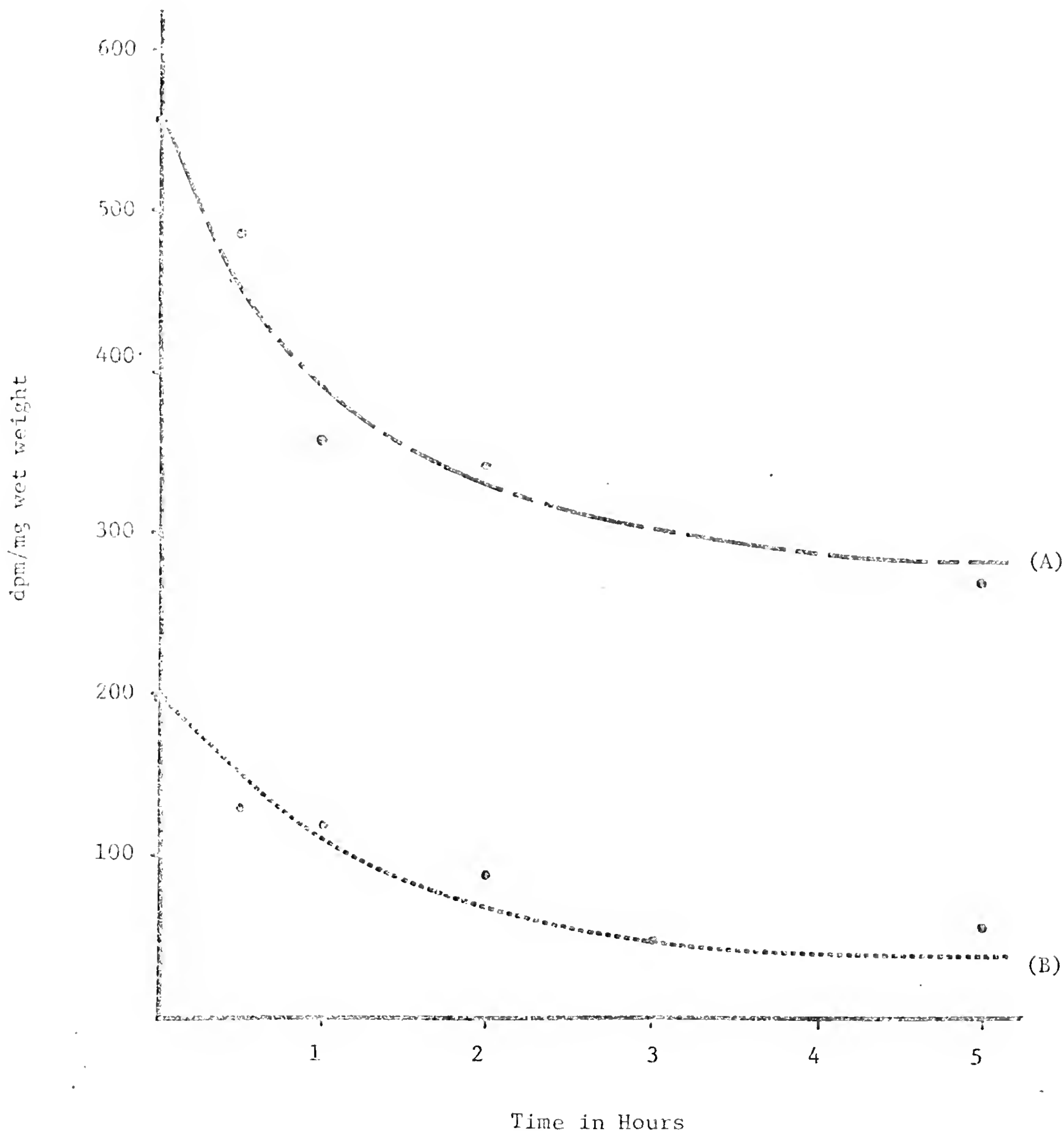


Figure 41. The Turnover of Lipid and Non-lipid Compounds Labled with [^{3}H]Acetate.

