AN ABSTRACT OF THE THESIS OF

Ronald Thomas Riley for the degree of Doctor of Philosophy in General Science (Biological Science) presented on July 6, 1978

Title: THE EFFECTS OF CHEMICAL PERTURBATION BY NAPHTHALENE ON GLUCOSE METABOLISM IN THE EUROPEAN FLAT OYSTER (OSTREA EDULIS): AN IN VIVO KINETIC ANALYSIS

Abstract approved: Redacted for privacy
Michael C. Mix

The purpose of this study was to evaluate the potential of utilizing an in vivo kinetic analysis of glucose metabolism as an approach for assessing the effects of chemical pollutants on bivalve mollusks.

Starved oysters were stressed in the presence of naphthalene in an open flow-through system that modeled the entry of the pollutant as if from a point source with the ambient pollutant concentration being zero at time zero and the eventual steady-state concentration approaching 90 ppb at the end of 72 hr.

Each 72-hr run consisted of exposing three separate groups of oysters to three different treatments. The first group, the "control-treated" (Ct) oysters, was never exposed to naphthalene; the second group was "naphthalene-treated" (Nt) and was exposed to unlabeled naphthalene dissolved in seawater; the third group was exposed to [1-14C] naphthalene dissolved in seawater. Oysters in the former two groups were utilized for measuring the pool sizes of the major precursors, intermediates, and end products of glucose

metabolism and for the in vivo kinetic analysis of glucose metabolism, and oysters in the latter group were used for measuring the naphthalene and naphthalene metabolite concentrations in the various tissues of the oysters.

The in vivo kinetic analysis involved tracing the carbon flow from D-[U-14C] glucose into the intermediates and end products of glucose metabolism in oysters, maintained in unstressed (control) and naphthalene stressed environments. Specific radioactivity-time curves for ethanol-insoluble polysaccharides (primarily glycogen), total protein, total polar lipids, total neutral lipids, neutral compounds (primarily glucose), free alanine, aspartate and glutamate, taurine, and total organic acids were determined for control and naphthalene-stressed oysters. Radioactivity-time curves for malate and succinate were also determined.

The water from the flow-through system was analyzed for dissolved oxygen, ammonia-nitrogen, and for the build-up of [1-14C] naphthalene from the initial zero concentration at time zero. The extent of bacterial metabolism of naphthalene, and the effects of the bacterial population on the dissolved oxygen concentration, and ammonia-nitrogen was also evaluated.

The results of this study indicated that there were three types of effects evident: effects attributable to starvation, effects attributable to either reduced oxygen concentrations in the flow-through system or difference in the glucose concentrations in the flow-through system and glucose incubation vessels, and effects due to the naphthalene treatment.

In each run approximately 150 μg of naphthalene entered the flow-through system containing the naphthalene-treated oysters during the 72-hr run. Of the naphthalene that entered only about 5.0% was recovered in oyster tissues. Of this 5.0%, about 5.0% of it was in the form of non-CO_2 saponifiable metabolites. Monohydroxylated naphthalene derivatives were the most commonly observed hexane extractable metabolites based on thin layer chromotographic procedures.

Increased catabolism of proteins and polar lipids, increased levels of amino acids and organic acids, increased initial rate of glucose uptake, and significant differences in the specific activity-time curves for alanine, aspartate, glutamate, protein, and polar lipids and radioactivity-time curves for malate and succinate, were effects attributable to naphthalene treatment.

The fact that total protein and total polar lipids were significantly reduced in the naphthalene-treated oysters suggested that naththalene treatment stimulated the catabolism of these compounds. The increased levels of amino acids and organic acids in naphthalene-treated oysters could have reflected either a disturbed protein metabolism or an increased dependence on anaerobic pathways.

In general, the specific activity-time curves for Ala, Asp and Glu and the radioactivity-time curves for malate and succinate suggested that the carbon flux through the Krebs cycle and associated amino acids was stimulated by naphthalene treatment. The fact that 14C-flux through the intermediates increased while 14C-flux into end

products may not have increased, suggested that the efficiency of assimilation into end products had been reduced by naphthalene treatment.

The Effects of Chemical Perturbation by Naphthalene on Glucose Metabolism in the European Flat Oyster (*Ostrea edulis*): An in vivo Kinetic Analysis

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I. INTRODUCTION

Many species of marine bivalve mollusks such as oysters, clams, and mussels are economically-important estuarine organisms. They are raised commercially or support substantial recreational fisheries in many accessible bays. Because of the increasing domestic and industrial use of coastal areas, shellfish are often exposed continuously to environmental contaminants. The effects of chronic environmental stressors on bivalve mollusks are now thought to be quite subtle. Since the future of marine ecosystems may depend to a great extent on the ability of these systems to cope with increasing chemical insults it is essential that information concerning the low level effects of chemical contamination be determined.

One of the major classes of chemical perturbants entering the marine environment is petroleum hydrocarbons. It was estimated that in 1973 6.1 million metric tons of petroleum hydrocarbons entered the marine environment (Clark and MacLeod, 1977). Over 40% of this was derived from land-based discharge of waste oils, run off, and sewage. Most of these hydrocarbons were transported by rivers and eventually reached estuaries and bays before entering the oceans. In the State of Washington (USA) alone, over 62,400 metric tons of waste oil from this coastal state was either discharged directly into the environment or was unaccounted for (Clark and MacLeod, 1977). Waste oils are for the most part highly refined oils

comprised primarily of spent lubricants (i.e. used crankcase oil). Refined oils, which are derived from the higher boiling range fraction of crude oils, usually contain a greater precentage of aromatic hydrocarbons and are generally more toxic to marine invertebrates than crude oils (Craddock, 1977, his Table 8). The greater toxicity of refined oils is due in part to the higher concentration of aromatic hydrocarbons, both in the unmodified oil and to a greater extent, in the water soluble fractions (WSF) (Neff, Anderson, Cox, Laughlin, Rossi, and Tatem, 1976).

Most studies evaluating the effects of oils have not measured the accumulation of oil in the tissues, rather, they have compared the effects observed to the initial concentration of oil or WSF dissolved in pure seawater at the beginning of the experiment. Since the more toxic aromatic hydrocarbons are easily volatilized from aqueous solution (Anderson, Neff, Cox, Tatem, and Hightower, 1974), studies which are conducted in static systems with continuous aeration usually show constantly declining concentrations of aromatic hydrocarbons in the water. This is especially true in studies with naphthalenes. Thus, when the toxicities of oils are being studies, 24-hr median tolerance limits (TLM's) are usually only slightly less than 96-hr TLM's (Rice, Short, and Karinen, 1976). The concentration that the organism is exposed to is maximum at the beginning of the experiment and declines constantly as the more volatile compounds evaporate. Neff and his co-workers (1976) stress that in most cases the concentration of dissolved hydrocarbons in

natural seawater is far below that required to elicit observable acute or sublethal effects. They further point out that the highest reported concentration of hydrocarbons from under an offshore oil spill was 200 ppb. However, Clark and MacLeod (1977, their Table 11) provide data which indicates that in bays and estuaries, the dissolved hydrocarbon concentrations may be much higher. Some examples are: Boston Harbor, Mass., 190-816 ppb; Narraganset Bay, R.I., 1000-12,700 ppb (sewage outfall); San Francisco Bay, Calif., 14-280 (paraffins) and < 5-59 ppb (aromatics). The levels in the sediments often reach even higher levels (VanVleet and Quinn, 1978). The tissue levels in bivalves from chronically-polluted harbors and estuaries are also very high (Clark and MacLeod, 1977, their Table 8).

In the estuarine marine environment, the route of entry of oils is primarily from point sources such as sewage outfalls, waste water from refineries, storm drains, runoff, etc. The contribution from sewage discharge alone is estimated at 8 g per capita per day from a major coastal city. The same amount per day enters from industrial sources (Clark and MacLeod, 1977). These sorts of discharges result in the continuous low level chronic exposure of benthic marine organisms. The level of exposure is dependent on numerous factors such as currents, wave action, tides, rate of evaporation, photo-oxidation, microbial metabolism, sinking, dissolution and the distance from the source. Assuming a continuous discharge and a constancy of the other factors, then the level of

exposure should reach some steady-state value which represents the balance of influxes and effluxes. A continuous flow-system best models the low level chronic pollution of estuarine environments. Surprisingly, few studies have been conducted on bivalves that utilize continuous flow-systems (Craddock, 1977, his Table 3).

Traditionally, attempts to determine the effects of exposure to chemical pollutants have been confined to monitoring of mortality rates, growth rates or histological abnormalities. The procedures associated with such analysis for shellfish are usually time consuming and often inconclusive. The lethal effects of numerous specific petroleum hydrocarbons have been studied extensively (Craddock, 1977). However, knowledge of the levels that cause extensive mortalities over a given period is of limited value for use in predicting the effects of chronic hydrocarbon pollution on the marine environment. Such information is of no value if there are critical processes which are adversely affected, at low pollutant dosages, and which do not result in immediate mortalities. These sublethal effects which occur at low pollutant dosages, may result in significant chronic effects if they persist. The sublethal effects must be studied further if an adequate understanding of the ability of the marine ecosystem to withstand petroluem-induced perturbation is to be attained (Johnson, 1977).

Several studies have recently been conducted on sublethal effects at the metabolic level. Most of these studies have been restricted to evaluating the effects of very high oil concentrations.

Avolizi and Nuwayhid (1974) found that 10,000-25,000 ppm (v/v) of Arabian crude oil in seawater inhibited the respiratory rate of Brachidontes variabilis and Donax trunculus. Dunning and Major (1974) found that 12% mixtures of the WSF of a premium lubricating oil in seawater and No. 2 fuel oil inhibited respiration. Gilfillan (1975) on the other hand, found that mixtures as low as 1% WSF of crude oil in seawater increased the respiratory rate of the mussel, Mytilus edulis and Modiolus demissus. There is little question that bivalves exhibit respiratory responses to sublethal concentrations of oils. However, the response is quite variable between species and studies. Recently it has been shown that in the soft shell clam, Mya arenaria, low levels of oil stimulate respiration whereas high levels inhibit respiration (Stainken, 1978). It has been suggested that petroleum hydrocarbons may influence respiration directly at the cellular level or by their interaction with cell membranes or indirectly by modifying behavior and activity (Neff et al., 1976). Coating the gills, interference with ciliary activity, and shell closure are effects or responses to petroleum pollution which could partially explain a reduction in respiratory rates.

There have been very few studies which have investigated sublethal metabolic effects at levels more basic than oxygen consumption. Gilfillan (1975) calculated the carbon budgets for M. edulis and M. demissus and found that in the presence of mixtures of only 1% WSF in seawater, both carbon consumption and carbon assimilation were considerably reduced. In 10% mixtures, the carbon flux was

reduced by 50% and at higher concentrations the carbon flux became negative. Gilfillan (1975) theorized that negative carbon flux would ultimately result in the mobilization of energy reserves which were normally required for gametogenesis. The results suggested that the effects of crude oil on these animals acted to reduce the amount of carbon available for growth and reproduction. It is interesting to note that salinity stress had a similar effect. The combined stressors of reduced salinity and crude oil served to enhance the effects of crude oil. A later study on softshell clams, M. arenaria (Gilfillan, Mayo, Hansen, Donovan, and Jiang, 1976) provided additional support for these theories for natural populations exposed to No. 6 fuel oil from an oil spill.

There are numberous approaches for studying sublethal effects at the level of metabolism. The most basic metabolic effect occurs at the level of the enzymes involved in metabolism. Enzymes are responsible for all physiologic functions. Any physiological effect observed as a result of exposure to low levels of oils would usually be preceded by changes in enzyme activity. It is therefore surprising that, to date, there has been only one published study reporting the effects of exposing a bivalve to oil, at the level of enzyme activity (Heitz, Lewis, Chambers, and Yarbrough, 1974). Of the enzymes assayed in that study, four were significantly affected by crude oil mixtures. Of those, two were related to intermediary metabolism, malate dehydrogenase and glutamate-pyruvate transaminase. One, B-glucuronidase, was possibly related to detoxification and one, was related to protein catabolism.

In light of the above study and the work by Gilfillan (1975), a study of the carbon flux through intermediary metabolism and into the end products of metabolism in a bivalve mollusk seemed warranted. Metabolic perturbation either precedes or is simultaneous with physiologic alteration. Theoretically, deviations at the level of enzyme activity, in vivo, should be rapidly and accurately determined by kinetic analysis using 14C-labeled precursors. The effect of a chemical perturbant at one end of a metabolic pathway should be directly conveyed to other pathways via stoichiometric linkage.

The purpose of this study was to evaluate the potential of utilizing an in vivo kinetic analysis of glucose metabolism as an approach for assessing the effects of chemical pollutants on bivalve mollusks.

Starved oysters were stressed in the presence of naphthalene in an open flow-through system that modeled the entry of the pollutant as if from a point source with the ambient pollutant concentration being zero at time zero and the eventual steady-state concentration approaching 90 ppb at the end of 72 hr.

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II. MATERIALS AND METHODS

Quantitative Methods

A. Fractionation and quantification of the intermediates and end products of oyster metabolism.

Except where noted, all water used in the analytical procedures was distilled and organic solvents were glass distilled from reagent grade solvents. Organic acids were reagent grade and all other reagents and standards were reagent grade or better and were obtained from the most economical supplier. Chloroform was distilled over sodium carbonate, acetone over potassium permanganate, and hexane over sodium wire. Phenol was distilled from 80% liquid phenol with an air condenser and stored under N_2 at -20°C in brown bottles to prevent decomposition. All screw-cap culture tubes had Teflon liners in the caps.

The freeze-dried gill was fractionated according to the procedure outlined in Figure 1. The details of the procedure are discussed below. The tissue was weighed and put into a 10-ml Vitro tissue grinding tube. The tube was first placed in a heavy-walled pyrex beaker and liquid N_2 poured into the beaker; after cooling, liquid N_2 was poured into the tube with the tissue. The tissue was then pulverized into a fine uniform powder with a 0.25-inch pyrex rod with an end that had been previously heated and formed into a ball-shaped knob. The powdered tissue was homogenized with ~ 1.0 ml of 80% ethanol (if the recovery of a substance was being determined

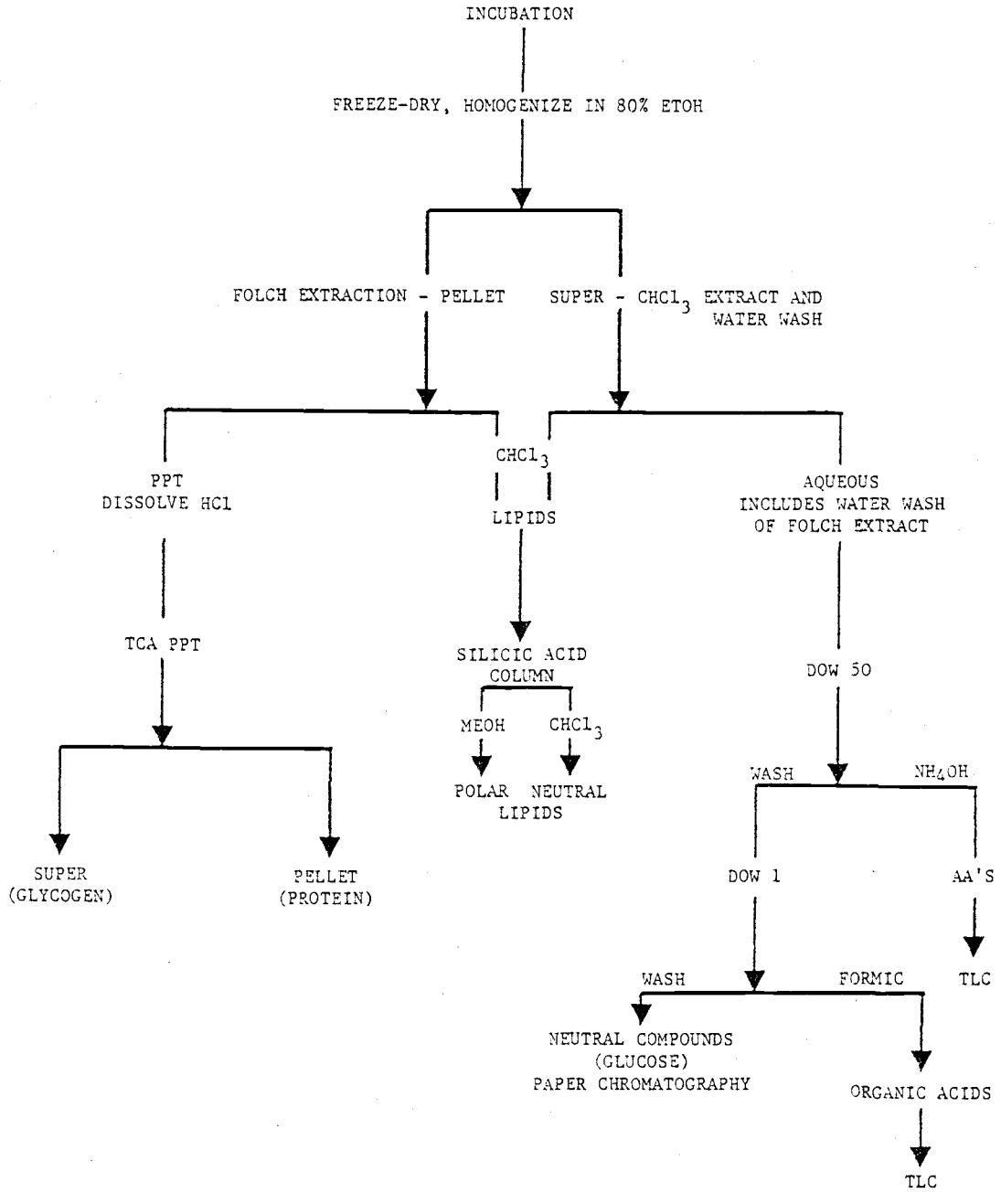

Figure 1. The fractionation protocol for isolating the end products and intermediates of glucose metabolism from oyster tissues.

by utilizing a radioisotope, then the isotope was added just prior to the ethanol) using a Teflon pestle and an electric power drive. The bottom of the tube was maintained in an ice bath to prevent warming and to increase the efficiency of homogenization. The pestle was rinsed with ~ 0.5 ml 80% ethanol and the homogenate was allowed to stand 1 hr at 4°C; it was then centrifuged at 4340 g at 4°C for 10 min and the supernatant pipeted off into a 18 x 150 mm screw-cap culture tube. The homogenization and centrifugation procedure was repeated twice and the final precipitate was washed with two 0.5 ml aliquots of 80% ethanol with vortex mixing between washes.

All the supernatants and washes were combined, two volumes chloroform and 2 ml of water were added and the culture tubes capped tightly and mixed throughly by 30 gentle inversions. The mixture was held 15 min at room temperature and then carefully centrifuged at low speed (1000 rpm) on a clinical centrifuge for 10 min to achieve phase separation. If that treatment was unsuccessful then standing overnight proved an effective method to achieve separation. The upper aqueous phase was pipeted off into a 25 ml Erlenmeyer flask and the water wash repeated three times; all washes were combined in the Erlenmeyer flask.

The precipitate from the original ethanol homogenization step was extracted by the method of Folch, Lees, and Sloane-Stanley (1957). A 5 ml aliquot of 2:1 chloroform:methanol was added to the tissue grinding tube containing the precipitate and homogenized. The pestle was washed with 1 ml of 2:1 chloroform:methanol and the

tube was centrifuged at 4340 g for 10 min. The supernatant was pipeted off into a 16 x 100 mm screw-cap culture tube and the 2:1 chloroform:methanol extract was then washed with 1.6 ml of water.

After phase separation, the aqueous phase was pipeted off and combined in the 25-ml Erlenmeyer flask with the water washes of the chloroform extract from the ethanol supernatant. The interface of the lower phase of the chloroform:methanol extract was washed with two 0.5 ml aliquots of pure solvent upper phase (prepared as described by Folch et al., 1957) which were also combined with the aqueous washes. The chloroform and chloroform:methanol extracts were pooled, made to one phase by the addition of methanol and then evaporated to dryness under N_2 at 50°C. The residue was brought up in ~ 0.5 ml chloroform and separated into polar and non-polar lipids by the method of Dittmer and Wells (1969). A Pasteur pipet with a glass wool plug at the bottom was loaded with ~ 0.3 g of silicic acid in a chloroform slurry and a wad of glass wool was placed on top of the column after packing. The column was washed with ~ 5 ml of chloroform and the sample was then transferred onto the column and washed with two 0.5 ml aliquots of chloroform and eluted with ~ 9 ml of chloroform. The effluent consisting of the original chloroform solution, the wash and the final eluting solvent was collected in a 10 ml glass-stoppered graduated tube. Polar lipids were eluted with ~ 10 ml of methanol. An aliquot of the chloroform eluate was placed in a scintillation vial and evaporated to dryness under N_2. The scintillation vial was filled with 5 ml of toluene scintillation fluor and counted on a Packard Tri-Carb LS

Spectrometer model 3330. Counting efficiencies were determined by internal standardization with 14C-toluene. An aliquot of the methanol eluate was also evaporated in a scintillation vial to remove any residual chloroform and then redissolved in a 2 ml volume of methanol and counted in PCS fluor (Amersham Co.). The total lipids present in each fraction were quantified by the charring technique of Marsh and Weinstein (1966) with tripalmitin used as a standard. The solvent was removed from an aliquot of the lipid sample in a 18 x 150 mm pyrex test tube under a flow of N_2 at 50°C. After cooling, 2 ml of concentrated sulfuric acid was added, the tube placed first in an aluminum heating block at 200°C for 15 min, and then into a 25°C water bath for 15 sec and finally into an ice bath for 5 min. When sufficiently cooled, 3 ml of water was added, the contents mixed thoroughly, and the tube replaced in the ice. After cooling, the tube was removed and left standing for 10 min or until all bubbles had disappeared. The absorbance at 375 nm was measured in a 1 cm quartz cuvet; standards were treated in a similar manner.

The combined aqueous extracts prepared previously, were partially evaporated under a flow of N_2 at 50°C and then evaporated to dryness over sodium sulfate in a sleeve-type pyrex desiccator under vacuum using a water aspirator vacuum pump which achieved a maximum vacuum of 29 mm of mercury. In order to prevent bumping, the vacuum was brought up very slowly and an infra-red heat lamp was used to speed evaporation. The dried residue was stored under N_2 at -20°C in a desiccator over sodium sulfate until ready for

fractionation into amino acids, organic acids and neutral compounds.

The lipid-free pellet from the Folch extraction was partially dried under a flow of N_2, homogenized in 2.0 ml of 0.01 N hydrochloric acid (HCl) and transferred to a 15 ml Corex centrifuge tube with four ~ 0.5 ml 0.01 N HCl washes of the pestle and homogenization tube. An equal volume (~ 5 ml) of 10% trichloroacetic acid (TCA) was added and the mixture held at 4°C for 1 hr; it was then centrifuged at 12,100 g at 4°C for 10 min. The TCA supernatant was pipeted off into a 15 ml graduated tube and the precipitate washed with two 0.5 ml aliquots of 5% TCA and recentrifuged between washes. The washes were combined with the supernatant in the 15 ml graduated tube. An aliquot of the TCA soluble substances was counted in PCS fluor and another aliquot was analyzed for total reducing sugars after acid hydrolysis by the method of Dubois (1956). The TCA solution was appropriately diluted and 1.0 ml of 5% phenol in water was added, followed by the rapid addition of 5 ml concentrated sulfuric acid. After cooling and mixing, the absorbance was determined at 490 nm in a 1 cm cuvet; glucose was used as a standard.

The TCA precipitate was washed with two 0.5 ml aliquots of 0.1 N potassium acetate in 80% ethanol. The ethanol washes were discarded and the precipitate dried under a flow of N_2 at room temperature. The dried precipitate was then dissolved in 2 ml of 0.1 N sodium hydroxide (NaOH) at 50°C. One aliquot was counted in 2:1 PCS and a second was analyzed for total protein by the method of Lowry, Rosebrough, Farr, and Randall (1951). Bovine serum

albumin (BSA) was used as a standard. The BSA stock solution was dissolved in 0.01 N HCl, precipitated with 10% TCA, washed with 0.1 N potassium acetate in 80% ethanol, dried under N_2 and then dissolved in 0.1 N NaOH at 50°C. The stock solution was diluted appropriately when determining the standard curve.

The dried residue from the water wash of the combined lipid extracts was dissolved in 1.0 ml of 0.01 N formic acid and then passed through a column of Dow 50 x 4, 200-400 mesh resin in the H^+ form, prepared as follows: a Pasteur pipet with a glass wool plug in the bottom was loaded with ~ 0.65 g of the resin and the surface covered with a wad of glass wool; the resin was converted to the NH_4^+ form with 2 ml of 4 N ammonium hydroxide washed to neutral (pH < 9.0) with ~ 3.0 ml of water, converted to the H^+ form with 2 ml of 0.1 N formic and then washed with 1 ml of water. The sample was then passed through the column and washed three times with 1 ml of 0.01 N formic acid. The sample solvent and washes were collected in a 25-ml Erlenmeyer flask. Amino acids were eluted with 4 ml of 4 N ammonium hydroxide and the eluate was collected in another 25-ml Erlenmeyer flask. The eluate and wash were frozen and then freeze-dried. Recoveries of L-[U-14C] glutamate and L-[U-14C] leucine spikes carried through from the homogenization step were 90.8% (s = 5.5, n = 4) and 90.7% (s = 1.2, n = 4) respectively.

The freeze-dried Dow 50 eluate was brought up in ~ 1.0 ml of 0.01 N HCl and transferred to a graduated tube along with two 0.5 ml rinses of the Erlenmeyer flask. An aliquot was then counted in 2:1 PCS and the eluate re-evaporated to dryness in the vacuum

desiccator described previously. The residue was dissolved in ~50 µl of 0.01 N HCl, and a 5 µl aliquot counted in 2:1 PCS. The exact volume of the concentrated eluate was calculated, based on the previously determined volume and activity. A 5-µl aliquot of the concentrated Dow 50 eluate was streaked with a 10 µl Hamilton microsyringe in a 2-cm scribed lane on a mixed layer (cellulose/silica gel) thin layer plate prepared as described by Turner and Redgwell (1966). A typical 20 cm x 20 cm plate accommodated nine samples. The plates were developed in glass distilled phenol:water (80:20 w/v) and air-dried overnight in a well ventilated hood. Lanes 1 and 9 were duplicates of lanes 2 and 8. The two end lanes were sprayed with 0.5% ninhydrin in 95% ethanol and the remaining lanes were stripped by the method of Redgwell, Turner, and Bieleski (1974). The areas corresponding to the amino acids alanine (Ala), aspartate (Asp), and glutamate (Glu), were removed and placed in scintillation vials containing 0.5 ml water and allowed to stand 15 min before 5 ml of 2:1 PCS was added. Ala, Asp and Glu were the only amino acids with detectable counts and they were well resolved in one dimension by the phenol:water solvent system. Fortunately, Ala, Asp, Glu, glycine (Gly), and serine (Ser) were the most abundant amino acids in the Dow 50 eluate; other amino acids were not present in significant amounts. Ala, Asp and Glu were well resolved, however Gly and Ser were not resolved from each other but were well resolved from Ala, Asp and Glu. Aspartate and asparagine were not resolved by the one dimensional method.

The Dow 50 amino acids were also quantified by TLC. A 2-4 μl aliquot of the eluate was streaked as before but in a 1-cm scribed lane. A typical 20 cm x 20 cm plate with every other lane blank, accommodated nine samples. The first, third, fifth, thirteenth, fifteenth, and seventeenth lanes were streaked with known standards of Ala, Asp and Glu. Each end had a matching set of standards estimated to cover the range of the unknowns from 1.25 to 20 nanomoles. In some instances preliminary analyses were required to match the appropriate set of standards with the samples. The plates were developed as before, air dried overnight then sprayed with 0.5% ninhydrin in 95% ethanol to completely saturate the plate with solvent. The ninhydrin positive substances (NPS) were developed at room temperature in the dark for a minimum of 8 hr. The plates were then scanned by dual beam reflectance spectrodensitometry with a Schoeffel SF 3000 spectrodensitometer (Figure 2). The illumination wave length was 585 nm and the reflectance wavelength 510 nm. A set of standard curves (Figure 3) were generated for each plate to compensate for variations between plates. (The reproducibility of this method was ± 4.0% (n = 42) for Ala, ± 4.6% (n = 42) for Asp, and ± 4.4% (n = 44) for Glu.)

The ninhydrin-developed amino acids for those samples with the greatest activity were stripped and counted as described previously. The purpose of this procedure was to ascertain to what extent the α-carboxyl group was labeled. The reaction of ninhydrin with α-amino acids results in the decarboxylation of the α-carboxyl group and the resultant loss of any activity in that

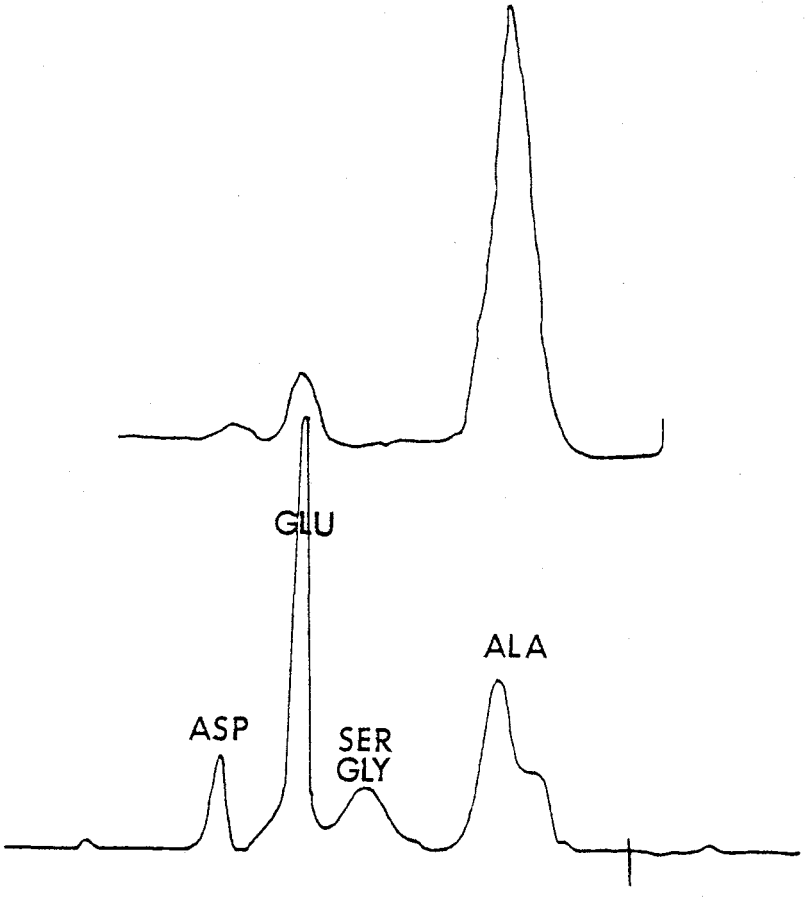

Figure 2. A typical spectrodensitometer TLC scan of a Dow 50 eluate with a radiochromatogram scan above.

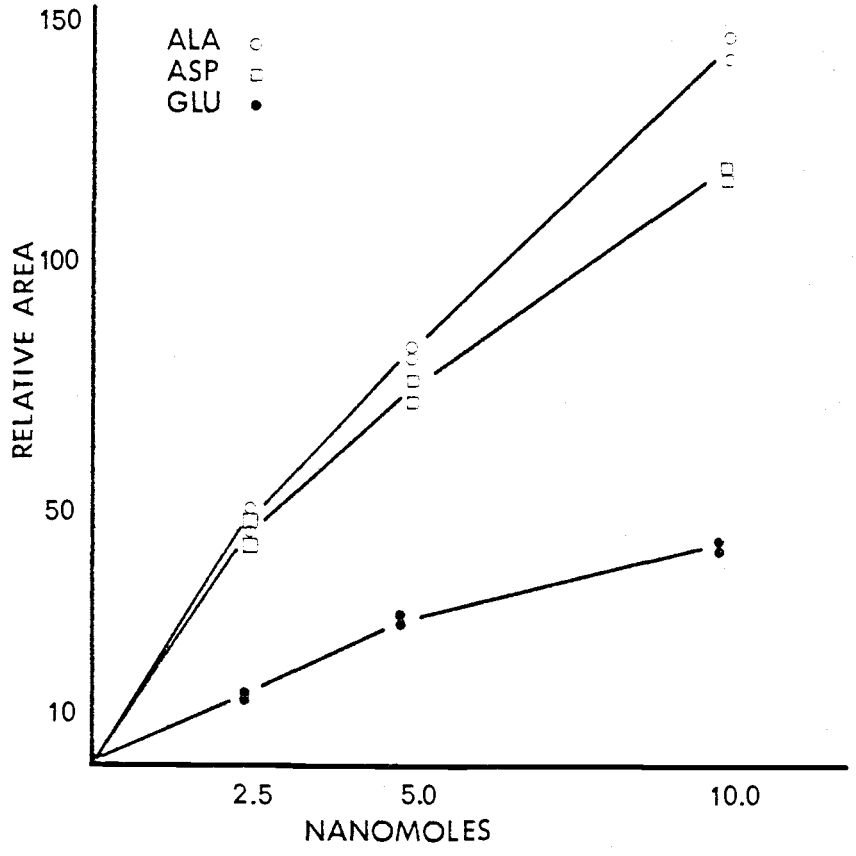

Figure 3. A typical set of standard curves for a spectrodensitometer TLC scan.

position, acquired from the [U-14C]-glucose precursor.

The freeze-dried Dow 50 wash was dissolved in ~ 1.0 ml of pH 6.0, 0.01 N Na formate buffer and then passed through a column of Dow 1 x 8, 100-200 mesh in the OH^- form, prepared as follows: a Pasteur pipet with a glass wool plug in the bottom was loaded with ~ 0.65 g of resin in the Cl^- form and the surface covered with a wad of glass wool; the resin was converted to the OH^- form by washing with 2 ml of 4 N ammonium hydroxide and then rinsed to neutral (pH < 9.0) with water. The sample was loaded on the column and the flask rinsed with four 0.5 ml water rinses, each of which was also passed through the column. All the original solvent and rinses were collected in a graduated tube, and subsequently referred to as "neutral compounds." The resin was eluted with 5 ml of 6 N formic acid and the eluate collected in a 25-ml Erlenmeyer flask and freeze-dried.

An aliquot of the neutral compounds was counted in PCS fluor and a second aliquot was analyzed for total reducing substances by the Dubois method (Dubois, 1956) with glucose as a standard. The remaining solution was evaporated in the vacuum desiccator as described previously, brought up in 25 µl of water and a 10-µl aliquot was spotted in a 2-cm lane on a 20 cm x 20 cm sheet of Whatman #1 paper (Clark, 1964), along with an authentic standard of glucose. The chromatogram was developed in isopropanol:acetic acid:water (3:1:1) then cut into 2.5-cm strips and scanned on a Packard model 7200 Radio-chromatogram Scanner; the radioactive peaks were compared to the standard. The paper strips were sprayed

with aniline oxalate reagent (0.9 g oxalic acid, dissolved in 200 ml water, then 1.8 ml aniline added) and developed at 100°C for 15 min. Reducing sugars appeared as greenish-brown spots. Also, strips were sprayed with 0.5% ninhydrin in 95% ethanol to identify NPS.

The freeze-dried Dow 1 eluate containing organic acids was brought up in ~ 1.0 ml of 50% acetone in water and the flask rinsed with four ~ 0.5 ml aliquots of 50% acetone. All washes were combined in a graduated tube and an aliquot counted in 5 ml 2:1 PCS. A 0.2 ml aliquot was diluted with 1.0 ml 50% acetone in a 12 x 75 mm culture tube containing a star-head stir bar. Three drops of phenol red indicator were added and the mixture titrated to a phenol red end point with 0.01 N NaOH utilizing a 100 µl Hamilton syringe and constant stirring with a magnetic stirrer. The NaOH solution was standardized against a 0.1 N K-biphthalate standard. The phenol red indicator was made by dissolving 0.1 g of phenol red in 28.2 ml of 0.1 N NaOH and made to a 500 ml volume with water. The Dow 1 eluate was then evaporated to dryness in the desiccator as described previously and brought up in a small volume of 50% acetone. The recovery of [1,4-14C] succinic acid and [2-14C] acetic acid spikes carried through from the homogenization step were 88.8% ($s = 4.6$, $n = 4$) and 1.3% ($s = 1.8$, $n = 4$) respectively. A 5 µl aliquot was counted and then 10 µl were spotted in the lower left corner of a mixed layer plate and the organic acids separated by two dimensional TLC. Authentic standards of pyruvate (sodium salt) citric, isocitrate (sodium salt), cis-aconitic,

α-ketoglutaric, malic, fumaric, succinic, and lactic acid were spotted over the unknown sample. The plates were then developed in the first dimension in 85% ethanol:1 N NH_4OH (4:1), run to within 0.5 cm of the top of the plate (4-6 hr) and air dried ~30 min. They were then developed in the second dimension in chloroform:tert amyl alcohol:100% formic acid:water (136:24:27:83), using the lower organic phase. The plates were developed to the top of the plate (~ 1 hr), air dried for ~ 30 min and then rerun in the second dimension in ethyl ether; formic acid; water (7:2:1) to within 1 cm of the top of the plate (~ 1 hr). The plate was air dried overnight and then sprayed with 0.04% bromcresol green with the pH adjusted with 0.1 N NaOH until a blue coloration just appeared. The organic acids appeared as yellow spots against a blue background (Figure 4). The plates were than coated with the strip mix formulation of Redgwell et al. (1974) and the spots cut out and counted in the same manner as the amino acids.

Acidic amino acids recovered from the Dow 1 eluate were separated, counted and quantified in the same manner as the Dow 50 amino acids.

B. Isolation and quantification of naphthalene and naphthalene metabolites in the tissues.

The oyster tissues exposed to [1-14C] naphthalene were analyzed for naphthalene and naphthalene metabolites. Oysters were placed on a bed of crushed ice and immediately dissected into three tissue components. The first was called "gill tissue" but in fact

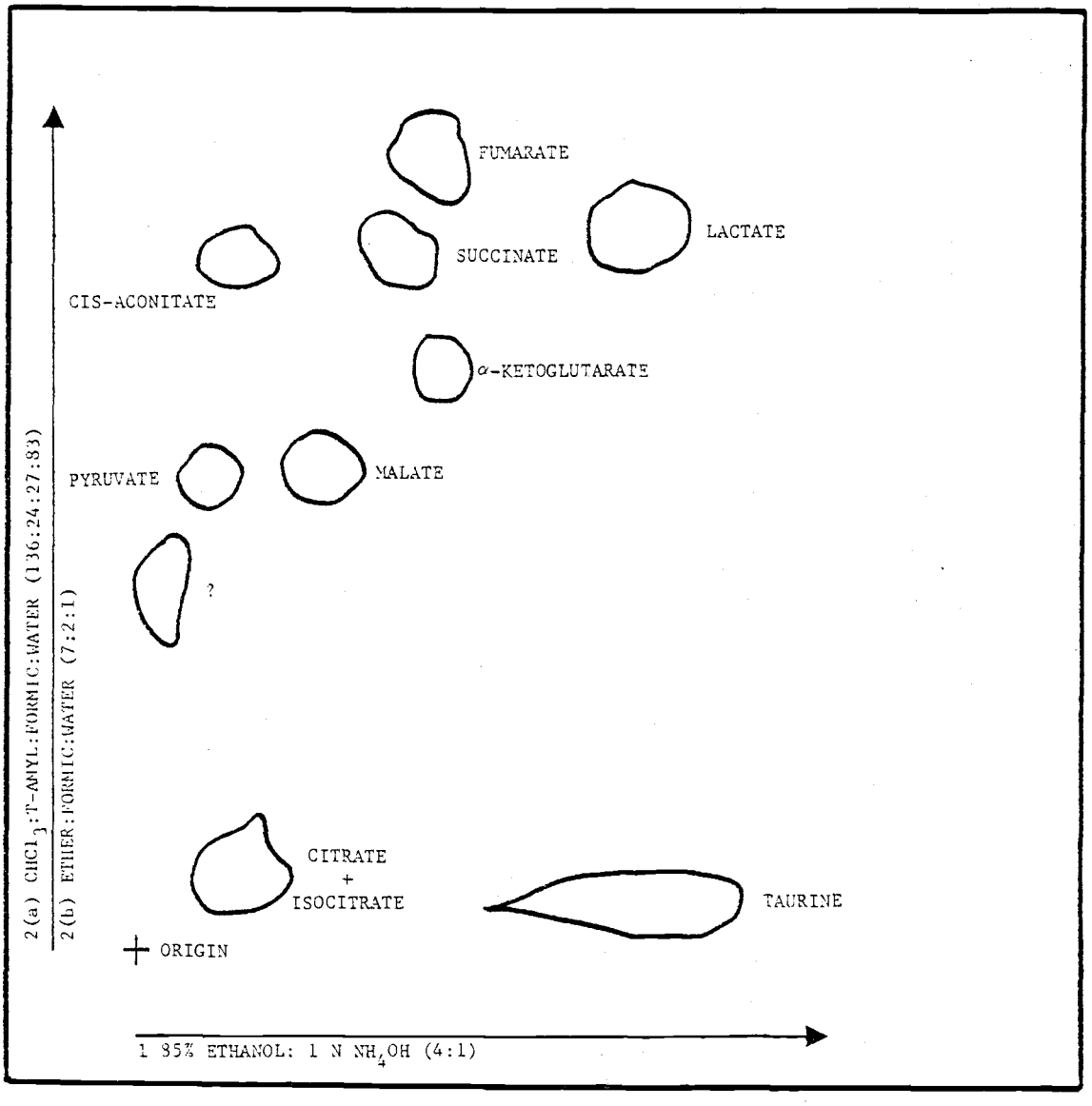

Figure 4. TLC of Krebs cycle intermediates, pyruvate and taurine. Acids are detected by acid-base indicator, and amino acids by ninhydrin reagent.

was comprised of the gills and overlying mantle; the second tissue component was called the "body" and included the digestive gland, gut, kidney, gonad and overlying mantle tissue; the final tissue component was the "adductor muscle" and included both the white and dark muscle fibers and the heart. Each tissue component was rinsed in three changes of sterile sea water, blotted dry, weighed, and then digested by the method of Roubals, Collier, and Malins (1977) in 2 ml of concentrated formic acid overlayed with 3 ml of hexane in a 18 x 150 mm screw-cap culture tube. The mixture was allowed to digest at room temperature for 72 hr and then a 0.2-ml aliquot of the formic acid phase and a 0.3-ml aliquot of the hexane phase were removed and combined in a 3-ml conical pyrex centrifuge tube to which ~ 0.4 ml of a saturated solution of NaOH in water was slowly added while the tube was held in an ice bath. The saponified digest was then extracted with three 0.3-ml aliquots of hexane using a vortex mixer; centrifuging between extractions aided in achieving phase separation. All the hexane extracts were combined in 10 ml of toluene fluor. The saponified layer was made to ~ 4 ml with water and then combined with 10 ml of PCS fluor. It was sometimes necessary to adjust the water volume in order to obtain a countable gel.

The remaining digests were pooled in order to reconstitute each oyster and the total digest of two separate oysters combined. The result was four pooled digests representing eight total oysters, two in each pool. Each pooled digest was roto-evaporated to ~ 0.50-0.75 ml, transferred to a glass-stoppered tube and extracted with

six 2-ml aliquots of hexane. Gentle hand-mixing of the extracts created firm gel-like emulsions which were broken by adding 10-20 drops of methanol followed by centrifuging at low g in a clinical centrifuge. The combined hexane extracts were evaporated under N_2 to dryness in order to volatilize the naphthalene, and then brought up in 50 µl of hexane. The recovery of 1-naphthol by this method, as determined by utilizing 1-[1-14C]naphthol was 46.3% (s = 1.5, n = 3). The concentrate was then streaked on a silica gel thin layer plate as a 2-cm band and developed in benzene. Authentic standards of 1-naphthol and 2-naphthol were run on each plate. The bands corresponding to the two unknowns were scraped and counted in toluene scintillation fluor. The areas above and below each known were also scraped and counted.

C. Isolation and quantification of naphthalene and naphthalene metabolites in seawater.

Periodic samples of the effluent from the flow system containing [1-14C] naphthalene were removed to determine the temporal changes in naphthalene concentration. A 2-ml sample of seawater was placed in a 16 x 100 mm screw-cap culture tube containing 1 ml of cyclohexane and 0.5 ml of 1 N sodium hydroxide. The seawater was extracted with three 1-ml aliquots of cyclohexane. All the cyclohexane extracts were pooled in a scintillation vial containing 10 ml of toluene scintillation fluor and counted. The recovery of naphthalene in the organic phase was 100.0% (s = 3.0, n = 5) and 1-naphthol was 2.7% (s = 0.8, n = 8). A 1-ml aliquot of

the lower saponified layer was counted in PCS fluor; recovery of naphthalene in this layer was 0.0% (s = 0.0, n = 5), 1-naphthol was 93.9% (s = 1.3, n = 8) and CO_2 was 101.0% (s = 5.0, n = 5). The quantities recovered were based on the recoveries of known amounts of 1-[1-14C] naphthol (Amersham 20.1 mCi/mmol), [1-14C] naphthalene and $14CO_2$ saturated seawater that was produced by first bubbling air through a culture tube containing an acid suspension of barium [14C] carbonate (Amersham, 50 mCi/mmol) and then passing the effluent air through a seawater trap.

In order to determine the quantity of non-CO_2 metabolites of [1-14C] naphthalene at the end of a 72-hr run, the water from the incubation vessel (~ 420 ml) was transferred to a 1-liter separatory funnel containing 10% (v/v) 6 N sulfuric acid and extracted three times with three 200-ml aliquots of hexane. The hexane extracts were pooled and roto-evaporated to a volume of ~ 10 ml, passed through a column of anhydrous sodium sulfate, evaporated to dryness in order to volatilize the naphthalene, and the residue brought up in 0.1 ml of hexane. The recovery of 1-naphthol by this method was 49.3% (s = 4.0, n = 3). The concentrate was streaked on a 250-μm-thick silica gel thin layer plate as a 2-cm band and developed in benzene. Authentic standards of 1-naphthol and 2-naphthol were run on each plate. The bands corresponding to the two knowns were scraped and counted in toluene scintillation fluor. The areas above and below each known were also scraped and counted.