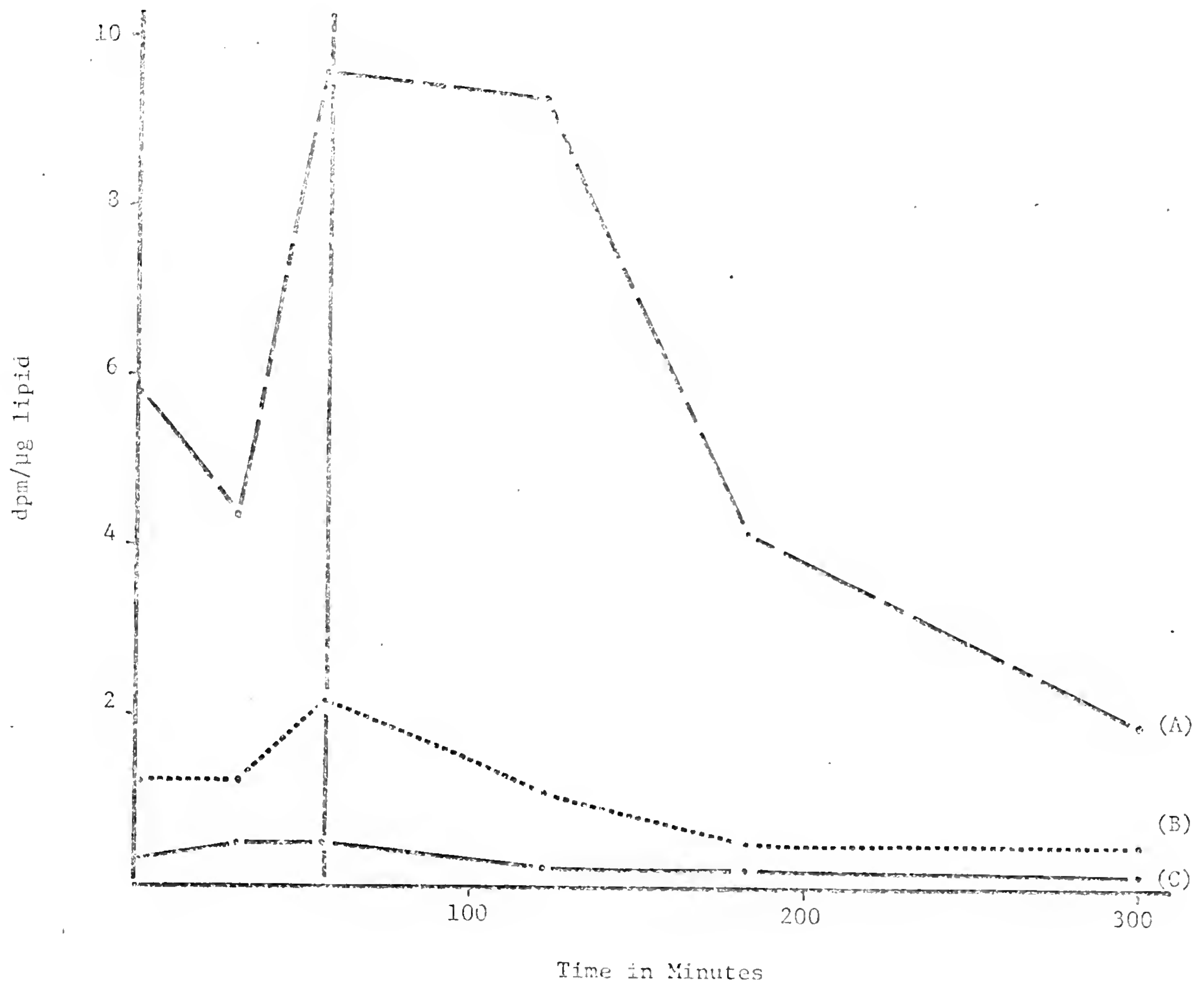
The animals were labeled for 18 hours in 4 liters of artificial sea water containing 8 m Ci^{3}H acetate at a concentration of 1.3 x 10^{-5} M. They were removed and placed in a non-labeled sea water medium. The radioactivity in the methanol (A) and chloroform (B) extracts of oyster tissue was plotted against time after removal from the non-labeled sea water.

leading to lipid synthesis, and that it has been metabolized to labeled products which are themselves incorporated into methanol extractable compounds.