D. Quantification of dissolved oxygen and ammonia-nitrogen in seawater.

The concentration of dissolved oxygen in seawater samples was determined by the Winkler method. A 20-ml sample of water was first removed, using a glass syringe, and 10 ml of this water was then transferred to a 10-ml plastic syringe. A 50-µl aliquot of manganese sulfate solution was first added through the syringe tip followed by the addition of 50 µl of alkaline potassium iodide; the solution was mixed by repeated inversion. Flocculent material was allowed to settle about a third of the way from the top and the mixing process was repeated two more times. The precipitate was dissolved by adding 75 µl of concentrated sulfuric acid. The sample was then transferred to a 20 ml vial and titrated with a standardized thiosulfate solution after adding 50 µl of a standard starch solution. Water samples to determine dissolved oxygen were taken at approximately 24, 48 and 72 hr during each run. The rate of O_2 uptake (QO_2) from the incubation vessel was estimated by the method described by Northby (1976). The measured quantities included the inlet and outlet dissolved oxygen (C_i, C_o), the flow rate f(t) and the flushing time defined as $T \cong V_s/f(t)$ where V_s is the volume of the incubation vessel. Assuming that mixing time was short relative to all other characteristic times then the concentration of dissolved oxygen in the vessel could be assumed to be uniform throughout the vessel and the QO_2 was calculated:

$$QO_2 = \left(f(t)\left((C_o(t)-C_i(t)) + T/2\Delta t\left[C_o(t-\Delta t)-C_o(t+\Delta t)\right]\right)\right)/g \text{ wet wt.}$$

Ammonia-nitrogen was determined by a method modified from Forman (1964). A 5-ml sample of water was transferred to a 10 ml polyethylene tube, cooled to 4°C and then, 0.5 ml of cold 1 N NaOH was added, the tube capped and the mixture centrifuged at high speed in a clinical centrifuge at 4°C for 10 min. After removal from the centrifuge the tube was decanted immediately into a plastic vial containing 0.33 ml of 0.6 N acetic acid. The NaOH treatment precipitated divalent cations and the immediate acidification (pH 5.0-6.0) prevented significant losses of ammonia. The sample was then passed through a column of pre-washed Dow 50 x 8 (> 400 mesh) resin packed in a 0.9 cm x 2.5 cm column which held ~ 1.5 ml of packed resin (MER Liquid Chromatography, Mountain View, CA, item TF-10) (Figure 5). The column was fitted with Teflon filtering discs which were covered with Whatman #1 filter paper to reduce the frequency of replacement. The ends of the column were fitted with 0.0625-inch miniature tube fittings (M.E.R. #301) and the column was connected to a Luer-Lok syringe fitting with 0.0625-inch OD heavy wall Teflon tubing. Liquids were forced through the column with plastic syringes and the effluent controlled with a small PVC valve. The resin column was prepared as follows: the resin was converted to the Na^+ form with 10 ml of 1 N NaOH, washed to pH < 9.0 with doubly distilled water (DDH_2O), converted to the H^+ form with 10 ml of 1 N HCl and then washed with 5 ml DDH_2O. After each use the resin was regenerated to the H^+ form with 5 ml of 1 N HCl

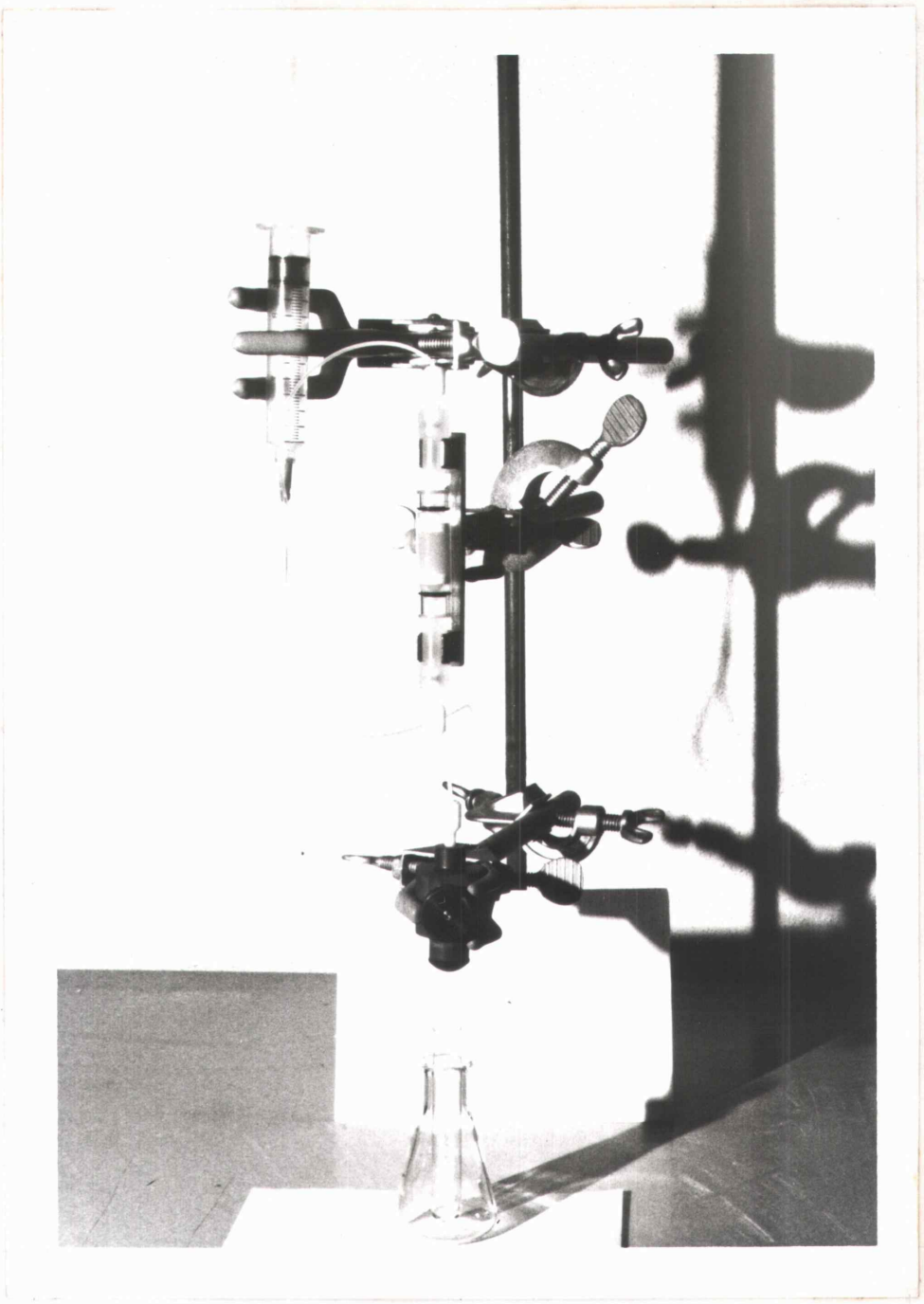

Figure 5. The packed Dow 50 column used for ammonia-nitrogen determination.

followed by 5 ml of DDH_2O. The column was then used until either results utilizing standard solutions became erratic or the filters became plugged.

The sample prepared as previously described was passed through the column at a rate of ~ 10 ml/min. The column was washed with 2 ml of DDH_2O and then eluted with 3 ml of Na-phenoxide solution (12.48 g NaOH, 25 g phenol, made to 1 liter in DDH_2O) discarding the first ml (pH < 9.0), saving the second (pH > 9.0) and discarding the third. To the saved second ml was added 0.2 ml of 0.01% Na-ferricyanide followed by 0.5 ml Of 0.04 N hypochlorite solution prepared as described by Forman (1964). The mixture was held in the dark at 37°C for ~ 30 min and then the absorbance read at 630 nm in a 1-cm cuvet. A standard curve (Figure 6) was constructed utilizing ammonium sulfate as a standard, an appropriate volume (< 100 µl) of ammonium sulfate stock solution in DDH_2O was made to 1.0 ml with Na-phenoxide reagent and then followed by the other reagents as usual. This approach was used in lieu of passing standard seawater solutions through the column since it saved considerable time. Absorbance values obtained by the latter approach were 10% greater ($\bar{x}$ = 10.2, s = 1.6, n = 5) than seawater standards run through the total procedure. The difference represented NH_3 losses due to the alkaline treatment, losses in precipitate, and ammonia-nitrogen losses on the column and during elution.

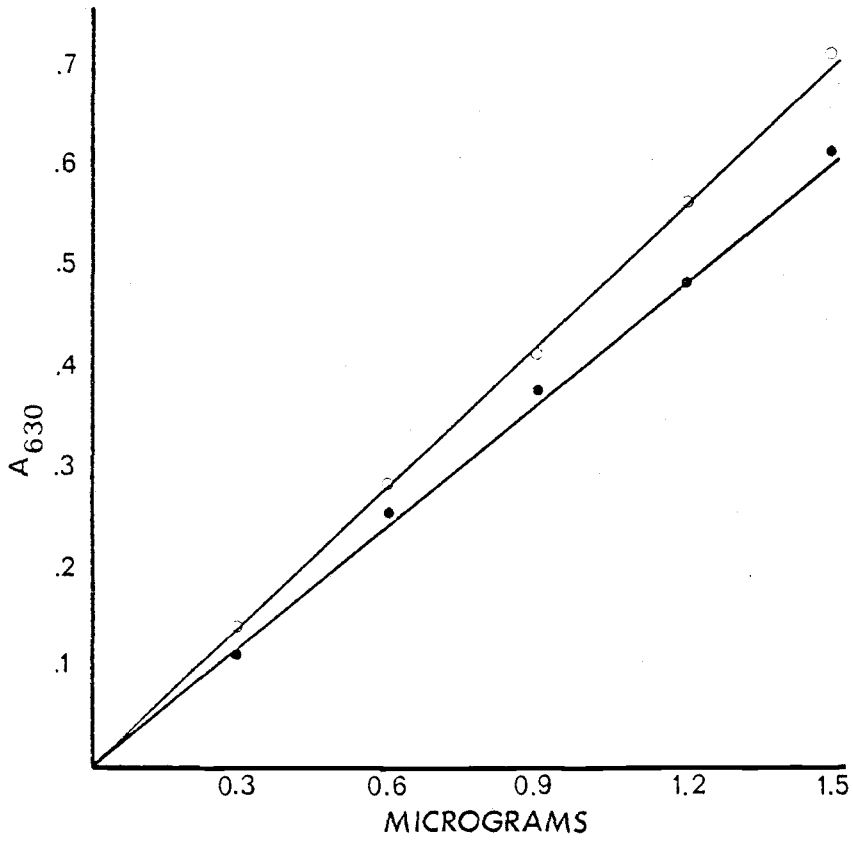

Figure 6. A typical standard curve for ammonia-nitrogen determination (○), and a standard curve generated by passing 5 ml of a standard seawater solution through the Dow 50 column (●). The quantities are total micrograms of ammonia-nitrogen added either to 1 ml of Na-phenoxide or to the standard seawater solution prior to passage through the column and the absorbances obtained at 630 nM.

The Flow System

A flow-through system was used to administer the naphthalene in solution because it most closely approximated the manner in which chemical pollutants are encountered by sedentary animals in the marine environment. The system (Figure 7) modeled the entry of a pollutant from a point source, with the time zero concentration of the pollutant being zero.

In order to prevent evaporative loss, the system was designed so that it was closed to the atmosphere whenever naphthalene was in solution. For this reason the system was composed, functionally, of two halves; one half was opened to the atmosphere and was designed for the handling of aerated seawater and the other half was designed for the handling of seawater which contained naphthalene in solution. The flow rate from each half was maintained by separate pumps. Mixing of seawater from the two pumps occurred at a tee, and the concentration of naphthalene in solution after mixing was a function of the two flow rates. All materials used in construction of the naphthalene half were inert, consisting of Teflon and glass with a few parts constructed of 316 stainless steel.

Aerated water was pumped from a common reservoir by a Buchler Polystatic pump. the water with naphthalene was pumped from a graduated 250 ml metering buret by a Buchler Mini pump constructed entirely of Teflon and glass. The liquid surface in the metering buret was covered with a glass float to prevent evaporative loss

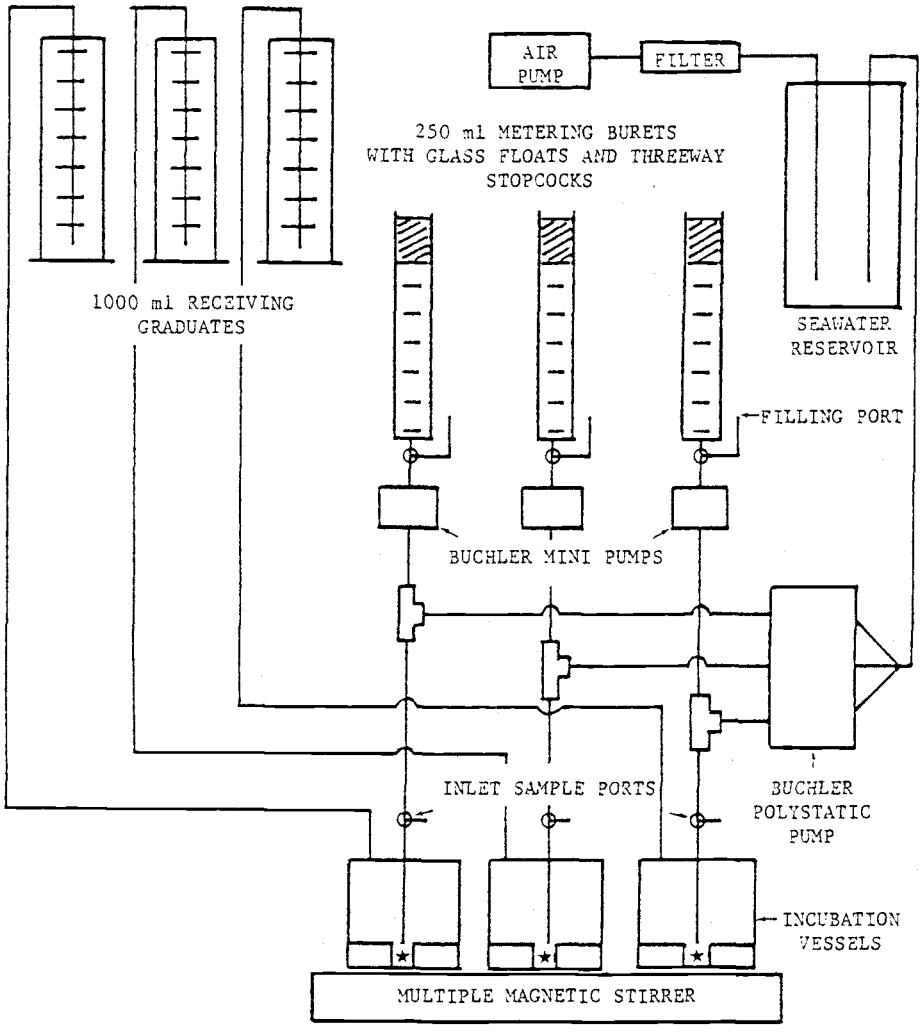

Figure 7. The flow-through system for administering naphthalene in solution. System is constructed of Teflon, glass, and 316 stainless steel.

(Figure 8). A 3-way stopcock permitted refilling the metering buret without exposing the solution to the atmosphere. Immediately after the Teflon tee was a sample port consisting of another tee with one branch made up of a Luer-Lock adapter and a 25-ml glass syringe which was connected only when removing water samples from the inlet side of the incubation vessel. The incubation vessel was a 500-ml wide-mouthed screw-cap vessel with a ground glass rim and a 0.0625-inch Teflon gasket. A length of 0.125-inch heavy wall Teflon tubing, held in place by two Teflon fasteners, passed through on either side of the gasket and into the incubation vessel. The outlet tubing of each vessel terminated with another Luer-Lok adapter which served as an effluent sampling port. The effluent was received in a 1000-ml graduated cylinder which was layered with 100 ml of cyclohexane. Incubation vessels were positioned on a magentic stirrer and a continuous mixing of the water in the incubation vessels was maintained by a recessed Teflon star head stir bar. The threads of the incubation vessel were wrapped with Teflon tape to prevent leakage.

The entire system was constructed in triplicate; there were three incubation vessels, metering burets, metering pumps and receiving graduates. However, all three incubation vessels received aerated seawater from the same reservoir. Incubation vessel #1 contained the control oysters which were never in contact with naphthlene. The metering buret for this vessel was spiked with an appropriate aliquot of carrier (10-20 µl) 95% glass distilled ethanol. Incubation vessel #2 contained one group of

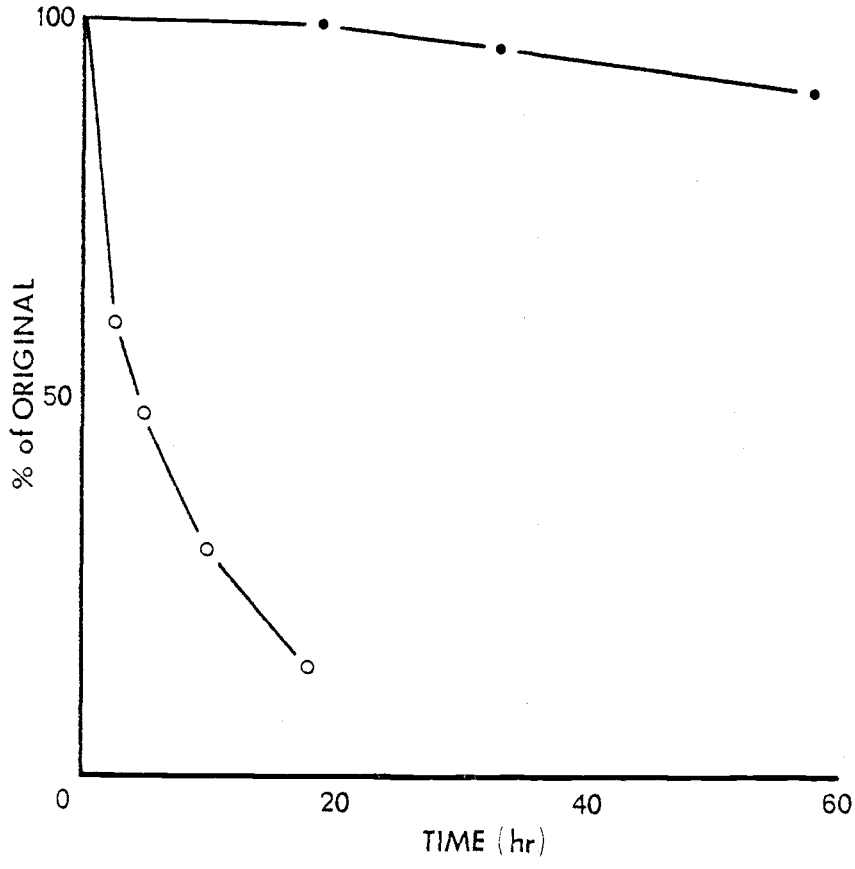

Figure 8. Naphthalene evaporative loss from a metering buret (●) and 250 ml beakers open to the atmosphere (○). Values from the buret represent only one measurement at each sample time; values from the open beakers represent estimated values from 4 independent beakers, $r^2 = .99$.

naphthalene exposed oysters. The metering buret for vessel #2 held seawater with dissolved reagent grade naphthalene carried in 95% glass distilled ethanol (10-20 µl). Similarly, vessel #3 contained naphthalene exposed oysters; however, radioactive [1-14C] naphthalene (Amersham, 36 mCi/mmol) was dissolved in the metering buret. The concentration of naphthalene was identical in #2 and #3. The flow rate through the system was ~ 0.40 ml/min with a flushing time of ~ 17.5 hr. The concentration of naphthalene entering vessels #2 and #3 after mixing was ~ 80-100 ppb. This value was chosen since it represented a conservative concentration expected for the water soluble fraction of various oils (Anderson, et al., 1974).

The seawater used in the system was made up using synthetic salts (Instant Ocean) to a salinity of 28.5 °/oo. The seawater was then held a minimum of 2 weeks in a 15°C holding aquarium, and continuously filtered through a biological filter to reduce the concentration of dissolved organics. Prior to each run, the water was filtered, first through a 0.80 µm filter and then a 0.20 µm filter.

The entire system as described was maintained in a 15°C constant temperature room. Between each run, all components and tubing were washed in scalding hot water, thoroughly rinsed with distilled water, wrapped in aluminum foil and heat-dried at ~ 100°C for 48 hr. All parts were handled in a manner to minimize the possibility of microbial contamination.

The flow rate through the system (f(t)) was calculated by the difference in the volume of the receiving graduated cylinder at time t (V(t)) minus the volume of the receiving graduated cylinder at some earlier time (V(0)), divided by the time difference Δt:

$$f(t) = V(t) - V(0)/\Delta t.$$

The amount of naphthalene that entered the vessel per unit time (q(t)) was similarly calculated by utilizing the volume difference in the naphthalene metering buret, and the known naphthalene concentration in the metering buret (C):

$$q(t) = (Vb(t) - Vb(0)/\Delta t)(C).$$

Assuming there was no loss, the ultimate steady state concentration of naphthalene (Qs) was calculated by determining the proportion of unflushed water remaining in the incubation vessel after one interval of duration t (x(t)), where x(t) is equal to one minus f(t) divided by the total volume of the incubation vessel (V_v):

$$x(t) = 1 - f(t)/V_v;$$

and

$$Q_s = (q(t))(x(t)/1 - x(t))/V_v.$$

a very close approximation of Qs can be made by dividing q(t) by f(t).

$$Q_s \cong q(t)/f(t)$$

The naphthalene concentration after any time t (Q(t)) was calculated utilizing the following model:

$$Q(t) = (1 - x\ (t)^{t})(Qs).$$

The model as described assumed no losses. However some loss did occur because of microbial uptake and metabolic transformation, evaporation, and adsorption. In order to determine the extent of these losses blank runs were made utilizing seawater treated as described previously, and unfiltered seawater.

Pre-conditioning of Oysters

Oysters (O. edulis), originally obtained as cultchless spat from International Shellfish Enterprises Inc., Moss Landing, CA, were purchased from Oregon Oyster Co., Yaquina Bay, OR on March 22, 1977 when they were approximately 10 months old. At that time they were transferred to a closed holding aquarium (synthetic seawater) where they were maintained at 28.5 °/oo salinity/15°C with no apparent source of nutrition. These oysters were ultimately used in experiments conducted during May 11, 1977 to July 5, 1977. Thus, they were starved between 50 and 105 days. Prior to their placement in the holding aquaria, each oyster was scrubbed with a stiff brush and all loose shell material and projections where sediments could lodge, were removed. Ten days prior to each run, the shell material of the left valve overlying the gills and cloaca was carefully removed with a very fine (32 teeth/inch) bone saw so as not to disturb the underlying tissues (Figure 9).

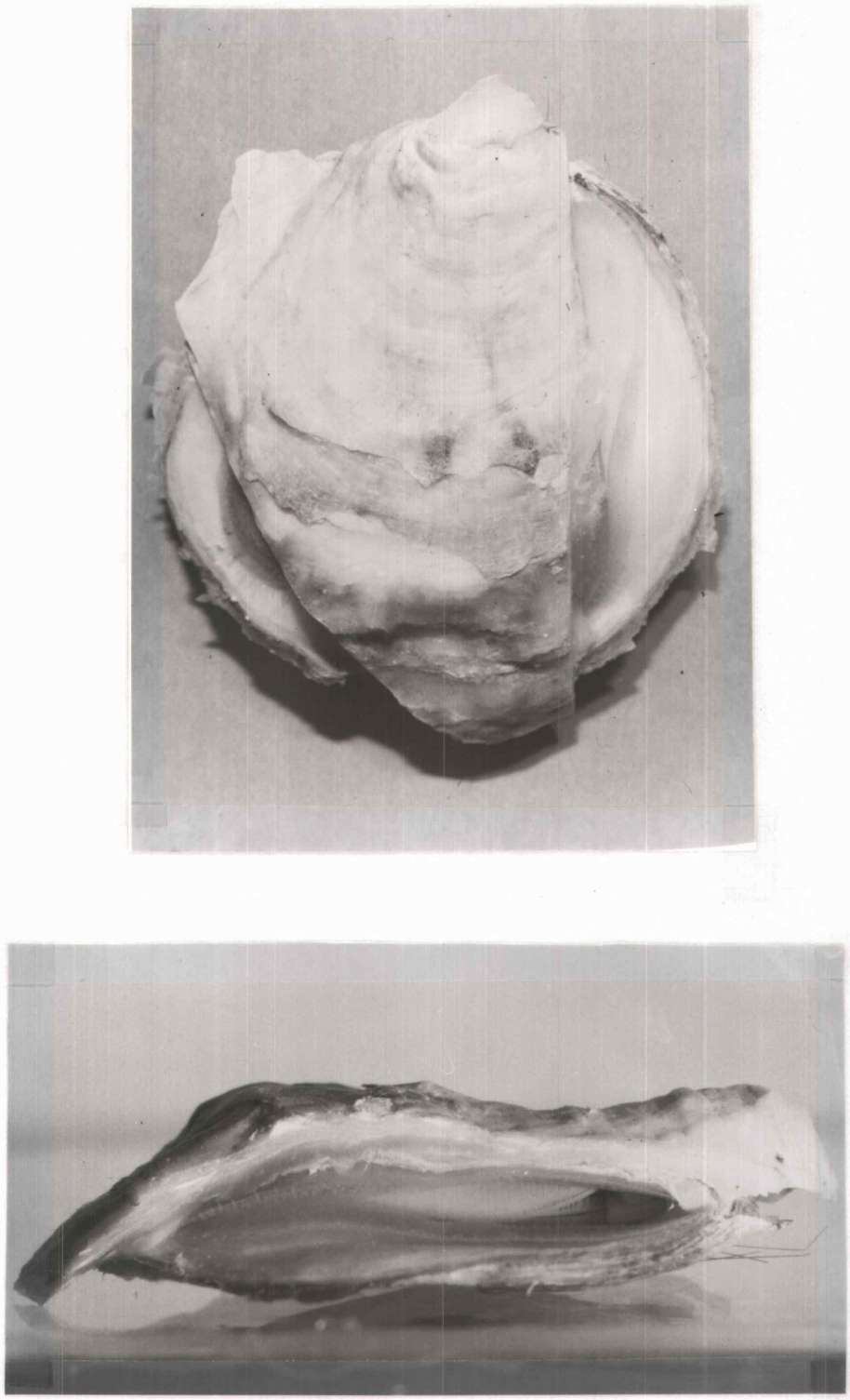

Figure 9. An oyster with the shell material from the left valve removed to allow the free flow of seawater through the gills.

After removal of the shell, oysters were returned to the holding aquaria until 24 hr prior to the run. It was noted that during the preceding 9-day period a delicate membranous shell material was secreted and usually extended a considerable distance from the cut portion of the valve. The secretion of this new shell was used as an indicator to confirm that the oysters were metabolically healthy. Oysters that did not show significant new shell growth after 9 days were not used. The reason for removing the shell from over the gill tissue was to ensure that frequent handling would not affect the uptake of naphthalene, glucose, and oxygen. Since the intent was to study aerobic glucose metabolism it was important to maintain the gill tissues in an aerobic environment.

At the end of 9 days, after removing the shell material, oysters were removed from the holding aquaria and placed in a 500-ml conditioning vessel containing glucose enriched (1 mM) sterile seawater containing 100 ppm streptomycin sulfate. During the 24-hr period prior to each run, the water was changed after 4 hr and again after 12 hr. Glucose was added to "prime" the glucose metabolism of the oyster prior to the metabolism experiments while streptomycin was added to control the bacterial flora from the oyster's shell and tissues. The filtered seawater was also glucose-enriched and contained 100 ppm streptomycin; the reservoir and burets of the experimental flow system were filled with this water prior to each run. After the 24-hr conditioning period, eight oysters were placed in each incubation vessel, the system was sealed, and all pumps were started with the initial

naphthalene concentration in the incubation vessel being zero at time zero. Each run lasted approximately 72 hr.

Experimental Protocol

A. Microbial metabolism

It was suspected that microbial metabolism would significantly alter the naphthalene concentration during normal conditions in the flow system unless precautions were taken to reduce the build-up of the microbial population. In order to assess the extent to which microbial contamination affected the naphthalene concentration, several blank runs were made with and without sterile seawater at two different concentrations of naphthalene ($\sim$ 4.0 ppb and $\sim$ 40 ppb).

To determine the nature of the naphthalene metabolites and the effects of glucose on the microbial metabolism of naphthalene, seawater from the holding aquaria was filtered first through a 0.80 µm filter, and subsequently through a 0.20 µm filter. The water was added to an incubation vessel with 6 oysters and stirred continuously for 24 hr. At the end of this period a portion of the water was removed, refiltered as before and then glucose was added to make a final glucose concentration of 1 mM. Half of the remaining water was also made to 1 mM with glucose, but was not refiltered. Glucose was not added to the remaining un-refiltered water. All three treatments were then spiked with ammonium sulfate to a concentration of 1.5 ppm ammonia-nitrogen, and [1-14C] naphthalene to a final concentration of 4.68 ppb. Each treatment

solution was drawn into a 50 ml all-glass syringe and two 2 ml samples were removed at 8, 16, 21, 28, 41, and 48 hr. One 2-ml sample was extracted as described previously after the addition of 0.5 ml of 1 N NaOH while the other was extracted following the addition of 0.2 ml of 6 N sulfuric acid. The acidified seawater was allowed to stand 10 min with occasional mixing and after 10 min the seawater was extracted with cyclohexane. All the cyclohexane extracts were then pooled and counted and 1.0 ml of the aqueous phase was also counted for each treatment. The recovery of naphthalene by the acid extraction method was 100.0% (s = 2.0, n = 5). The recovery of $^{14}CO_2$ in the aqueous phase by the acid method was 0.0% (s = 0.0, n = 5) and in the organic phase 5.8% (s = 0.4, n = 5). Recovery of $^{14}CO_2$ in the saponified phase of the base extraction method was 101.0% (s = 5.0, n = 5), and in the organic phase 0.0% (s = 0.0, n = 5).

The effect of streptomycin on the metabolism of naphthalene was determined in static assays. Unfiltered synthetic seawater (28.5 °/oo) taken from the holding aquaria, was made to concentrations of 0, 25, 50, 100, and 200 ppm streptomycin and 1.5 ppm ammonia-nitrogen with ammonium sulfate. Incubation vessels were filled with this water and broken pieces of oyster shells were added to ensure microbial activity. Each vessel was spiked with [1-14C] naphthalene to a concentration of 120 ppb. After 120 hr of continuous stirring, the vessels were opened and the total saponifiable metabolites in the water were determined by the methods described previously.

In order to quantify the microbial population of an incubation vessel during a normal run with oysters present, water samples from the inlet and outlet sample ports were streaked with a calibrated loop on seawater agar plates. The seawater agar plates were prepared using standard microbiological practices and formulated as follows: 1 g bacto-peptone, 0.5 g bacto-yeast extract, 0.5 g dibasic potassium phosphate, 15.0 g agar, made to 1 liter in filtered synthetic seawater from the holding aquaria. The streaked plates were incubated at 15°C in the dark for 1 week and then the colonies were counted.

To measure the effects of the microbial activity on the oxidation of naphthalene, glucose loss, QO_2, and excretion of ammonia-nitrogen, a run was made with only shells present, with and without streptomycin in the water. The shells were treated as previously described for oysters, except that the tissues were removed. Vessels #2 and #3 contained shells with and without streptomycin treatment respectively and therefore, separate aerated seawater reservoirs were required and both metering burets contained [1-14C] naphthalene. Vessel #1 was used as a control without naphthalene but was similar to #2 in all other respects. The extent of naphthalene oxidation was determined as described in the previous section. The extent of glucose oxidation was determined by monitoring changes in the glucose concentration in the seawater effluent by the method of Dubois (1956). One ml of 5% phenol in water was added to a 1 ml aliquot of appropriately diluted seawater followed by 5 ml of concentrated sulfuric acid. An appropriate

standard curve was constructed utilizing glucose. The uptake of dissolved oxygen and excretion of ammonia-nitrogen were also determined as described previously.

B. Uptake and metabolism of naphthalene by oyster tissue

A total of four runs were made with oyster tissue present as a sink for [1-14C] naphthalene. The first run (R-0) was intended to evaluate the function of the flow system with oysters present and to gather preliminary data concerning the accumulation of naphthalene by the gill tissue after being exposed for 72 hr to [1-14C] naphthalene. The second and third runs (R-1 and R-2) were designed to determine the concentration of [1-14C] naphthalene in each of the three tissue components after 72-hr exposure to [1-14C] naphthalene. The fourth run (R-3) was similar to the second and third except that 12 oysters rather than 8 were incubated and groups of 3 oysters were removed at 15, 27, 49, and 72 hr.

The extent of non-CO_2 metabolites in the three tissue components were determined as outlined in the previous section describing the isolation and quantification of naphthalene and naphthalene metabolites except that in run R-3, three oysters were pooled for the TLC of non-CO_2 metabolites as opposed to two oysters in runs R-0, R-1, and R-2.

Temporal changes in the [1-14C] naphthalene and in saponifiable metabolites in the seawater effluent from vessel #3 were monitored during each run as described in the section on the isolation and quantification of naphthalene and naphthalene

metabolites in seawater. Also, non-CO_2 metabolites were separated by TLC as described, except in the case of run R-0 when the metabolites were not separated by TLC.

C. Effects of naphthalene on oxygen consumption and ammonia-nitrogen excretion

The rate of oxygen consumption and ammonia-nitrogen excretion for control and naphthalene-treated oysters were determined as described in the sections on quantification of dissolved oxygen and ammonia-nitrogen in seawater, for runs R-0, R-1, R-2, and R-3.

D. Effects of naphthalene on the uptake of D-[U-14C] glucose and $^{14}CO_2$ production

At the end of a 72-hr run the naphthalene-treated (Nt) oysters from vessel #2 were removed and each was placed into a separate incubation vessel (Figure 10) containing 25 ml of filtered synthetic seawater which had been spiked with approximately 4 µCi of D-[U-14C] glucose (274 mCi/mmol, Amersham); the solution did not contain streptomycin or 1 mM unlabeled glucose. Each vessel was sealed with a Teflon gasket and aerated with the effluent air bubbled through a trap containing 3 ml of 10% NaOH. Mixing was effected by a multiple magnetic mixing apparatus. A 50 µl water sample from vessels 1, 3, 5, and 7 was counted in 2:1 PCS:xylene at t = 0 prior to the addition of the oysters. The water was again sampled from the appropriate vessel when an oyster was removed, usually at intervals of approximately 30 min during a 240-min period. When an oyster was removed it was quick-frozen on a bed of

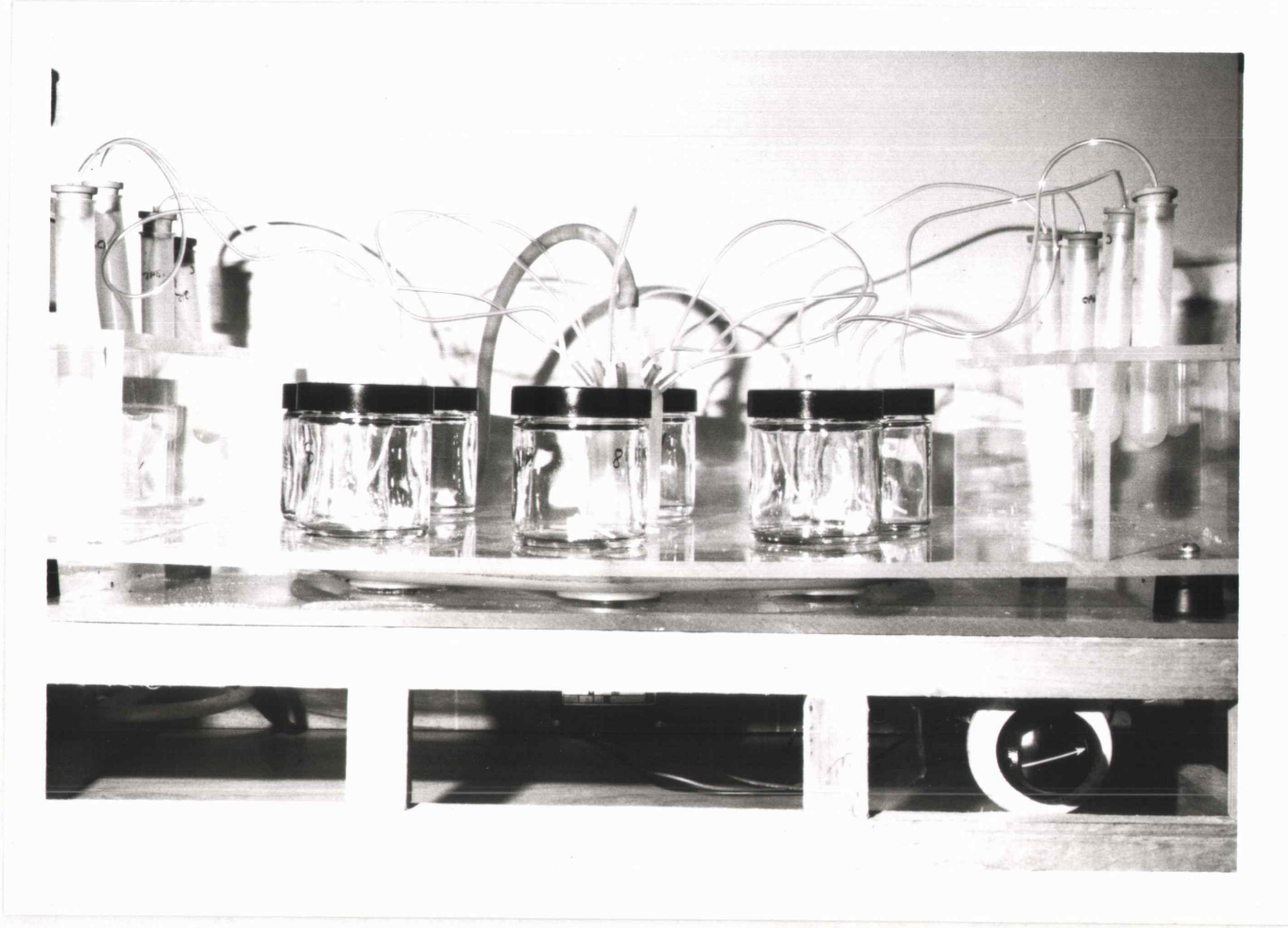

Figure 10. The glucose incubation vessels, associated CO_2 traps, and multiple mixing apparatus.

dry ice. The gill tissue and mantle edge was then removed, weighed, freeze-dried, and stored under N_2 at -20°C in a desiccator over anhydrous sodium sulfate. After the last oyster was removed at 240 min, 2.5 ml of 6N sulfuric acid was added to each vessel, the vessels resealed and air bubbled through for an additional hr. At the end of this period aeration was stopped and a 50-µl aliquot from each vessel and CO_2 trap was counted in 2:1 PCS:xylene. The control oysters (Ct) were treated similarly, immediately after counting the CO_2 traps. A total of four runs were made when oyster tissue was present to serve as a sink for naphthalene. At the end of the first run (R-0), the three (Ct) oyster tissue components were digested at 50°C for 48 hr in NCS tissue solubilizer (Amersham) and then counted in toluene fluor. No Nt oysters were used in the glucose uptake experiment during this run. The other three runs (R-1, 2, and 3) were designed to compare the incorporation of glucose into metabolic end products and intermediates in the gill tissue of Ct and Nt oysters.

E. Effects of naphthalene on the carbon flow into the intermediates and end products of glucose metabolism

The pool sizes of ethanol insoluble polysaccharides (primarily glycogen), total protein, total polar lipids, total neutral lipids, free neutral reducing sugars, free alanine, aspartate and glutamate, taurine, and total organic acids were determined for Ct and Nt oysters.

Specific radioactivity-time curves for ethanol insoluble polysaccharides (primarily glycogen), total protein, total polar lipids, total neutral lipids, neutral compounds (primarily glucose), free alanine, aspartate and glutamate, taurine, and total organic acids were determined for Ct and Nt oysters. Radioactivity-time curves for malate and succinate were also determined.

Statistical Methods

Interval estimates and testing of differences between means ("t" test) of the pool sizes of the end products and intermediates for Ct and Nt oysters were accomplished as described in Petersen (1973). Curve fitting was accomplished by linear regression analysis with a search for the best fit of the data using linear ($y = b_1x + b_o$), exponential ($y = ae^{bx}$, $a > 0$), logarithmic ($y = a + b \ln x$ or $y = a + b \log_{10} x$), and allometric ($y = ax^b$) models. The appropriate transformations were made to linearize the models. The best fit was judged by visual inspection and the magnitude of the coefficient of determination (r^2). The comparison of regression lines was accomplished as described in Neter and Wasserman (1974). Regression lines were compared only if error terms for the regressions were equal. The F^* statistic was used to test for differences. The regression parameters were also compared but in this case an indicator variable and design matrix were used and this allowed for the selective testing of the significance of each parameter using the F^* statistic. The details of this analysis will be covered in the results section.

The following abbreviations are used throughout the text for convenience:

s = standard deviation

C.I. = confidence interval

C.V. = coefficient of variation

r^2 = coefficient of determination

α = significance level

P = probability

III. RESULTS

A total of five runs were made with oysters or shell material present in the experimental system. This number does not include blank control runs or runs required for testing certain specific features of the flow system.

Four of the runs were conducted with oyster tissues present. The mean wet tissue weight of the oysters was 291.5 ± 21.5 mg (± 95% C.I., n = 91). Gill tissue accounted for 136.3 ± 9.35 mg (± 95% C.I., n = 91), about 46.8% of the total wet wt. Since each run was done sequentially, the total length of starvation increased between each run. It has been shown previously that as the length of starvation increases and polymeric reserves are depleted, the percent water content of the tissues increases and the percent dry wt decreases (Riley, 1976). Of all the tissues, the gills are the least affected by starvation in the Pacific oyster, Crassostrea gigas (Riley, 1976). In this study the percent dry wt of the gill tissue decreased consistently between runs but was only statistically significant ($P < .05$ between runs 1 and 3 (Table 1).

The flow system was maintained in a 15°C constant temperature room. Unfortunately, after the experiments began substantial fluctuations in the temperature were discovered and a backup system was then utilized. The accumulation of naphthalene determined during runs R-0, R-1, R-2 and R-3, were conducted at 14.0 ± 2.0°C. The glucose uptake experiments were carefully controlled, with the temperature stabilized at 15.0 ± 0.2°C at least 5 hr prior to each

Table 1. Decrease in the percent dry wt of the gill tissue between runs and the length of starvation.

	R-1	R-2	R-3
Days of starvation	67	85	105
% dry wt.	17.0	16.3	15.6
95% C.I.	± 0.9	± 0.8	± 0.9
n	16	16	16

run. During run R-1, the temperature control mechanism failed when measuring the uptake of glucose by the Ct oysters; the temperature fell to 12.8 ± 0.6°C. The uptake of glucose by Nt oysters for run R-1 was conducted at 15.0 ± 0.2°C. All static assays of microbial activity were carried out at 15.0 ± 0.1°C.

Microbial Metabolism

Evaporative losses of naphthalene were greatly reduced by the design of the flow system (Figure 8). Construction of the system with inert materials precluded any adsorptive losses. Nevertheless, considerable deviations from the expected concentrations, based on calculations from the quantitative model, indicated that significant losses had occurred in the flow system even when there was no apparent sink for the naphthalene. The fact that most of the loss could be accounted for by an acid-volatile, saponifiable metabolite implied that the observed deviations were due to microbial oxidation. The complete oxidation of simple aromatic hydrocarbons to carbon dioxide and water by certain strains of bacteria has been well established (Gibson, 1976).

When seawater which had been filtered previously through a 0.20 μm filter was used in the flow system with no tissues or shells present, the calculated rate of naphthalene accumulation was closely approximated by the experimentally observed values (Figure 11a). The use of unfiltered seawater resulted in considerable deviations from the calculated values (Figure 11b). These deviations were especially noticeable at the lower naphthalene concentration

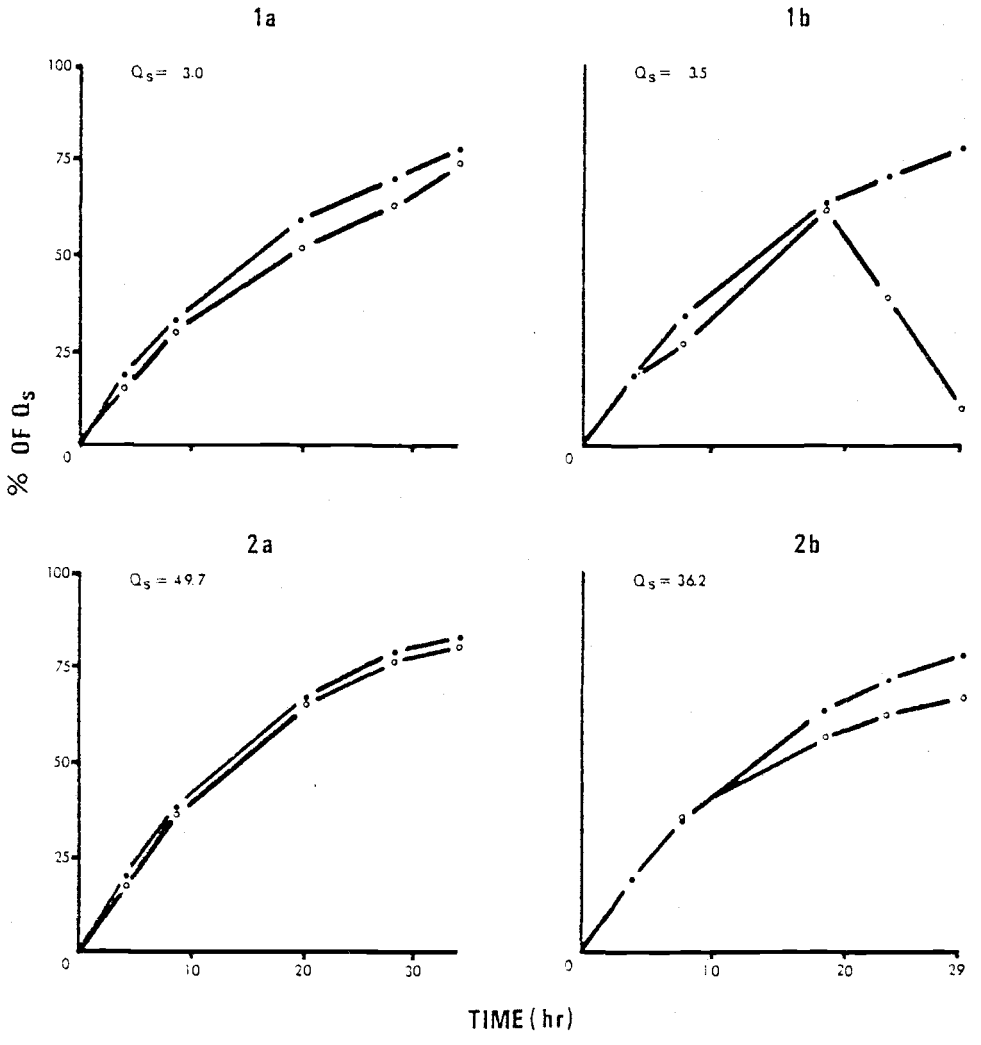

Figure 11. The effects of seawater filtration on the accumulation of naphthalene in the flow system comparing the expected values based on the quantitative (•) model with the experimentally derived values (∘) for treatments; (1a and 2a) utilizing filtered seawater, and (1b and 2b) unfiltered seawater. Q_s is given in ppb.

(3.5 ppb). At the higher concentration, the losses were less evident (36.2 ppb). In both cases, including that where filtered seawater was used, the model overestimated the actual naphthalene concentration.

In static assays, unfiltered water which had been in contact with oysters and was subsequently spiked with [1-14C] naphthalene, lost considerable naphthalene over a 34-hr incubation period (Figure 12a). Filtered or autoclaved water showed no losses with 99.6% of the counts being recovered in the cyclohexane phase ($s = 4.0$, $n = 36$). In unfiltered seawater, there was a lag period prior to the onset of the maximum rate of naphthalene oxidation. The total recovery of counts, calculated as the sum of the counts in the organic phase plus the counts in the aqueous phase, decreased with time; the greater the extent of oxidation, the lower the total recovery. Counting the water directly in PCS at the end of 34 hr resulted in the recovery of significantly more counts, 83.8% ($s = 4.2$, $n = 5$) vs 71.4% ($s = 4.7$, $n = 5$); nevertheless, all the original activity could not be accounted for. The differences were not due to adsorption on the glass surfaces of the syringes ($\bar{x} = 0.010$, $s = 0.004$, $n = 4$). Since the total activity recovered was directly correlated with the extent of oxidation, it appeared that the loss was related to the volatilization of metabolites during or after the extraction process. Recently, it has been demonstrated that counting $^{14}CO_2$, solubilized in NaOH, in xylene base scintillation fluors resulted in losses of activity up to 36% less than the initial spike (Iverson, Bittaker, and Myers, 1976).

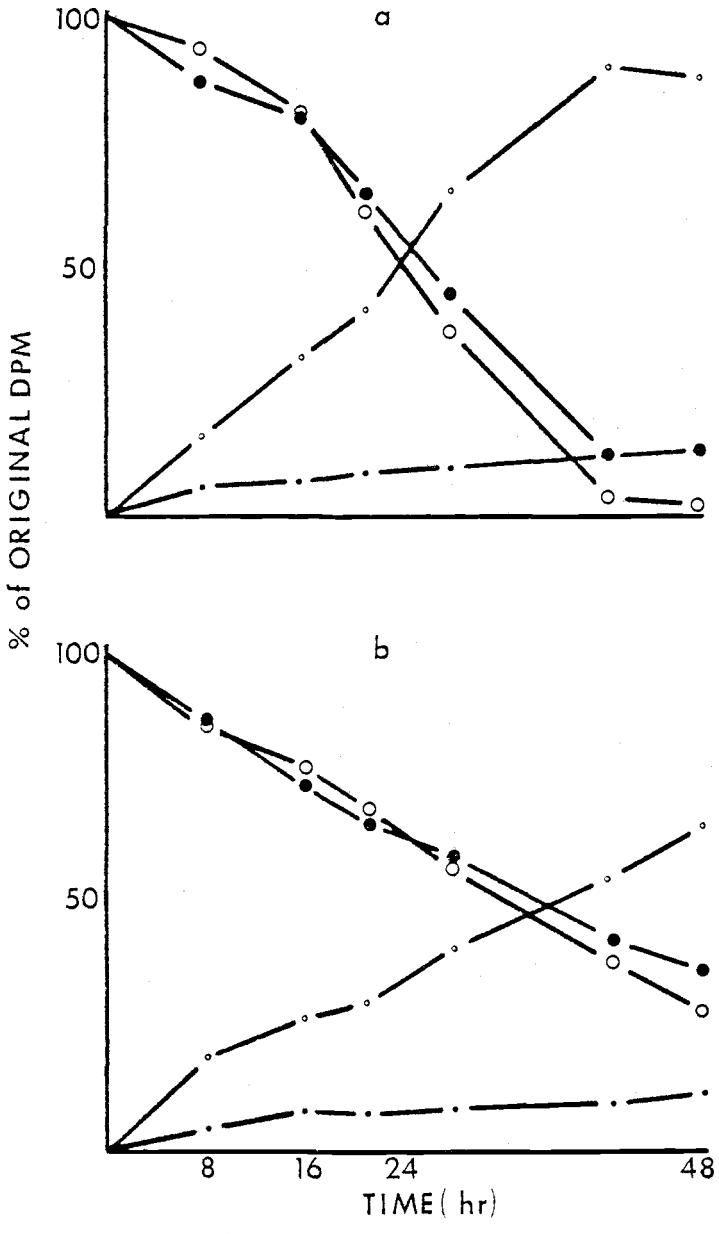

Figure 12. Naphthalene oxidation and metabolite production in static assays without (a) and with (b) 1 mM glucose present. ○ , ∘ cyclohexane extractable substances, and metabolites by the base extraction technique; ● , • cyclohexane extractable substances, and non-acid-volatile metabolites by the acid extraction technique.