The isolated lipid classes of triglycerides, total polar lipids, and cholesterol were the only compounds with sufficient specific activity to permit determinations of turnover rates. Figure 42 shows decrease in the specific activity of each class versus time after the animals were removed from the acetate labeled sea water and placed in unlabeled sea water. There is a short lag of 60 minutes during which the maximum incorporation occurs. This is due to the time required for the acetate to enter the metabolic pools following its assimilation from the external medium. The curve decreases in 5 hours to that turnover times may be determined. The triglycerides are the most metabolically active lipid class in the animal indicating that they represent the major energy storage form in the oyster. The polar lipids are metabolically active and important in the quantitative amounts which they represent, for up to 60 percent of the lipid material in oysters is the polar lipid fraction (Watanabe and Ackman, 1972). The large incorporation into the phospholipid shown in Figure 34 may reflect both the turnover activity and the large weight percentage that the phospholipids contribute. The sterols and other neutral lipid compounds are not very active and have a low rate of turnover.

Figure 42. The Turnover of Specific Lipid Classes in the Chloroform Extracts of Oysters Labeled with [^{3}H]Acetate.

The decrease in the specific activity of isolated lipids was plotted against time after removal from a sea water chase solution. The labeling sea water solution was the same as described in Figure 40. The lipids were separated on the neutral lipid TLC system. The curves were (A) triglycerides, (B) total phospholipids, and (C) cholesterol.



10
8
6
4
2
dpm/µg lipid
100
200
300
Time in Minutes
(A)
(B)
(C)

CONCLUSIONS

The presence of amino acids and carbohydrates in sea water, and their uptake by soft-bodied marine invertebrates have been demonstrated for at least 5 different animal phyla (Stephens, 1964). The uptake of lipids, specifically free fatty acids, has only been shown for 2 nereid species (Testerman, 1972) and 2 pogonophoran species (Southward and Southward, 1972). The concentrations of free fatty acid used were 0.06 - 6.0 μM which approximated the range of concentrations of free fatty acid found in the sea waters in which the animals lived.

In the present work, we have demonstrated that the American oyster, *Crassostrea virginica*, can remove palmitic and stearic acids from sea water at concentrations as low as 0.07 μM. The naturally occurring concentrations of lipids that we determined for the sea water from the Shell Mound estuary were 280 μg/liter total lipid including up to 77 μg/liter of total free fatty acid (equivalent to a 0.3 μM solution of palmitate).

The uptake of palmitic acid was shown to be completely inhibited by 200 mM sodium cyanide, indicating an energy dependent step in the process. We have shown that the loss of labeled palmitate from sea water is physiological and due, only in a small part, to chemical adsorption of the fatty acid onto the shell of the animals and glass walls of the experimental apparatus. The loss in label from the sea water occurred rapidly, within the first 60 - 90 minutes, and was concurrent with the appearance of radioactivity in the animal extracts.

The concentration dependent uptake experiments revealed that palmitate and stearate are assimilated by saturable uptake systems, but oleate is not. At concentrations above the saturated level (0.5 - 0.6 μM), the uptake of palmitate and stearate abruptly increases. This increased uptake may be due to self-aggregation of the fatty acid molecules into large micellar particles which are then filterable by the oysters. Fatty acids in sea water at concentrations in the range of 0.1 μM will occur in the form of small molecular aggregates since lipids are hydrophobic and have natural tendencies to aggregate in aqueous media; but these aggregates are too small to be filterable by the oysters' normal filter-feeding apparatus. At artificially increased concentrations (0.5 - 0.6 μM), these molecular aggregates increase in size and become greater than 0.5 μm approaching the lower size limit for the oysters' ciliary-mucoid filtration system, thus increasing the uptake.