Complete recoveries can be achieved if the CO_2 is reacted with phenethylamine to form carbamates which are stable in xylene base scintillation fluors. The recoveries of $14CO_2$ by the base extraction procedure were reported as 100% in the Materials and Methods section. However, that figure may be misleading since losses of the original spike and comparable losses in the saponified phase may have occurred when the $14CO_2$ saturated seawater standard was counted in PCS, a xylene base fluor. That could account for the reduced recovery of counts in seawater where extensive metabolism to $14CO_2$ occurred.

Many bacteria partially oxidize aromatic hydrocarbons when an alternative growth substrate is available (Gibson, 1976). Glucose was added to the seawater during each run to prime the pathways of glucose metabolism in the oyster. In order to determine if the presence of glucose resulted in significantly more polar metabolites, unfiltered seawater with and without glucose was compared to determine if microbial metabolism resulted in greater quantities of polar metabolites in the glucose-treated samples.

In static assays, the presence of 1 mM glucose reduced the extent of naphthalene oxidation. There was an apparent sigmoidal nature to the plot of naphthalene loss and metabolite production when glucose was absent which was not apparent when glucose was present (Figure 12); for the latter treatment, the plot is essentially linear. The sigmoidal nature of the former curves suggest that there was a lag period in the metabolism of naphthalene which was eliminated when glucose was present. Besides eliminating

the lag period, the presence of glucose reduced the maximal rate of naphthalene oxidation which followed the lag period. Linear regression of a plot of the percent naphthalene oxidized against the percent metabolites (Figure 13a) indicated that statistically, the two regressions were significantly different ($P < .025$). Regression of the percent non-acid-volatile polar metabolites plotted against the percent naphthalene oxidized suggests that the glucose treatment increased the rate of production of non-acid-volatile metabolites. The two regression lines were significantly different ($P < .001$). However, considering the small amount of data points and large error variance, the conclusion that the presence of glucose stimulates the partial oxidation of naphthalene should be considered tentative until further data are available.

In static assays, streptomycin concentrations ranging from 25-200 ppm (Figure 14) resulted in a significant reduction of naphthalene oxidation. However, even at 200 ppm some oxidation occurred. If no streptomycin was added to the incubation mixture the seawater rapidly became cloudy, a condition which was not evident in any of the streptomycin treated samples. Cloudiness was considered indicative of microbial blooms.

Whenever streptomycin was not added to the 1 mM glucose seawater in the flow system, the water quickly (< 24 hr) became clouded and nearly opaque. Cloudiness was noted only once in all the runs when streptomycin was present and that occurred when the flow system was run with only oyster shells ($R\text{-}S_w$) in the incubation vessels. In this case the cloudiness appeared near the end of the

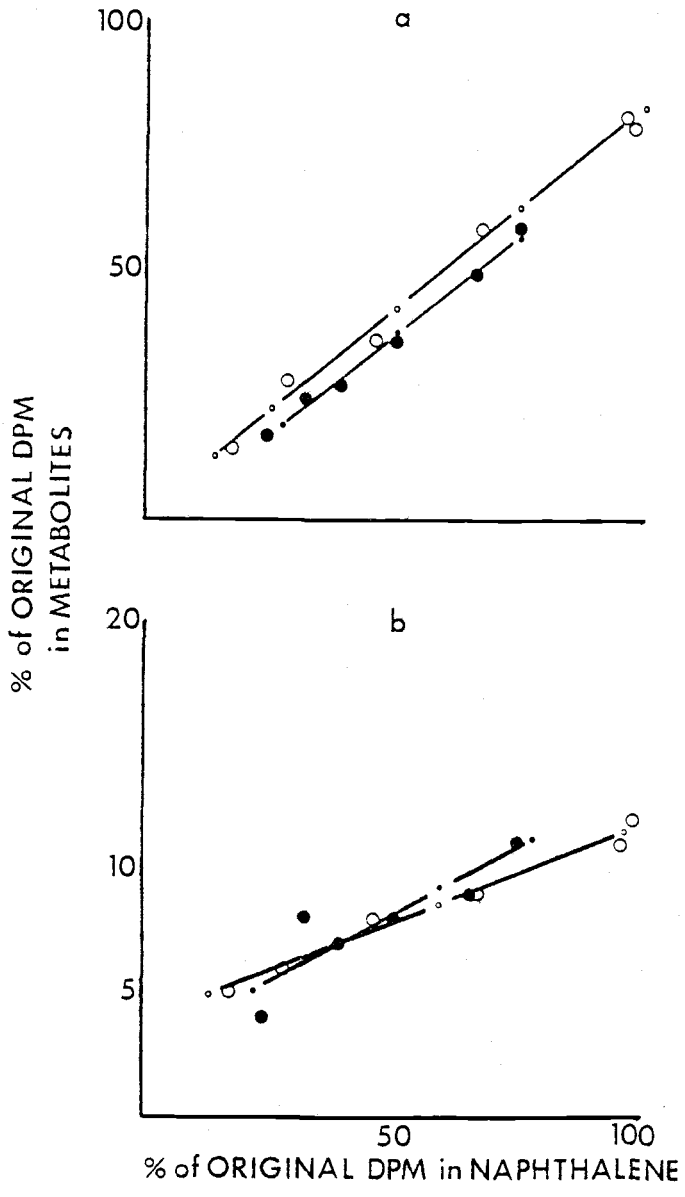

Figure 13. Regression analysis to demonstrate the effects of 1 mM glucose on the alkali soluble (a), and non-acid-volatile (b) metabolite production in untreated (○) and 1 mM glucose treated (●) seawater assays.

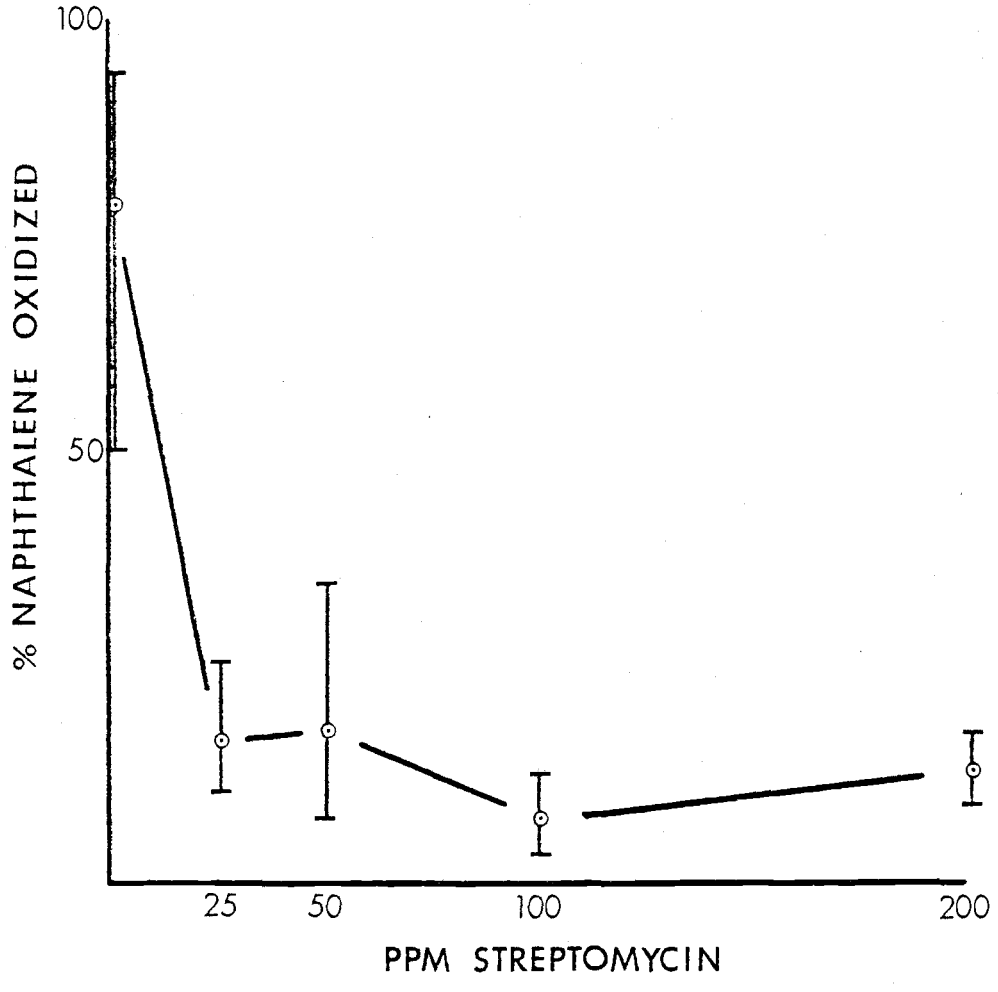

Figure 14. The effects of various concentrations of streptomycin on the oxidation of naphthalene. Vertical bars represent range of three measurements.

run (> 60 hr) and was minimal. The cloudiness was never evident when both oyster tissues and streptomycin were present which suggests that the oysters themselves somehow influenced the number of bacteria present, either through removal by filtration (Zobell and Feltham, 1938) or through some antimicrobial activity (Li, Prescott, Jahnes, and Martino, 1962). Streptomycin greatly reduced the growth of bacteria in the flow system.

In order to evaluate the growth of bacteria during a normal run with tissues present, the water from the outflow and inflow ports was sampled during run R-3 and analyzed for the total number of bacteria present (Figure 15). The bacterial population increased during the run. The fact that the increase occurred both at the inlet and outlet indicated that bacterial contamination was from sources other than the oysters alone. The final bacterial population at the outlet was 9800 ± 3110/ml (± 95% C.I., n = 15).

When only oyster shells were in the incubation vessel without streptomycin ($R\text{-}S_o$), the incubation vessels, seawater reservoir, and metering burets all became extremely cloudy (< 24 hr). With streptomycin present ($R\text{-}S_w$) a slight cloudiness was noted after 60 hr but was confined to the #1 and #2 incubation vessels and to the metering buret for #2. The common seawater reservoir did not appear clouded.

Streptomycin reduced the extent of naphthalene oxidation in the flow system when only shells were present ($R\text{-}S_w$) (Figure 16). However, oxidation was still extensive in both treatments. The initial rate of increase in saponifiable metabolites was

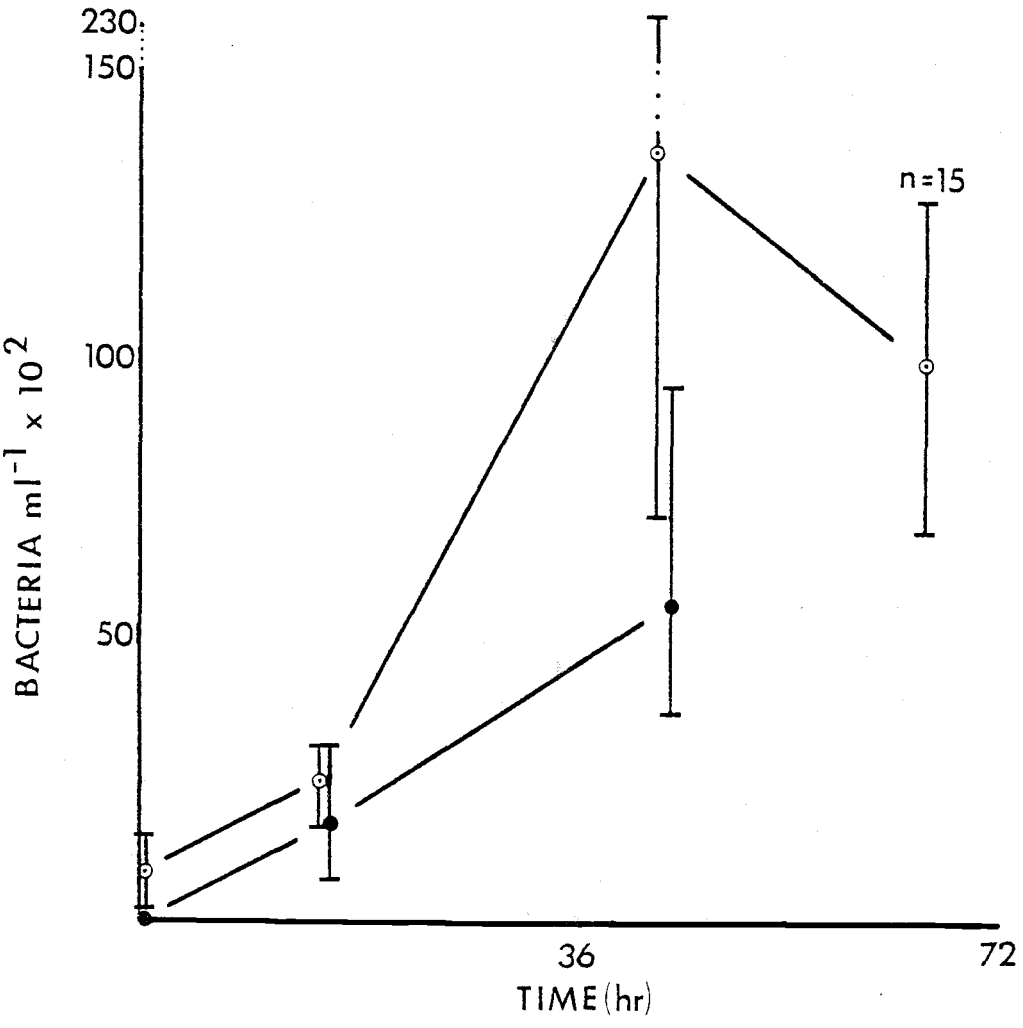

Figure 15. The bacterial concentration at the outlet (⊙) and inlet (●) port of the incubation vessels during run R-3. For the first three sets of measurements the vertical bars represent the range of three determinations. The final measurement includes the mean ± 95% C.I.

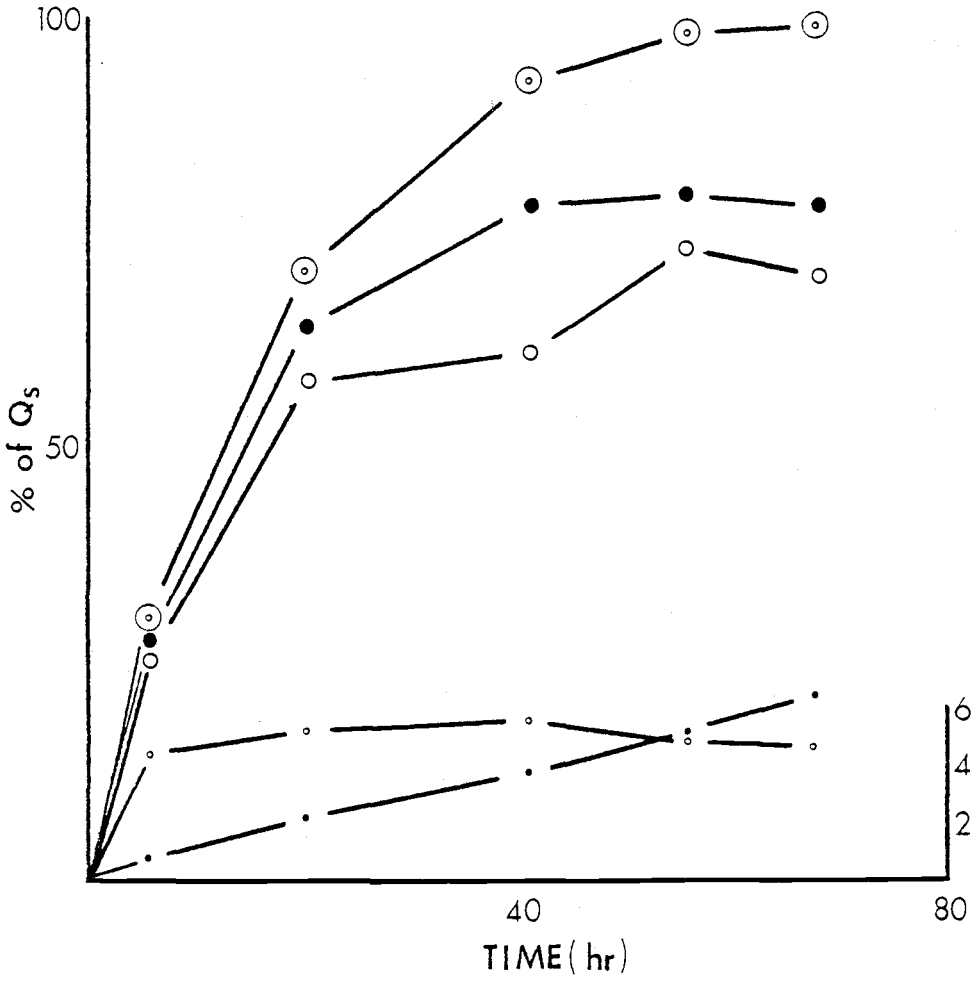

Figure 16. The accumulation of naphthalene (●, ○) and naphthalene metabolites (•, ∘) in the flow system with only shells present, with (●, •) ($R\text{-}S_w$) and without (○, ∘) ($R\text{-}S_o$) streptomycin present and the comparison to the calculated accumulation based on the quantitative model (◎).

considerably less for the streptomycin treatment ($R\text{-}S_w$) but by the end of the run the actual quantity of saponifiable metabolites was greater.

Thin layer chromatography revealed that there were no non-CO_2 naphthalene metabolites present in the hexane extracts of the seawater from the streptomycin free treatment ($R\text{-}S_o$) (Table 5). In the seawater from the streptomycin-treated flow system ($R\text{-}S_w$), an unidentified metabolite was extracted and determined by TLC to have a mobility intermediate to 1-naphthol and 2-naphthol.

Within 24 hr, 95% of the glucose was degraded in the streptomycin-free seawater from the flow system with shells only ($R\text{-}S_o$) (Table 2). The streptomycin-treated systems ($R\text{-}S_w$) showed no glucose oxidation until after 50 hr. At the last sample period (67 hr), more than half the glucose loss had occurred prior to entry into the incubation vessels indicating again, that the source of bacterial contamination was not due exclusively to the shells.

Oxygen consumption in the streptomycin-free flow system ($R\text{-}S_o$) occurred primarily before the seawater entered the incubation vessel (Table 6). The dissolved oxygen concentration fell to a very low value within 24 hr. In vessel #2 ($R\text{-}S_w$) the oxygen consumption again occurred primarily before passage through the incubation vessel. In system #1 ($R\text{-}S_w$) the oxygen consumption appeared to be confined to the incubation vessel. At the final sample interval, oxygen consumption attributed to microbial uptake in vessel #1 (- 0.48 µl/ml/g) was 40% of the consumption observed when tissues were present (average value of all oxygen uptake values). In

Table 2. Glucose oxidation before and after passage of seawater through the incubation vessels during run R-S, expressed as percent of the original 1 mM glucose concentration.

Time (hr)	Vessel #1[a]		Vessel #2		Vessel #3	
	Outlet	Inlet	Outlet	Inlet	Outlet	Inlet
24	100	100	100	100	5	5
51	100	100	100	100	5	5
67	91	95	78	88	0	8

[a]Vessel #1, #2, and #3 contained, respectively, 100 ppm streptomycin and 1 mM glucose; 100 ppm streptomycin, 1 mM glucose, and [1-14C] naphthalene; and 1 mM glucose and [1-14C] naphthalene.

system #2, the final value (- 0.09 µl/ml/g) was only 8%. This difference was due primarily to the considerable oxygen consumption that took place prior to entry of the water into the incubation vessel in system #2. Since both vessel #1 and #2 drew seawater from the same reservoir, the major site of oxygen consumption prior to entry into incubation vessel #2 was probably the metering buret. It was noted previously that the metering buret for vessel #2 during this run showed some cloudiness indicative of bacterial contamination. Metering buret #1 did not have any cloudiness. It is possible that cross contamination of metering burets #3 and #2 could have occurred as a result of using the same syringe when refilling these burets with [1-14C] naphthalene seawater.

Ammonia-nitrogen excretion decreased steadily in the streptomycin-treated system ($R\text{-}S_w$) (Table 7). Negative values indicated that the level of ammonia-nitrogen entering was greater than the amount leaving the incubation vessel. In the streptomycin-free system ($R\text{-}S_o$) there was extensive loss of ammonia-nitrogen following passage through the incubation vessel. The levels of ammonia-nitrogen were low in both the outlet and inlet ports at the final sampling period and the net flux was zero.

Uptake and Metabolism of Naphthalene

For convenience, all radioactivity in cyclohexane or hexane extractable substances was reported as being naphthalene. Similarly, all metabolite activity was reported as naphthalene equivalents. Based on Rf values from thin layer separations, the

assumption that naphthalene was the sole major constituent of cyclohexane and hexane extracts of saponified digests was, in fact, warranted.

In run R-0, only the gill tissue was analyzed for naphthalene and its metabolites. The mean naphthalene concentration in the gill for run R-0 was 2.41 ± 0.17 ppm. Saponifiable metabolites accounted for 0.15 ± 0.02 ppm (± 95% C.I., n = 7). In all other runs the body tissue component possessed the greatest naphthalene concentration (Table 3). Naphthalene concentration in the adductor muscle did not follow any consistent pattern. In runs R-1 and R-2, the adductor muscle concentration was greater than the gills but in R-3 the concentration was greater in the gills. In runs R-1, R-2, and R-3 the naphthalene concentration in the gills increased with the length of starvation.

The bioaccumulation factor for each tissue component was calculated by dividing the concentration of naphthalene in the tissue by the concentration of naphthalene in the seawater at the time of sampling (Table 3). The body consistently had the highest bioaccumulation factors. There were no significant differences at the $\alpha < .05$ level between the mean bioaccumulation factors in the body for any of the runs. The bioaccumulation factors between runs in the adductor muscle were all significantly different ($P < .05$); however, there was no consistent pattern that would explain the differences. The mean bioaccumulation factors in the gills were not significantly different between run R-0 and R-1 but were significantly different between all other runs ($P < .05$). The

Table 3. Naphthalene concentration (hexane extractable substances [HES] and saponifiable metabolites [MET]) in the tissues.[a]

Run		Gills		Body		Adductor	
		HES	MET	HES	MET	HES	MET
R-1 n = 8	PPM	2.19	0.10	4.40	0.16	2.50	0.10
	95% C.I.	0.25	0.03	0.56	0.04	0.27	0.03
	B.F.	31		62		35	
R-2 n = 8	PPM	2.69	0.18	4.33	0.26	3.84	0.14
	95% C.I.	0.10	0.02	0.62	0.04	0.70	0.03
	B.F.	36		58		52	
R-3 n = 3	PPM	2.84	0.26	4.03	0.36	1.65	0.24
	95% C.I.	0.27	0.01	0.32	0.09	0.12	0.04
	B.F.	42		59		24	

[a]Abbreviations: B.F. = bioaccumulation factor; n = number of tissues sampled; PPM = parts per million as naphthalene equivalents, based on the wet wt.

differences in the gills may have been associated with starvation effects. In run R-3 the naphthalene concentration in each tissue increased in a manner corresponding to the increase in the naphthalene concentration in the flow system (Figure 17). Also, in run R-3 the bioaccumulation factors for naphthalene increased with time in the body and gills, but decreased in the adductor muscle (Figure 17).

With the exception of run R-0, the non-CO_2 saponifiable metabolite concentration increased significantly ($P < .05$) between each run for every tissue component. These differences suggest that the increases were associated with starvation (Table 3). In run R-3 the accumulation of non-CO_2 saponifiable metabolites increased in a manner corresponding to the increase of unmodified naphthalene in the tissue (Figure 18). The adductor muscle showed very high initial concentrations of metabolites in run R-3.

The thin layer separation of hexane extractable metabolites from the tissue digests revealed that the 15-hr sample from R-3 was the only tissue sample, from all of the runs, that did not have any radioactivity in 2-naphthol (Table 4). Activity was occasionally recovered in 1-naphthol and in very polar metabolites located near the origin (Table 4). In run R-3, the abundance of these very polar metabolites was greatest in the first sample (15 hr) and least in the final sample (72 hr). Conversely, activity in 2-naphthol and 1-naphthol was not detected in the first sample but was highest in these metabolites in the last sample.

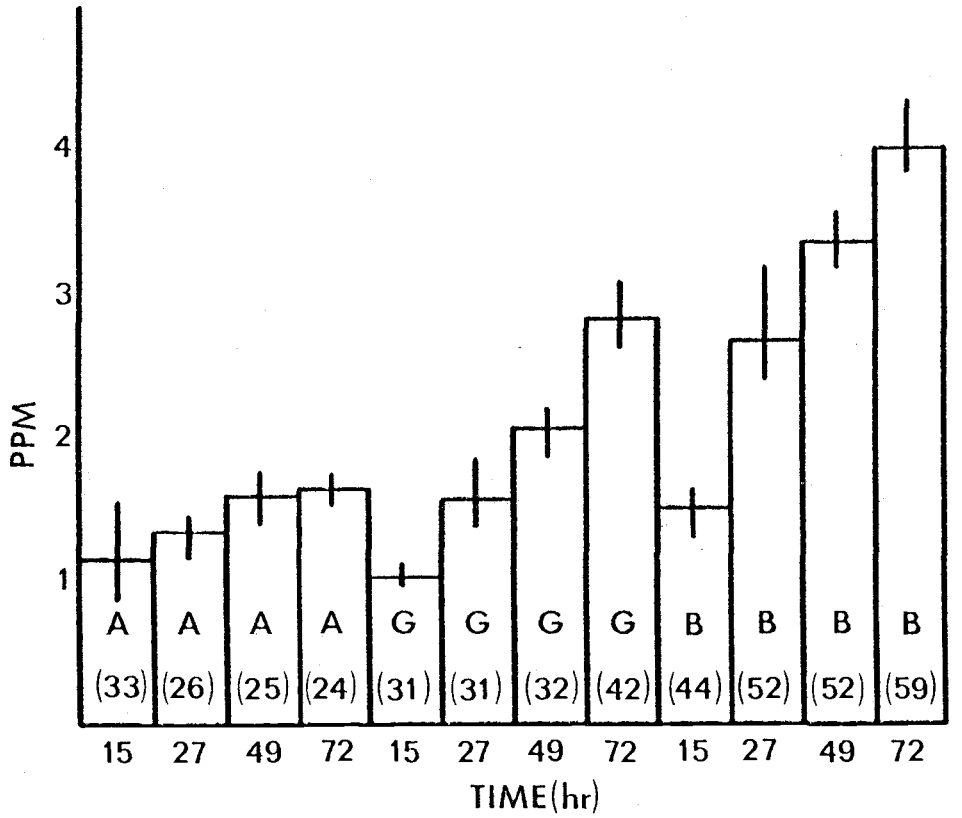

Figure 17. The accumulation of naphthalene in the various tissue components; A = adductor, G = gills, B = body. Vertical bars represent the range of three samples. The number in parentheses are the bioaccumulation factors.

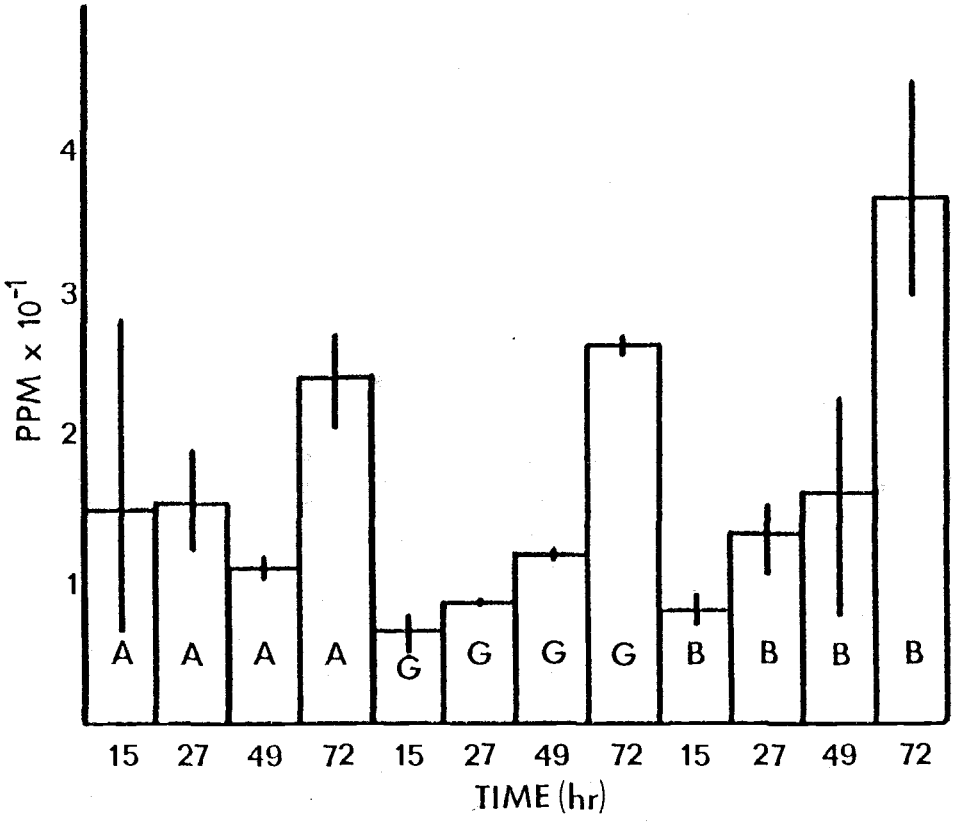

Figure 18. The accumulation of saponifiable naphthalene metabolites in the various tissue components expressed as naphthalene equivalents. Abbreviations same as Figure 17.

Table 4. Non-CO_2 naphthalene metabolites in the pooled formic acid tissue digests.[a]

Run	Origin			1		2-Naphthol		2		1-Naphthol		3	
	n[b]	hr	PPB[c]	PPB	R	PPB	R	PPB	R	PPB	R	PPB	R
R-0	2	73	17	n.d.	0.33	32	0.67	n.d.	0.89	n.d.	1.00	n.d.	1.35
	2	73	n.d.	n.d.	0.44	44	0.67	n.d.	0.89	n.d.	1.00	n.d.	1.21
	2	73	n.d.	n.d.	0.44	29	0.66	n.d.	0.89	n.d.	1.00	n.d.	1.21
	2	73	13	n.d.	0.44	17	0.67	n.d.	0.89	n.d.	1.00	n.d.	1.21
R-1	2	72	-	n.d.	0.43	36	0.61	n.d.	0.78	n.d.	1.00	n.d.	1.23
	2	72	-	n.d.	0.41	42	0.65	n.d.	0.81	n.d.	1.00	n.d.	1.30
	3	72	-	n.d.	0.50	34	0.65	n.d.	0.83	n.d.	1.00	n.d.	1.11
R-2	2	72	n.d.	n.d.	0.45	9	0.69	-	-	2	1.00	n.d.	1.21
	2	72	n.d.	n.d.	0.41	9	0.64	-	-	n.d.	1.00	n.d.	1.24
	2	72	2	n.d.	0.48	8	0.71	-	-	n.d.	1.00	n.d.	1.21
	2	72	n.d.	n.d.	0.43	12	0.64	-	-	2	1.00	n.d.	1.19
R-3	3	15	95	n.d.	0.60	n.d.	0.73	-	-	n.d.	1.00	n.d.	1.29
	3	27	15	n.d.	0.48	8	0.76	-	-	5	1.00	n.d.	1.24
	3	49	8	n.d.	0.50	6	0.73	-	-	2	1.00	n.d.	1.23
	3	72	10	n.d.	0.60	20	0.80	-	-	8	1.00	n.d.	1.20

[a] Samples were spotted with authentic standards of 1-naphthol and 2-naphthol and developed on silica gel plates in benzene solvent. Areas corresponding to the standards and the areas above and below each standard (1,2,3) were scraped and counted by LSC.

[b] Symbols and abbreviations: - = area not counted; n.d. = no detectable activity; R = position of the band scraped relative to 1-naphthol; n = number of oysters pooled; hr = sample time in hours.

[c] Values calculated as total DPM extracted/specific activity/g wet wt.

Because of the manner of extraction, as described in the Materials and Methods section, it was unlikely that any highly oxygenated or otherwise highly substituted polar metabolites would have been extracted into the hexane. Even low quantities of 1-naphthol were recovered (< 50%). However, the hexane extracts were relatively clean in comparison to extracts using more polar solvents and separated (by TLC) cleanly with little tailing.

Temporal changes in naphthalene concentration in the flow system generally followed the predicted values although considerably reduced in magnitude (Figure 19). In run R-3, the rate of increase in naphthalene decreased considerably after the second measurement. In other runs the decrease was apparent but not so abrupt. That was partially because the incubation vessel had to be opened three times prior to the end of the run in order to remove samples. In runs R-0, R-1, and R-2 the rate of naphthalene increase was actually greater than the predicted rate near the end of the run.

The metabolite concentration in the water quickly reached a plateau and then decreased towards the end of each run. The percent metabolites in the water never exceeded 0.5% of the steady-state value when tissues were present. In run R-S, the concentration exceeded 6.0%. The increased rate of naphthalene accumulation in the seawater and the decrease in metabolites in the late part of each run correlated well with the apparent decrease in the bacterial population observed in run R-3 during the final stages of that run (Figure 15). The accumulation of naphthalene with oysters present did not differ much from that observed for run $R\text{-}S_w$

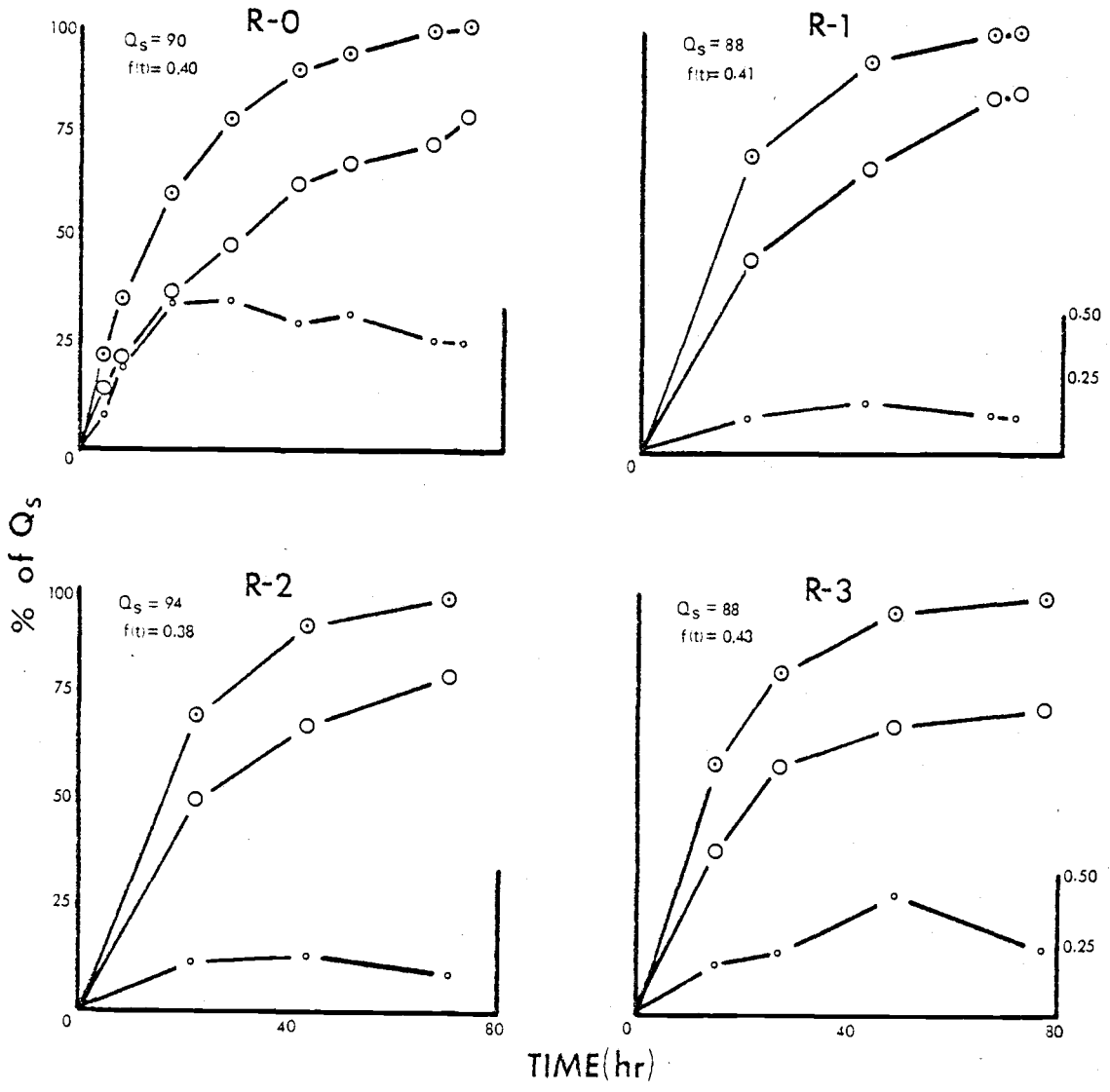

Figure 19. The accumulation of naphthalene (○) and metabolites (∘) in the seawater of the flow-through system and the comparison to the calculated values for the quantitative model (⊙). Q_s is given in ppb; f(t) in ml/min.

($\sim$ 75-80% of Q_s); however, in run R-S_w the naphthalene concentration actually decreased during the final sample period, an event that never occurred when tissues were present. The fact that the metabolites (which were primarily $^{14}CO_2$) were $\sim$ 10-30 times greater in run R-S suggested that CO_2 was being quickly removed from solution when oysters were present. This observation is consistent with the fact that rapid shell deposition was occurring in the live oysters.

Of the hexane extractable metabolites isolated from seawater and separated by TLC, 2-naphthol was the most abundant based on the recovery of 14C-label from areas corresponding to known standards. In run R-3, considerable activity was recovered from very polar metabolites near the origin and from the area corresponding to known standards of 1-naphthol (Table 5).

The [1-14C] naphthalene used to spike the seawater in runs R-0 and R-1 was used directly as supplied from Amersham Co. The reported purity was 99% by gas-liquid radiochromatography. However, TLC analysis revealed that three contaminants were consistently present in the [1-14C] naphthalene obtained from Amersham Co. The first contaminant was totally immobile in the benzene solvent system and accounted for 0.02% of the total activity applied to the plate. The second and third had mobilities of 0.30 and 0.87 respectively, relative to 1-naphthol. Each accounted for 0.02% of the total activity. One and 2-naphthol were well separated from the contaminants in runs R-0 and R-1 and activity was not detected in the bands above and below 2-naphthol. The [1-14C] naphthalene

Table 5. Non-CO_2 naphthalene metabolites extracted from seawater and separated by thin layer chromatography.[a]

Run	Origin	1		2-Naphthol		2		1-Naphthol		3	
	PPB[b]	PPB	R[c]	PPB	R	PPB	R	PPB	R	PPB	R
R-1	-	n.d.	0.46	0.124	0.63	n.d.	0.80	n.d.	1.00	n.d.	1.21
R-2	n.d.	n.d.	0.52	0.029	0.68	n.d.	0.81	n.d.	1.00	n.d.	1.25
R-3	0.775	n.d.	0.46	0.305	0.68	n.d.	0.81	0.481	1.00	n.d.	1.25
$R\text{-}S_w$	0.009	n.d.	0.53	n.d.	0.67	0.025	0.83	n.d.	1.00	n.d.	1.14
$R\text{-}S_0$	n.d.	n.d.	0.53	n.d.	0.69	n.d.	0.86	n.d.	1.00	n.d.	1.14

[a]Samples were spotted with authentic standards of 1-naphthol and 2-naphthol and developed on silica gel thin layer plates in benzene solvent. Areas corresponding to the standards and the areas above and below each standard were scraped (1,2,3) and counted by LSC.

[b]Values calculated as: total DPM extracted/specific activity/ml water extracted.

[c]Symbols and abbreviations: - = area not counted; n.d. = no detectable activity; R = position of the band scraped relative to 1-naphthol.

used in runs R-2 and R-3 was pre-purified by TLC on silica gel.

Effects of Naphthalene on Oxygen Consumption and Ammonia-Nitrogen Excretion by Oysters

The average dissolved oxygen concentration of seawater in the system was 5.2 ml O_2/l of seawater (Table 6). This was very close to the saturation value at 28 °/oo and 15°C. By the time each run was completed, the concentration was reduced to approximately 2.5 ml/l. During run R-0, only the final oxygen concentration was determined. The samples from run R-2 were accidentally lost. The rate of oxygen uptake (QO_2 in µl/hr/g) was calculated as outlined in the Materials and Methods section. The oxygen consumption during the second sample interval was determined (Table 7). Assuming that by the time of final sampling, the rate of change of dissolved oxygen was zero, then the rate of oxygen consumption at that sample time was:

$$QO_2 = f(t)(C_o(t) - C_i(t))/g \text{ wet wt.}$$

The results of these calculations suggested that the control oysters had consistently higher respiratory rates. But because accurate estimates of the bacterial contribution were not possible, interpretation of these results was tenuous.

Ammonia-nitrogen excretion peaked very early and then decreased consistently to near zero and in some cases to negative values (Table 8). The rates of ammonia-nitrogen excretion did not follow any distinguishable pattern (Table 9). There was no

Table 6. Comparison of the dissolved oxygen uptake by control (Ct) and naphthalene-treated (Nt) oysters (R-0, R-1, R-3), and control and naphthalene-treated shells with and without streptomycin (R-S).[a]

Time Interval		R-0			R-1			R-3		R-S		
		Vessel										
		#1	#2	#3	#1	#2	#3	#1	#2	#1	#2	#3
1	μC_i[b]	-	-	-	4.99	5.06	4.85	5.13	5.13	4.40	4.22	0.98
	C_o	-	-	-	2.45	2.95	2.60	2.11	2.53	4.22	4.36	0.70
	1/ml/g	-	-	-	-0.99	-0.97	-0.95	-1.26	-1.06	-0.07	+0.06	-0.12
	t(hr)	-	-	-	18	18	18	19	19	24	24	24
2	μC_i	-	-	-	4.96	4.96	5.06	5.37	5.34	4.39	4.08	1.90
	C_o	-	-	-	2.76	2.95	2.81	2.25	2.25	3.59	4.01	0.70
	1/ml/g	-	-	-	-0.88	-0.93	-0.95	-1.31	-1.26	-0.34	-0.03	-0.51
	t(hr)	-	-	-	40	40	40	49	49	42	42	42
3	μC_i	6.19	5.76	5.76	5.00	4.90	4.90	5.41	5.41	4.50	3.16	1.41
	C_o	2.53	2.95	2.81	1.94	2.42	2.28	2.22	2.46	3.37	2.95	1.21
	1/ml/g	-1.22	-1.18	-1.14	-1.19	-1.14	-1.11	-1.34	-1.21	-0.48	-0.09	-0.08
	t(hr)	72	72	72	64	64	64	68	68	67	67	67

[a] For R-0, R-1 and R-3, vessel #1 was the control and vessels #2 and #3 naphthalene-treated; both R-S #1 and #2 contained 100 ppm streptomycin while #3 did not.

[b] C_i = inlet dissolved oxygen concentration in µl/ml; C_o = outlet concentration; µl/ml/g = C_o-C_i/g wet wt. In run R-S the tissue wt was the wet wt of the tissues that were removed.

Table 7. Comparison of the rate of oxygen uptake (µl/hr/g) by control and naphthalene-treated oysters.

Time Interval		R-0			R-1			R-3	
		Vessel							
		#1	#2	#3	#1	#2	#3	#1	#2
	f(t)	-	-	-	24.6	25.2	22.8	33.0	24.0
2[a]	T	-	-	-	17.1	16.7	18.4	12.7	17.5
	QO_2	-	-	-	22.8	25.6	22.9	42.0	30.5
	f(t)	26.4	23.4	24.0	25.8	24.6	25.8	33.0	25.8
3[b]	T	15.9	18.0	17.5	16.3	17.1	16.3	12.7	16.3
	QO_2	32.1	27.6	27.3	30.6	28.0	28.6	44.2	31.2

[a] f(t) = ml/hr; T = hr; $QO_2 = \left[(f(t)\left((C_o(t) - C_i(t)) + (T/2\Delta t)\left(C_o(t-\Delta t) - C_o(t+\Delta t)\right)\right)\right]$/g wet wt.

[b] $QO_2 = f(t)(C_o(t) - C_i(t))$/g wet wt; calculation assumes $dC_o/dt = 0$.

Table 8. Comparison of ammonia-nitrogen excretion by control (Ct) and naphthalene-treated (Nt) oysters (R-0, R-1, R-3), and control and naphthalene-treated shells with and without streptomycin (R-S).[a]

Time Interval		R-0			R-1			R-3		R-S		
		Vessel										
		#1	#2	#3	#1	#2	#3	#1	#2	#1	#2	#3
0	ppb	76	67	71	-	-	-	-	-	-	-	-
	ppb/g	25	28	27	-	-	-	-	-	-	-	-
	t(hr)	5	5	5	-	-	-	-	-	-	-	-
1	ppb	64	67	102	51	50	28	122	93	15	12	22
	ppb/g	21	28	39	20	23	12	47	38	6	5	9
	t(hr)	19	19	19	18	18	18	14	14	27	24	24
2	ppb	63	58	61	27	33	-6	66	67	7	-6	-105
	ppb/g	22	24	23	10	15	-2	28	27	3	-2	-45
	t(hr)	45	45	45	36	36	36	43	43	41	41	41
3	ppb	0	-19	76	-7	18	7	19	22	5	-13	0
	ppb/g	0	-8	29	-3	8	3	8	9	2	6	0
	t(hr)	66	66	66	64	64	64	65	65	65	67	67

[a] Same as in Table 5; - = no measurement taken.

[b] ppb calculated as the difference between the inlet and outlet concentrations; ppb/g wet wt. In run R-S the tissue wt was the wet wt of the tissues that were removed.

Table 9. Comparison of the rate of ammonia-nitrogen excretion by control and naphthalene-treated oysters.[a]

Time Interval		R-0			R-1			R-3	
		Vessel							
		#1	#2	#3	#1	#2	#3	#1	#2
	f(t)	26.4	23.4	24.0	24.6	25.2	22.8	33.0	24.0
2	T	15.9	18.0	17.5	17.1	16.7	18.4	12.7	17.5
	ng/g/hr	+5.2	+3.5	+9.1	-0.3	+3.4	-2.7	+9.6	+7.3

[a]Same as Table 6.

evidence that naphthalene treatment affected ammonia-nitrogen excretion. Again, the fact that accurate estimates of bacterial metabolism were not possible, made interpretation of these results difficult.

Effects of Naphthalene on the Uptake of D-[U-14C] Glucose and $14CO_2$ Production.

The 14C-label was rapidly lost from the seawater medium in the glucose incubation vessels during the glucose uptake experiments. Regression of the rate of 14C-label loss from the incubation media (Figure 20) expressed as a percent of the original activity and calculated as:

$$((\mu Ci/g \text{ wet wt at time } t)/(\mu Ci/g \text{ wet wt at } t = 0))(100)$$

for control (R-0, R-2, R-3) and naphthalene-treated oysters (R-1, R-2, R-3) revealed that there were significant differences in the loss of 14C-label from the glucose incubation vessels for Ct and Nt oysters ($P < 0.01$). Neither parameter (constant a, coefficient b) alone was able to account for the differences between the two treatments. The initial rate of 14C-label loss from the seawater was greater for Nt oysters.

The $14CO_2$ production was not so easily evaluated (Table 10). There was no consistent level of $14CO_2$ production that indicated regression of the data would be of any value. In run R-2, the control oysters evolved considerably more $14CO_2$ than the naphthalene-treated oysters. That situation was reversed in R-3.

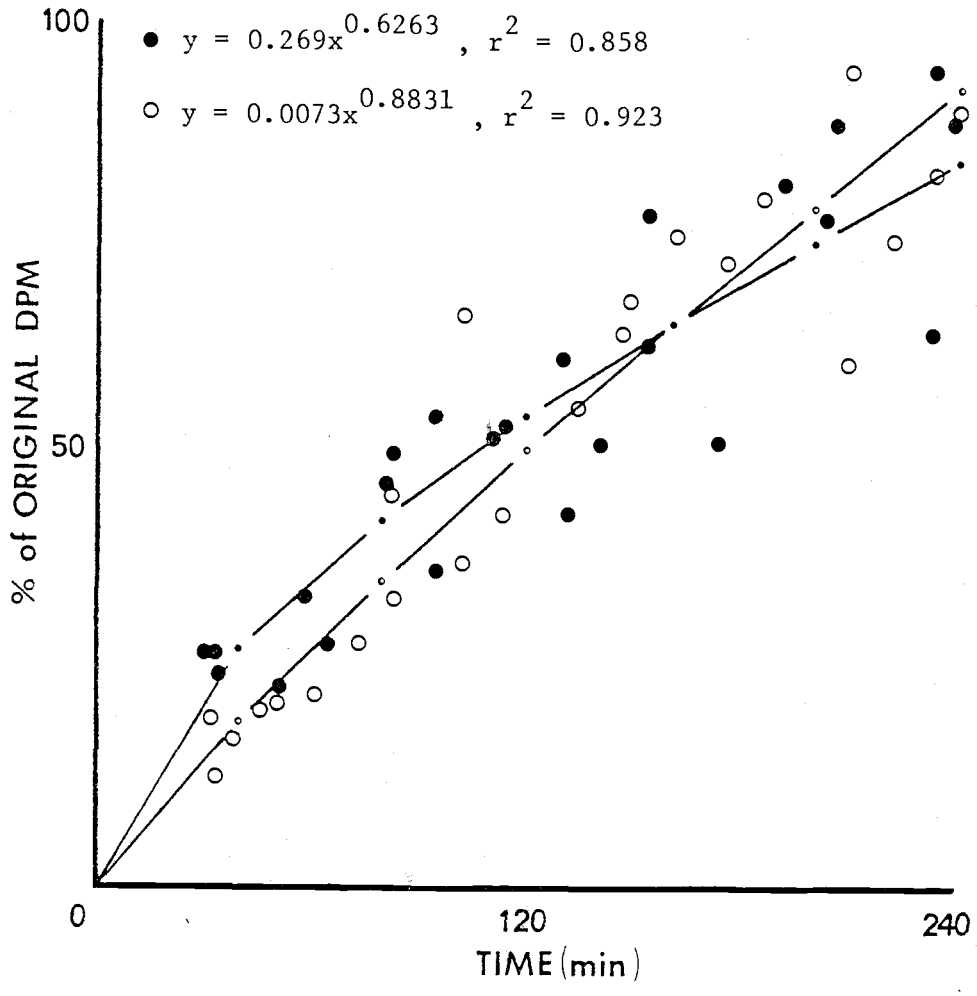

Figure 20. Regression analysis of the loss of 14C-label from the incubation seawater for control (○) and naphthalene-treated (●) oysters after $14CO_2$ had been driven off by acidification. The regression is of the pooled data for control (R-0, R-2, and R-3) and naphthalene-treated (R-1, R-2, and R-3) oysters. Values are expressed per g wet wt.