We have shown that the uptake of radioactively labeled celite particles of sufficient size, 50 μm, to be filtered by the oysters' filter-feeding system differs from the uptake of freely soluble fatty acid in the time sequence involved. Soluble fatty acid can begin to accumulate in the lipid pools during the initial 15 minutes of exposure, but the celite filtration requires more than 30 minutes before incorporation is seen. This observation, along with our findings on the time course of uptake of celite containing adsorbed aniline dye, confirms the autoradiographic observations made on ^{14}C amino acid uptake by another lamellibranch species (Péquignat, 1973).

Our results with the temperature dependence of the uptake process indicated a depressed uptake rate at intermediate temperatures and a totally negative uptake at 20°C, a temperature to which the animal would

be exposed environmentally. These results may indicate a physiological reaction of the animals to temperature rather than a metabolic one. In shelled animals, such as oysters, which can seal themselves off from their milieu, the investigation of processes requiring exposure of the animal to the media is dependent upon the physiological stimuli to which the animal normally responds.

The experiments on the inhibition of uptake by competing fatty acids revealed that stearate uptake can be inhibited by low concentrations of oleate. Investigations into the effect of oleate upon palmitate uptake showed no inhibition up to a 1/1 oleate/palmitate molar ratio, but at a 2/1 ratio, the uptake of palmitate was promoted. We showed that the rate of oleic acid uptake was very small in comparison to that of palmitate and stearate, but that by adding unlabeled palmitate to labeled oleate, the rate of uptake of oleate could be increased. The results seem to indicate, once again, the recurring observation that the rate of uptake of dissolved material can occur in the absence of filtration feeding, but that when a concentration dependent micellar aggregation occurs, an increase in the assimilation rate due to filtration feeding is seen. The results of any inhibition studies at elevated concentrations should, therefore, be interpreted carefully; inhibition of the uptake systems for dissolved lipids may be masked by the promotion effects due to particle formation.

The results of the incorporation experiments show that palmitate and stearate are major fatty acids in the esterified lipids of the oyster. The labeled fatty acid removed from the sea water by the animal is esterified immediately into the complex lipids, for the animal does not have a large free fatty acid pool. The fatty acid is incorporated into

all the lipid classes, but the major incorporation occurs into the phospholipids, primarily phosphatidyl choline, and into the triglycerides. The presence of label in the cholesterol fraction indicates that the animals were viable and metabolically active for the fatty acid must be broken down to acetate before steroid synthesis can occur. The levels of incorporation into the triglycerides varied from one experiment, and even from one group of animals, to the next. The large turnover rate seen for the triglycerides helps to explain this variation; the triglycerides are the major lipid energy storage form in the oyster. Therefore, the concentrations of triglycerides would depend upon the length of time the animals had been without adequate food. In negative energy debt, the fatty acids being assimilated would be used for energy and not the synthesis of a storage form.

The importance of the uptake of freely dissolved lipid in the form of fatty acids for the energetic needs of the animal can be determined from the maximum velicity of uptake. We found in open shell experiments with palmitate that 0.26 μmoles of fatty acid are lost from the sea water in 2 hours and that 0.147 μmoles are taken up into the lipid extracts of the animals. This uptake represented incorporation of the palmitate removed into esterified lipid, since no free fatty acid was found in the lipid extracts. A small amount of the label lost in the experiment is lost due to adsorption onto the glass surfaces and the shells of the animals, but the majority is lost due to adsorption onto the feces and pseudofeces of the animals and onto the surface of the water itself. The uptake into the chloroform extracts of the animal and the small amount of non-lipid incorporation seen in the methanol extracts account for over 50 percent of the label lost from the sea water during the experiments. If the maximum rate of uptake is 2.30 pmoles/gr/hr, as measured from our experiments, and

the average oyster weight is taken as 3.5 grams, then the uptake rate per oyster per hours would be 8.05 pmoles/animal/hr. If this is converted to weight/animal/hr for palmitic acid, the rate would be 2.1 μg/gr/hr. This is small relative to the 0.16 mg carbon/hr that an oyster normally removes from the sea water for its metabolic needs (Nicol, 1970), but when one considers all the lipid available to the animal, the accelerated rate when particulate matter is formed, and the range of concentrations found in natural waters, this pathway becomes more important energetically.