Table 10. $14CO_2$ production in control and naphthalene-treated oysters.[a]

Time Interval		R-0	R-1		R-2		R-3	
		Ct	Ct	Nt	Ct	Nt	Ct	Nt
1	µCi/g	0.157	0.037	0.024	0.143	0.047	0.015	0.005
	t(min)	32	34	34	32	33	33	30
2	µCi/g	0.094	0.041	0.092	0.152	0.168	0.042	0.015
	t(min)	56	59	58	60	64	50	50
3	µCi/g	0.058	0.052	0.076	0.223	0.121	0.041	0.057
	t(min)	71	86	80	81	87	82	82
4	µCi/g	0.214	0.105	0.307	0.133	0.185	0.083	0.072
	t(min)	102	109	94	101	100	112	113
5	µCi/g	0.199	0.053	0.331	0.370	0.254	0.095	0.138
	t(min)	148	139	130	133	130	145	140
6	µCi/g	0.539	0.159	0.254	0.840	0.308	0.266	0.195
	t(min)	184	164	152	160	152	174	172
7	µCi/g	0.316	0.113	0.250	0.546	0.204	0.172	0.251
	t(min)	221	205	205	209	190	208	201
8	µCi/g	0.712	0.220	0.410	1.383	0.515	0.106	0.583
	t(min)	284	238	237	240	230	232	232

[a]Values expressed per g wet wt; t is the sample time.

The treatments from R-1 were not comparable because of the difference in the incubation temperature for control (12.6 ± 0.6°C) and naphthalene-treated oyxters (15.0 ± 0.2°C).

After the oysters were removed from the glucose incubation vessels the media in vessels 1-4 was counted and then after ~ 4 hr recounted, acidified and counted again. The results indicated that after removing the oysters, only a very small amount of label was lost from the remaining incubation medium ($\bar{x}$ = 3.4%, s = 2.0, n = 12), probably as $14CO_2$, and that acidification did not drive off any additional counts from these four vessels ($\bar{x}$ = 3.4%, s = 2.0, n = 12). This latter finding suggested the count loss that did occur was not a function of time and that after removal of the oysters from the incubation media, the oxidation of glucose ceased. The oysters and their shells and associated bacteria were the sinks for all glucose loss and the source of all $14CO_2$ production.

In run R-0, the oyster tissues from the control oysters were immediately dissected out, digested in NCS tissue solubilizer and counted. The activity expressed as μCi/g wet wt, was regressed against time for each tissue component. The results of these regressions indicated that there was no significant difference between the activity-time curves for any of the tissue components at the $\alpha < 0.05$ level. The best fit of the pooled data was obtained with a first-order linear model (Figure 21). The results suggested that the 14C-label was quickly and evenly mixed throughout the oyster.

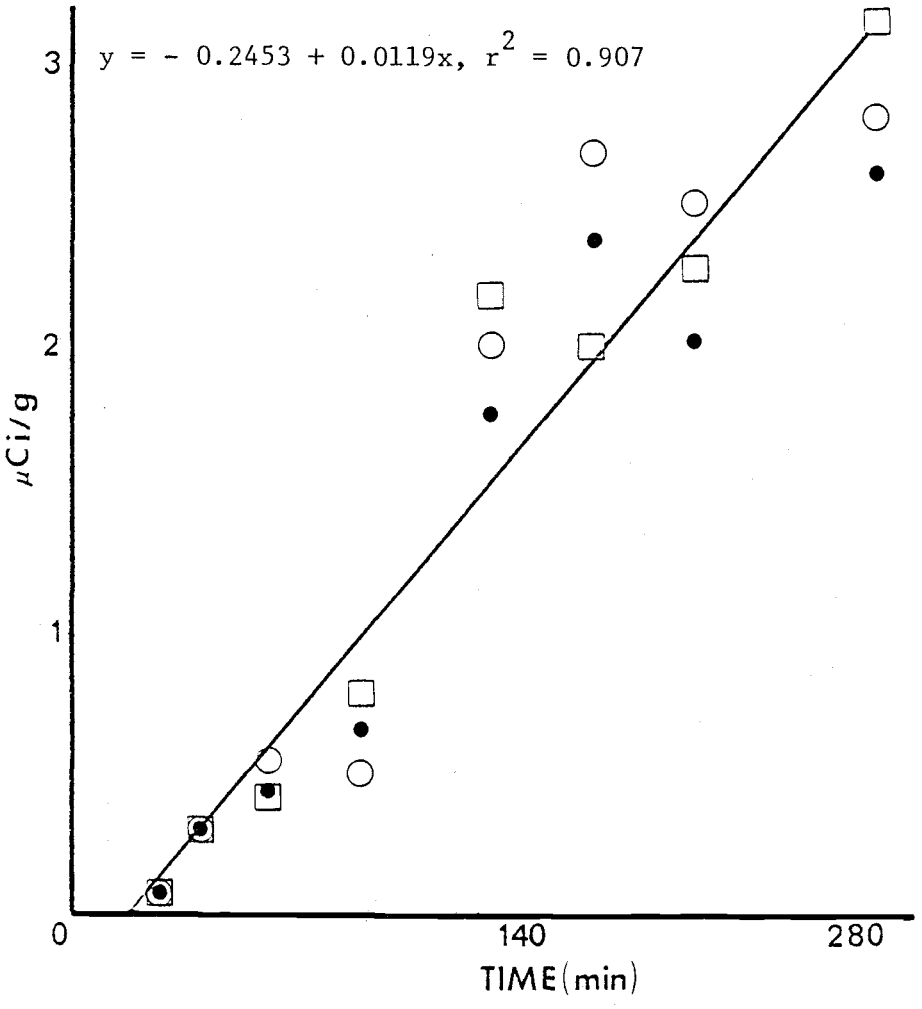

Figure 21. The incorporation of 14C-label from [U-14C] glucose into the various tissue components during run R-0. ● body, ○ gills, and □ adductor muscle. Solid line is the fitted regression. Values expressed per g wet wt.

A plot of the regression lines for the loss of 14C-label from the incubation media, evolution of $14CO_2$, and incorporation of 14C-label into the whole oyster for run R-0, revealed (Figure 22) that there were extensive amounts of radioactivity which were not accounted for. The formation of $14CO_2$ and incorporation of the 14C-label into tissues accounted for only about 38% of the label lost from the medium. It seems highly probable that the recoveries of $14CO_2$ were low due to considerable $14CO_2$ fixation for the purpose of shell renewal. The shell material was not counted.

The 14C-label that flowed into the intermediates and end products of glucose metabolism was summed and the data expressed as the total μCi/g dry wt in control and naphthalene-treated oysters pooled from R-2 and R-3 and regressed against the sample time in hr (Figure 23). The oysters from R-1 could not be compared because of the temperature fluctuations. The plot was similar to that described previously for run R-0 (Figure 21) except that only control oysters were used in the case of R-0, and the tissues were digested in NCS and the results expressed as μCi/g wet wt. The tissues were never freeze-dried or fractionated as was the case for R-1, R-2 and R-3. The results of the pooled regression of 14C-label incorporation into the gill tissue in runs R-2 and R-3 was very similar in appearance to the pooled regression of the loss of 14C-label from the incubation media (Figure 20). The crossover point for both plots occurred at 160 min and the 14C-label incorporated into tissue and that lost from the media, were both greater prior

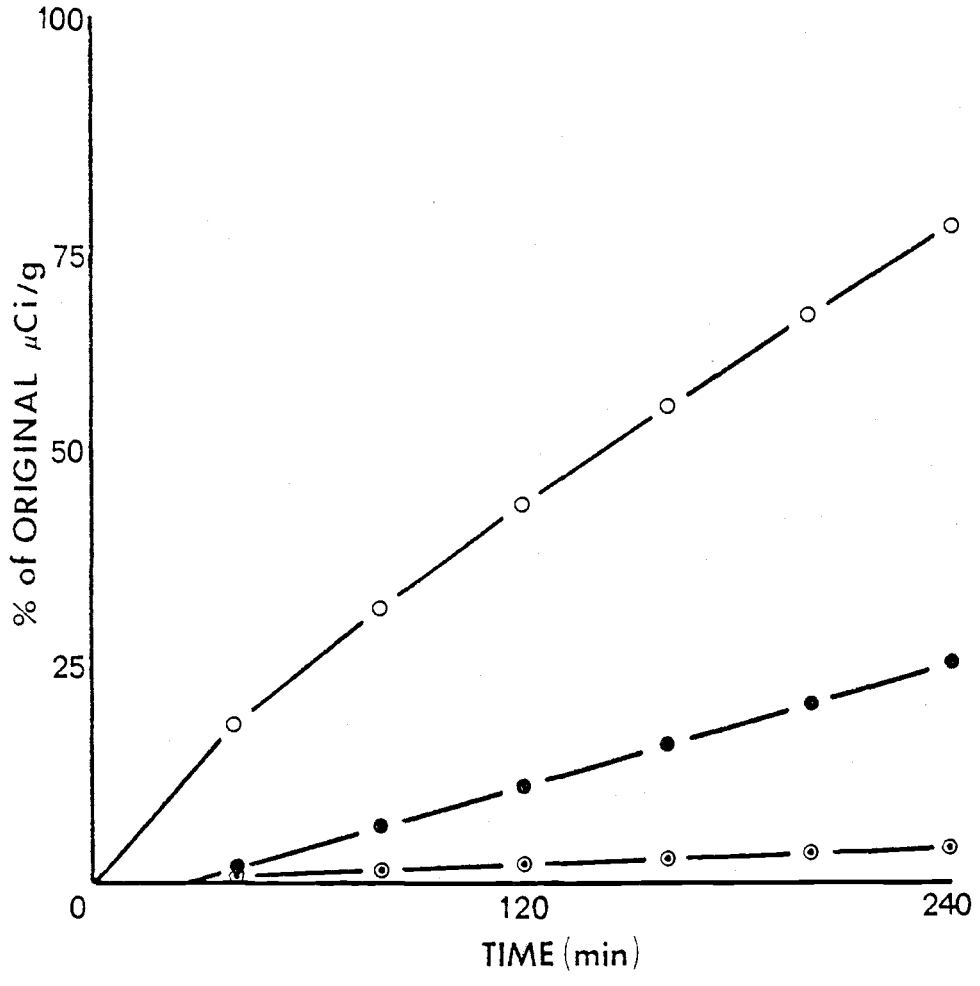

Figure 22. Regression of the loss of 14C-label from the incubation seawater (○), accumulation of 14C-label in the whole body (●), and evolution of $14CO_2$ (⊙) by the whole body for run R-0. Values expressed per g wet wt.

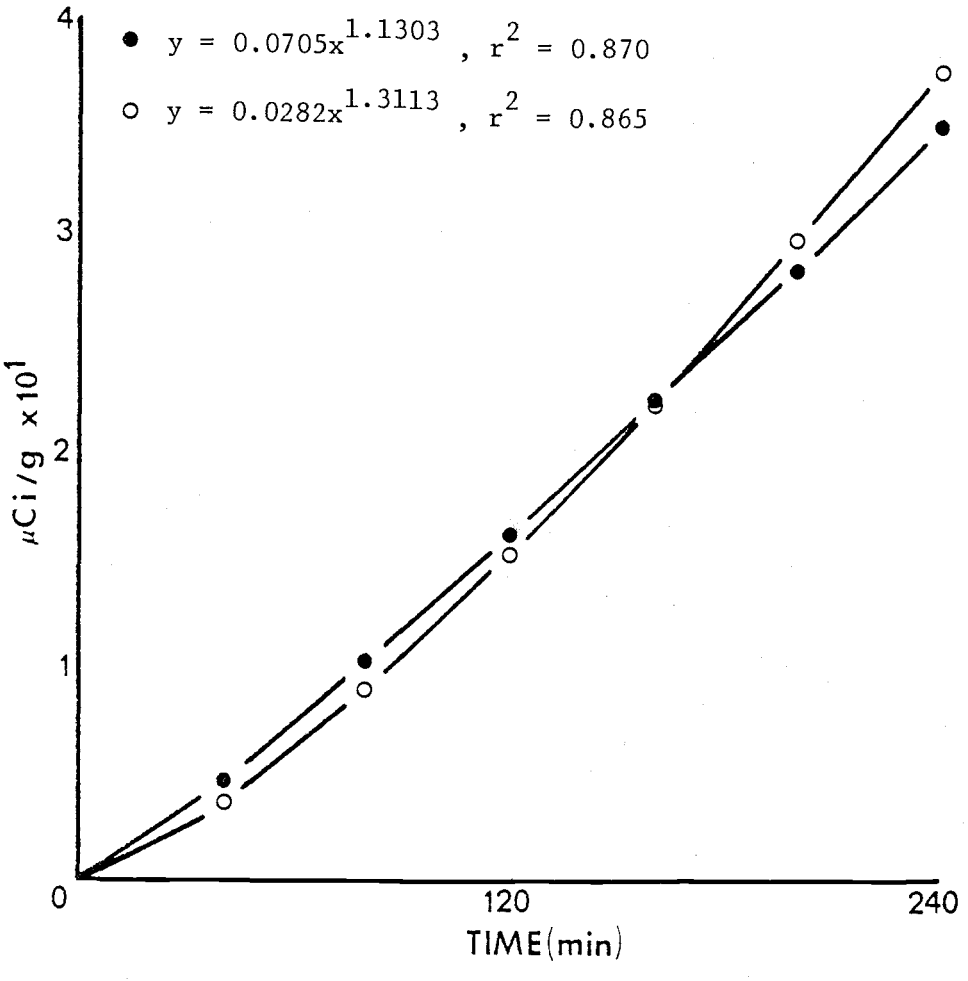

Figure 23. Regression of the incorporation of 14C-label into the total end products and intermediates of glucose metabolism for control (○) and naphthalene-treated (●) oysters. Values expressed per g dry wt.

to this time and less after 160 min. In the former case however, the two regressions were not significantly different at the $\alpha < .05$ level.

Effects of Naphthalene on Carbon Flow into the Intermediates and End Products of Glucose Metabolism

The t statistic for two means was used as the test statistic for evaluating the effects of naphthalene treatment on the pool sizes of the end products and intermediates from runs R-1, R-2 and R-3. The pool sizes for Nt and Ct oysters from R-1 were assumed to be comparable, despite the reduced temperature during the glucose uptake experiment with the control oysters. This assumption was based on the fact that the temperature effect was primarily a rate effect and that the rate differences over the short period of the glucose uptake experiment were insufficient to affect the absolute pool size which was a result of the carbon flux during the prior 72-hr period in the flow system. The pool sizes during the glucose incubation period (< 4 hr) were assumed to be in the steady-state. The steady-state assumption was verified for all pools except the amino acids Ala and Asp. There was no apparent net change in the pool sizes during the sampling period except as noted for Ala and Asp, which will be discussed later.

The mean total pool size (the average concentration for all Nt runs) for proteins and polar lipids was greater for the Ct oysters than the Nt oysters ($P < .01$ and $P < .005$, respectively) (Table 11). In the case of total proteins this difference was

Table 11. Comparison of the percent glycogen, protein, neutral lipids, and polar lipids in the gills of control (Ct) and naphthalene-treated (Nt) oysters.

Run		Glycogen		Protein		Neutral lipids		Polar lipids	
		Ct	Nt	Ct	Nt	Ct	Nt	Ct	Nt
1	% of dry wt	5.31	5.68	59.10	58.31	4.30	4.20	5.03	4.95
	95% C.I.	±0.21	±0.37	±2.44	±1.92	±0.23	±0.24	±0.26	±0.14
	n	8	8	8	8	8	8	8	8
2	% of dry wt	5.61	5.16	63.13	54.69	4.03	4.41	5.36	4.91
	95% C.I.	±0.45	±0.66	±2.54	±4.25	±0.25	±0.24	±0.08	±0.21
	n	8	8	8	8	7	8	7	8
3	% of dry wt	5.55	5.56	61.88	60.19	5.13	4.90	5.41	5.01
	95% C.I.	±0.46	±0.42	±2.86	±4.09	±0.26	±0.34	±0.28	±0.22
	n	8	8	8	8	8	8	8	8
Totals	% of dry wt	5.49	5.47	61.38	57.96	4.50	4.50	5.26	4.96
	95% C.I.	±0.23	±0.30	±1.66	±1.95	±0.25	±0.20	±0.15	±0.11
	n	24	24	24	24	23	24	23	24
	t*[a]	0.135		2.694		0.001		3.331	
	P	>.500		<.010		>.500		<.005	

[a]Symbols: t* = computed value of the t statistic for testing the hypothesis that the difference in the mean values were equal to zero for Nt and Ct oysters; P = probability that there are no differences; negative t* values indicate that the Ct mean was less than the Nt mean.

attributable primarily to R-2, but the protein concentration was consistently greater in the Ct oysters for all runs although the differences were not statistically significant. Similarly, the difference in mean polar lipids was significantly greater only in R-2 and R-3 but was also higher for Ct oysters in R-1.

There were no statistically significant differences for glycogen or neutral lipids at the $\alpha < .05$ level.

The total percent lipids (neutral and polar) increased consistently between runs: R-1 = 9.33 and 9.15; R-2 = 9.39 and 9.32; and R-3 = 10.44 and 9.91 for Ct and Nt respectively. These values correlated with the consistently increasing naphthalene bioaccumulation factors observed in Table 3 and may have been associated with starvation effects.

The mean Ala, Asp, and Glu concentrations (sum of Ala, Asp, and Glu concentrations) in the Dow 50 eluate decreased significantly between each run; R-1 vs R-2, $P < .02$; R-2 vs R-3, $P < .001$. The decrease in the free amino acid pool during starvation was a typical manifestation in oysters (Riley, 1976). The difference between the mean Ala, Asp, and Glu concentrations for the Nt and Ct oysters for the individual runs were not significant at the $\alpha < .05$ level (Table 12), although the Nt oysters had consistently higher levels of amino acids than the Ct. Because of the starvation effect, direct comparison of the mean total Ala, Asp, and Glu concentrations for the pooled runs (R-1 + R-2 + R-3 Nt vs R-1 + R-2 + R-3 Ct) was not directly possible. The data was transformed by expressing the concentration for each oyster (x) during each run as a percent

Table 12. Comparison of the total alanine, aspartate, and glutamate in the Dow 50 eluate from the gills of control (Ct) and naphthalene-treated (Nt) oysters.[a]

	R-1		R-2		R-3	
	CT	Nt	Ct	Nt	Ct	Nt
$\bar{x}$	49.1	54.2	37.6	40.8	19.0	22.5
95% C.I.	±4.4	±8.3	±12.3	±12.9	±4.3	±3.8
n	8	8	8	8	7	8
t*[b]	-1.25		-0.42		-1.40	
P	<.40		>.50		<.20	

[a]Values expressed as total μM/g dry wt/n.

[b]Symbols: t* and P same as in Table 10.

of the mean concentration for that run:

$$x_{transformed} = (x/((x_{Nt} + x_{Ct})/(n_{Nt} + n_{Ct})))(100)$$

The validity of this transformation was verified by the fact that the calculated t statistics for the comparison of Nt and Ct oysters for each run (Table 12) were exactly the same whether the data were transformed or not. The comparison of the pooled treatments (total mean Nt vs total mean Ct) was not significant at the $\alpha < .05$ level, $(.20 < P < .10)$.

Glutamate was consistently the most concentrated amino acid and also the least variable (Table 13). With the exception of the control oysters in R-1, Asp was the next most concentrated and Ala the least. In R-1, Ala was significantly greater in the Ct oysters and Asp significantly greater in the Nt oysters, $P < .025$ and $P < .010$ respectively. In all other treatments and runs, the relative concentrations were not significantly different at the $\alpha < .05$ level. The relative Ala and Asp concentrations were consistently more variable than the relative Glu concentration. A plot of the ratio of Ala:Glu normalized to 1.0 and divided by the ratio of Asp:Glu normalized to 1.0, revealed that the reason for the variability of Ala and Asp was that the Ala concentration was decreasing with time while the Asp concentration was increasing (Figure 24). The pooled regression, utilizing a logarithmic model, revealed that there was no significant difference between Ct and Nt oysters at the $\alpha < .05$ level $(.05 < P < .10)$.

Table 13. Comparison of the relative concentrations of alanine, aspartate and glutamate expressed as a percent of the total alanine, aspartate and glutamate from the Dow 50 eluate for control (Ct) and naphthalene-treated (Nt) oysters.

	R-1						R-2						R-3					
	CT			Nt			Ct			Nt			Ct			Nt		
	Ala	Asp	Glu	Ala	Asp	Glu	Ala	Asp	Glu	Ala	Asp	Glu	Ala	Asp	Glu	Ala	Asp	Glu
$\bar{x}$	33.2	22.5	44.3	23.5	32.3	44.2	19.9	31.4	48.7	19.9	29.5	50.6	19.7	29.9	50.4	18.5	31.8	49.9
95% C.I.	±7.2	±5.5	±2.5	±3.3	±5.0	±3.2	±7.1	±9.0	±4.3	±6.6	±7.1	±5.1	±2.9	±8.5	±6.1	±3.3	±8.2	±7.4
C.V.[a]	21.2	24.4	5.6	14.0	15.5	7.2	35.7	28.7	8.8	33.2	24.0	10.1	14.7	28.4	12.1	17.8	26.3	14.8
n	8	8	8	8	8	8	8	8	8	8	8	8	7	7	7	8	8	8
t*[b]	2.88-3.07		0.00				0.01	0.38-0.66					0.61-1.08		0.14			
P	<.20	<.01	>.50				>.50	>.50	>.50				>.50	<.40	>.50			

[a] C.V. = coefficient of variation = (s/x) 100.