An important implication of a free fatty acid uptake system is in the physical similarity of the fatty acid and other lipid material to the hydrocarbon pollutants found in our coastal waters. Oysters are known to concentrate petro-hydrocarbons (Stegeman and Teal, 1973) from sea water and store them for several months. Very few metabolic interconversions occur during this time and it appears that the petro-hydrocarbons are merely dissolved in the lipid pools of the animal. The uptake of these compounds must occur by a pathway similar to that utilized for free lipid uptake. Long after an oil slick on the surface has dissipated, the animals can still remove hydrophobic material dissolved in sea water.

The latest research into the *in vivo* and *in vitro* uptake of dissolved organics by lamellibranch molluscs (Bamford and McCrea, 1975) indicates that these animals may remove a certain percentage of particulate-adsorbed organic material by extra-brachial enzyme secretion, breakdown, and uptake directly acorss the gill surface, rather than ciliary transport of the particles to the mouth. Future work on the mechanisms of uptake of esterified materials is certainly indicated.

Work done by Ryther and his colleagues at Woods Hole Marine Biological Laboratory has shown that the American oyster, *Crassostrea virginica*, is a good candidate for exploitation by aquaculture technology (Ryther *et al.*, 1972;

Tenore *et al.*, 1973). In their work with tertiary treatment of municipal sewage by algal farming, the oyster was used as a primary consumer of algal material grown in diluted sewage effluent. The oysters grew to full harvestable size in a matter of nine months on this algal diet (Ryther *et al.*, 1972). The apparent efficiency of the animal in converting nutrients to body mass may be due in a large part to direct uptake pathways involving the elevated concentrations of dissolved nutrients that would be in the sewage effluent, which may not be completely utilized by the algal cultures. This pathway of direct assimilation of lipid material which we have demonstrated may be very important to the future farming of animals in our coastal waters.

The appearance of such an assimilatory pathway in marine invertebrates has been demonstrated. The presence of dissolved organic material in fresh and brackish waters and its utilization by fresh water lamellibranchs should be investigated, for it may reveal information on the universality of these processes in all soft-bodied aquatic invertebrates.

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BIOGRAPHICAL SKETCH

Terry Allan Bunde was born on January 11, 1947, in Orlando, Florida. He was raised in Orlando and following his graduation from high school he entered Rollins College, Winter Park, Florida where he majored in pre-medical science. He received his Bachelor of Science degree in 1968.

He entered the Department of Biochemistry in the graduate school at the University of Florida in 1968 and worked toward his degree until he was drafted in 1969. After two years in the United States Army, he reentered the Department of Biochemistry in the graduate school of the University of Florida in September, 1971. Since then, he has pursued his work toward the degree of Doctor of Philosophy in the Biochemistry Department.

He was married to the former Pamela Sue Riess in August, 1971.

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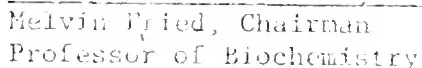

Melvin Fried, Chairman
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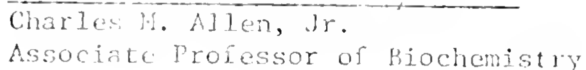

Charles M. Allen, Jr.
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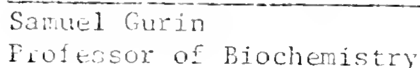

Samuel Gurin
Professor of Biochemistry

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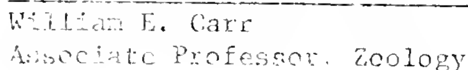

William E. Carr
Associate Professor, Zoology

This dissertation was submitted to the Graduate Faculty of the Department of Biochemistry in the College of Arts and Sciences and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June, 1975

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Dean, Graduate School