[b] Symbols: t* and P same as in Table 10; value in each column is value calculated from the comparison of the means of the two treatments

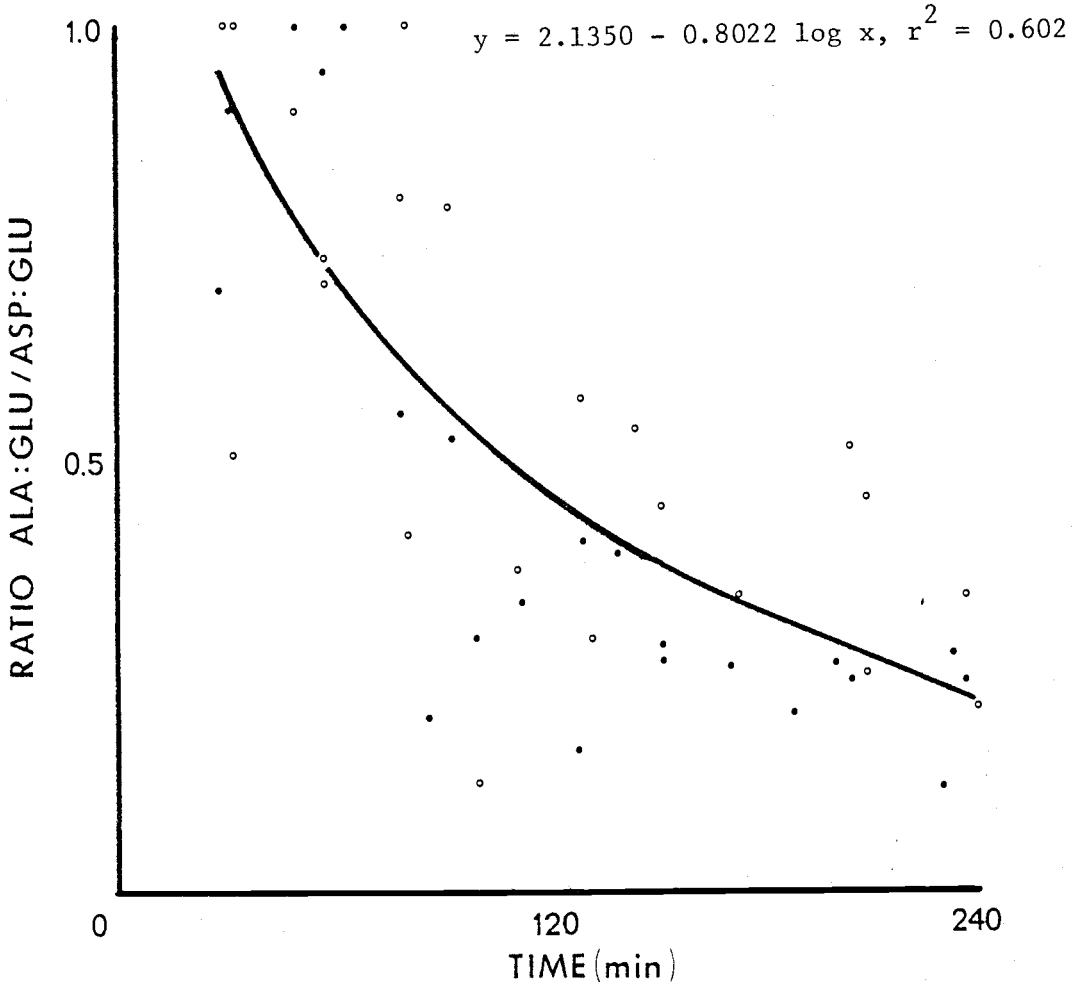

Figure 24. Regression analysis of the changes in the Ala:Glu/Asp:Glu ratio during the glucose incubation period. (∘) control; (•) naphthalene-treated.

The mean Dow 1 acids for the R-2 and R-3 control oysters were significantly different, $P < .01$. There was a consistent decrease in the mean Dow 1 organic acids, for each treatment between each run; again, a possible starvation effect. The difference between the mean organic acids for Ct and Nt oysters for each run were not significant at the $\alpha < .05$ level (Table 14). However, the Nt oysters had consistently higher levels of organic acids. As with the amino acids, the starvation effects described above prevented a direct comparison of the mean total organic acids for the pooled runs. The data were transformed as described for amino acids and retested. Again, as with the amino acids, the difference was not significant at the $\alpha < .05$ level ($.05 < P < .10$). The differences that did exist were attributed mainly to the differences in R-3.

With the exception of the CT oysters from R-1, the mean free reducing sugars (neutral compounds) from the Dow 1 wash were not significantly different between runs or treatments (Table 15). The free reducing sugars in the Ct oysters from R-1 were significantly higher than any other run or treatment, $P < .0001$. Since the system was primed with 1 mM glucose it was possible that the reduced carbon flux attributable to the reduced incubation temperature for control oysters could account for the higher levels of reducing sugars in the Ct oysters in R-1.

Taurine was the major free amino acid in gill tissues. It was 3 to 8 times more abundant than the total concentration of Ala, Asp, and Glu. Variations in the taurine concentration from sample to sample were very large (Table 16) and the mean taurine

Table 14. Comparison of total acids recovered from the Dow 1 eluate from the gills of control (Ct) and naphthalene-treated (Nt) oysters.[a]

	R-1		R-2		R-3	
	Ct	Nt	Ct	Nt	Ct	Nt
$\bar{x}$	0.199	0.219	0.188	0.198	0.117	0.156
95% C.I.	±0.039	±0.058	±0.034	±0.039	±0.029	±0.046
n	7	8	8	7	8	8
t*[b]	-0.63		-0.45		-1.62	
P	>.50		>.50		<.20	

[a]Values expressed as total Meq/g dry wt/n.

[b]Symbols: t* and P same as in Table 10.

Table 15. Comparison of the total free reducing sugars in the Dow 1 wash from the gills of control (Ct) and naphthalene-treated (Nt) oysters.[a]

	R-1		R-2		R-3	
	Ct	Nt	Ct	Nt	Ct	Nt
$\bar{x}$	0.441	0.333	0.333	0.285	0.304	0.336
95% C.I.	±0.019	±0.037	±0.046	±0.092	±0.045	±0.042
n	8	8	8	8	8	8
t*[b]	5.99		1.08		-1.23	
P	<.0001		<.400		<.400	

[a]Values expressed as total % dry wt/n.

[b]Symbols: t* and P same as in Table 10.

Table 16. Comparison of the total taurine in the Dow 1 eluate from the gills of control (Ct) and naphthalene-treated (Nt) oysters.[a]

	R-1		R-2		R-3	
	Ct	Nt	Ct	Nt	Ct	Nt
$\bar{x}$	164.2	205.9	321.9	250.0	153.8	158.2
95% C.I.	±42.9	±52.8	±144.6	±45.9	±48.1	±69.2
n	8	8	8	8	8	8
t*[b]	-1.41		1.07		-0.12	
P	<.200		<.400		>.500	

[a]Values expressed as total μM/g dry wt/n.

[b]Symbols: t* and P same as in Table 10.

concentration exhibited no consistent difference between runs. The difference between the mean total taurine concentration for Nt and Ct oysters for each run was not significant at the $\alpha < .05$ level. The oysters from R-2 had considerably higher levels of taurine than either R-1 or R-3.

Only 15.2% (s = 4.2, n = 32) of the original 14C-label that was lost from the media, was recovered in the gill tissue (Ct and Nt, R-2 and R-3). The flow of 14C-label was directed primarily into metabolic end products. Total protein accounted for 5.63% (s = 2.94, n = 48), ethanol insoluble polysaccharides 62.30% (s = 9.99, n = 48), total neutral lipids 0.03% (s = 0.02, n = 47), and total polar lipids 0.2% (s = 0.13, n = 47) of the total recovered 14C-label. The intermediates, amino acids and organic acids, accounted for 9.16% (s = 4.81, n = 48) and 5.40% (s = 4.60, n = 48) respectively. Neutral compounds, primarily glucose, accounted for 17.38% (s = 12.16, n = 48).

In general, the percent activity in end products increased with time while for neutral compounds (primarily glucose), there was a concomitant decrease. The intermediates neither increased nor decreased (Figure 25).

Paper chromatography of the neutral compounds revealed that all the label was present in two well-separated compounds, one of which was identified as glucose, based on the R_f value. The unidentified compound separated considerably below glucose (Figure 26). Within the neutral compound pool the glucose activity increased with time while the activity in the unidentified compound

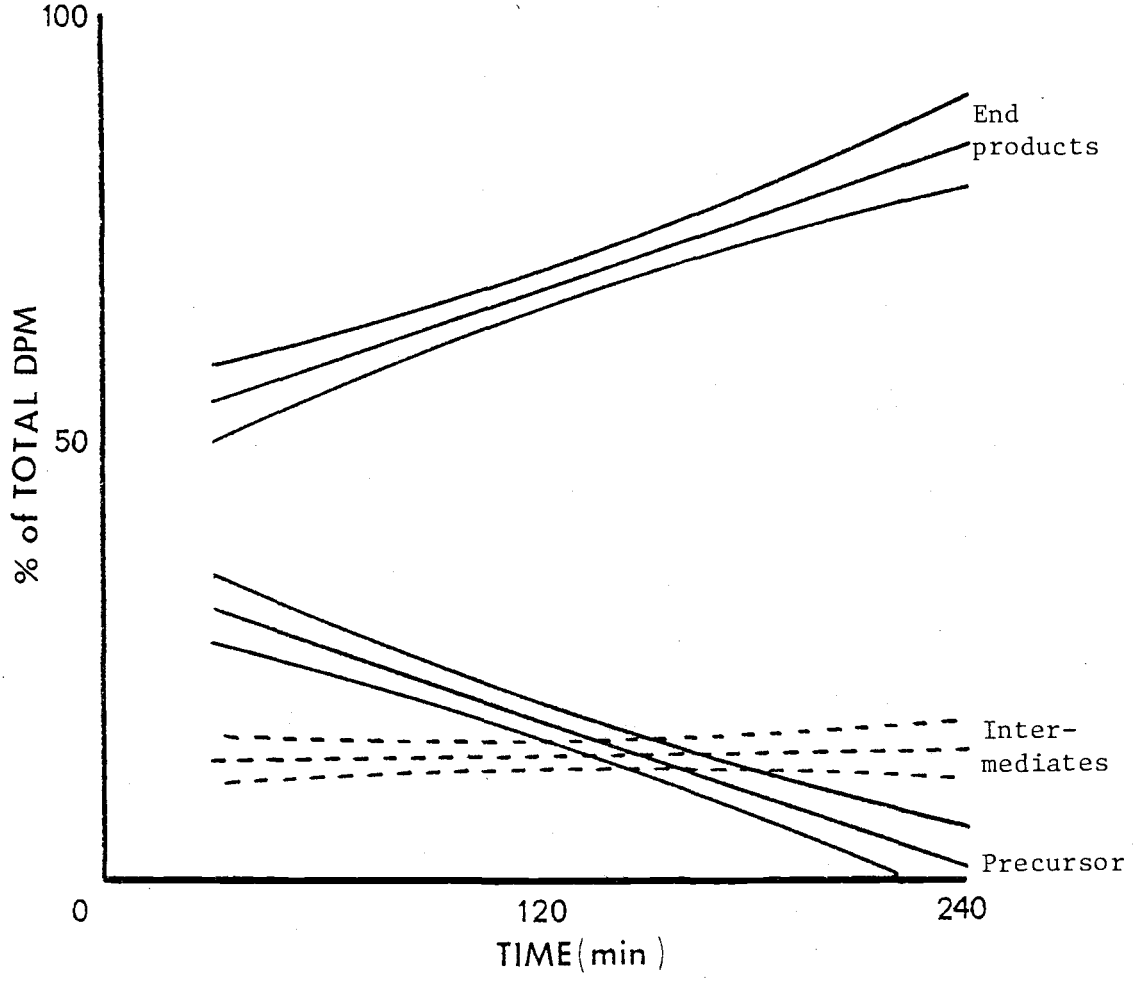

Figure 25. Regression of the changes in the percent 14C-label incorporated into end products, and intermediates, and the 14C-label loss from the precursor pool (neutral compounds). The curves are fitted to a first-order model ± 95 C.I.

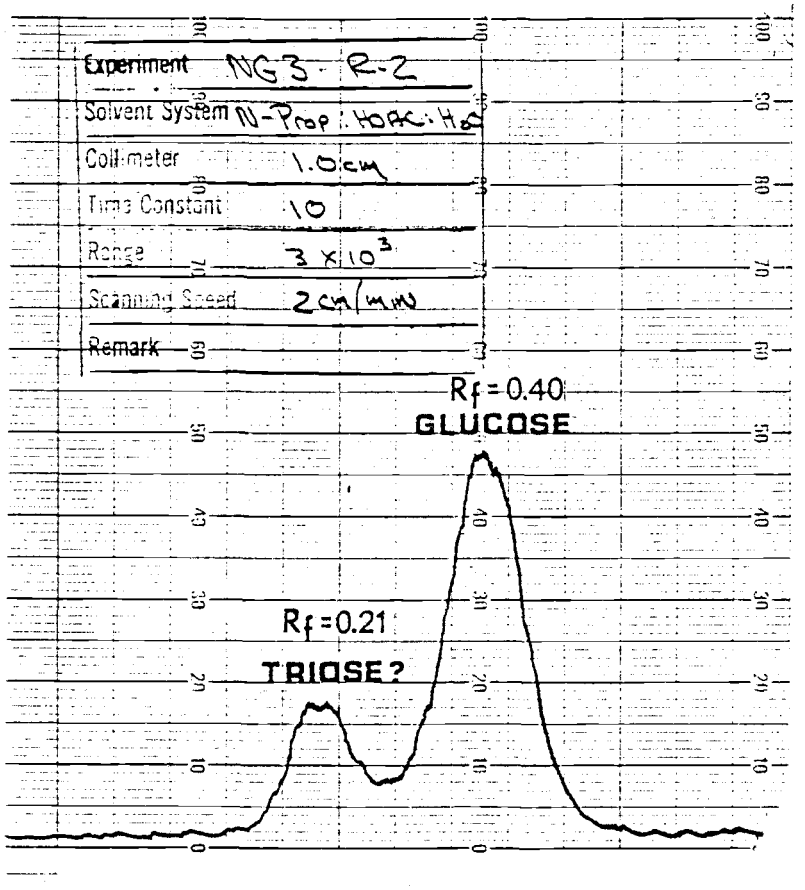

Figure 26. Radiochromatogram scan of the paper chromatography separation of the neutral compounds fraction showing glucose and an unidentified neutral compound, possibly a triose sugar.

increased (Figure 27). This suggested that the unidentified compound was in fact a product of glucose metabolism. Unfortunately only 17 samples were analyzed by paper chromatography and comparison between Nt and Ct oysters for each run was not possible because of the small sample size.

Almost all of the activity from the Dow 50 eluate was recovered in Ala, Asp, and Glu ($\bar{x}$ = 96.8%, 95% C.I. = ± 5.0, n = 47).

TLC of the Dow 1 eluate revealed that the majority of the activity was in unidentified organic acids and taurine ($\bar{x}$ = 71.56% ± 5.45, 95% C.I., n = 16). Malate and succinate were the only Krebs cycle intermediates that were consistently labeled in detectable quantities ($\bar{x}$ = 2.11% ± 0.79, 95% C.I., n = 15) (Figure 28).

Specific radioactivity-time curves for total protein, ethanol insoluble polysaccharides (primarily glycogen), total neutral lipids, total polar lipids, free alanine, aspartate, and glutamate, were determined (Figures 29 to 35). Radioactivity-time curves expressed as activity/Meq acid were also determined for malate and succinate (Figures 36 and 37).

In principle, the flow of 14C-label from precursor to product, after a single administration of the precursor, is a linear process (Berman, 1969). There is a linear dependence of enzymatic velocities on substrate concentration. When an organism is in the steady-state, the enzyme velocities between precursors and products should, theoretically, be constant and the incorporation of

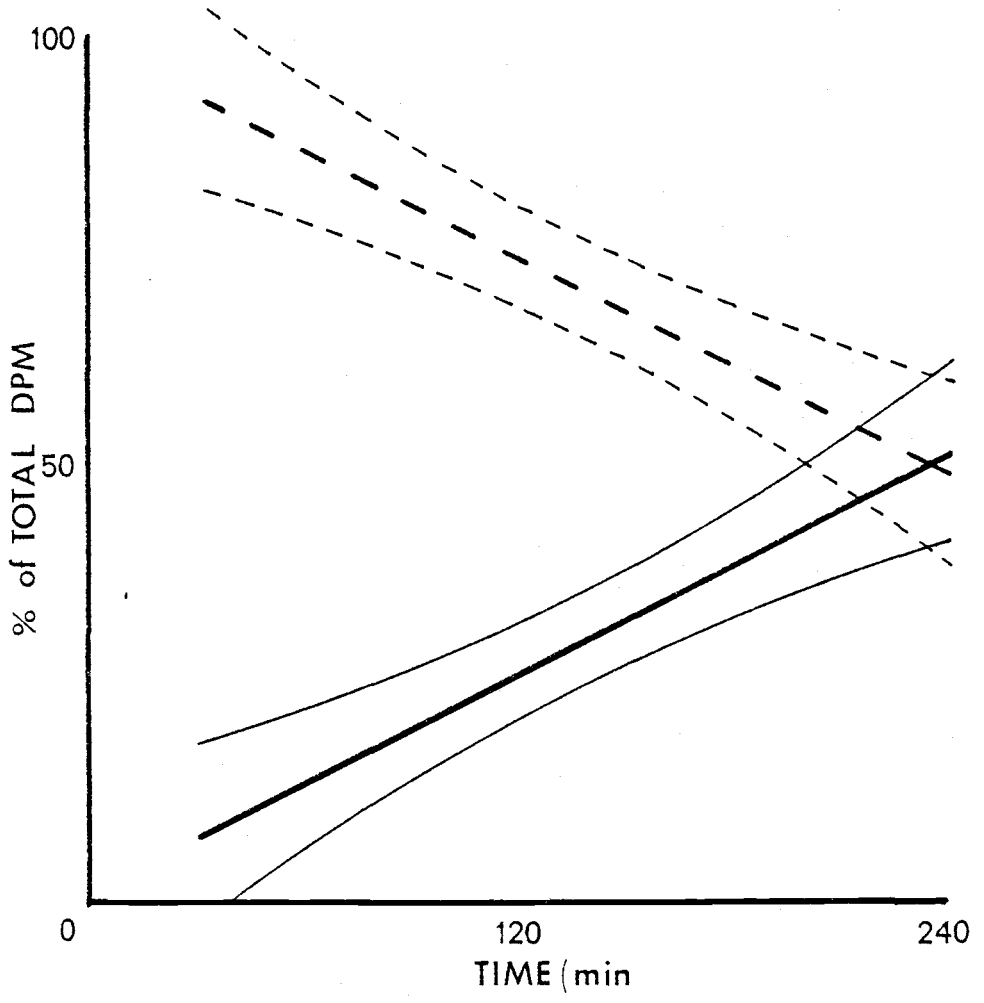

Figure 27. Regression of the change in the percent 14C-label incorporated into glucose (dashed lines) and an unidentified neutral compound (solid lines) in the neutral fraction. The curved lines are the 95% C.I.

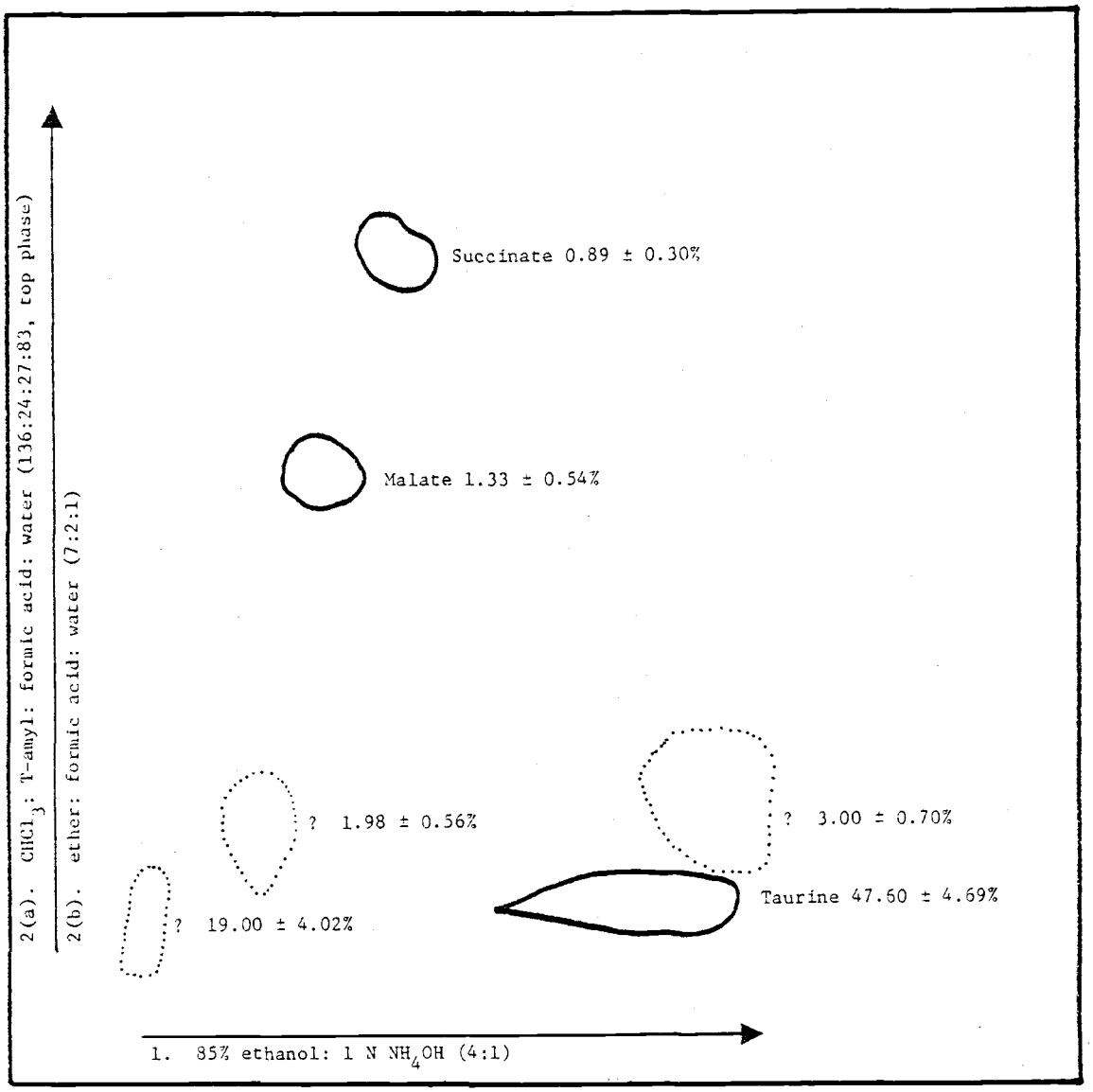

Figure 28. Two-dimensional TLC of a Dow 1 eluate showing mean ± 95% C.I. recovery of activity in succinate (n = 15), malate (n = 15), and taurine (n = 16), and three unidentified organic acids (n = 16) from the oyster gill.

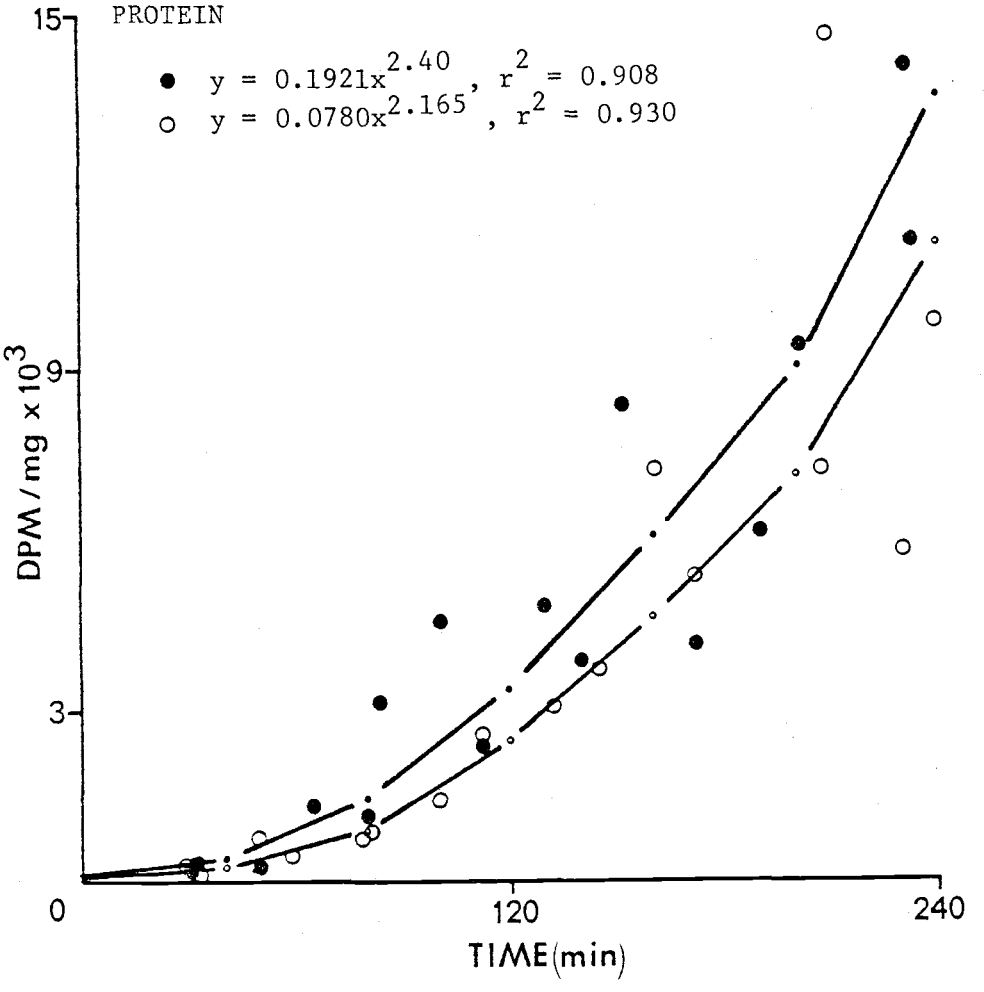

Figure 29. Specific activity-time curve for total protein expressed per mg dry wt in BSA equivalents. (○) control; (●) naphthalene-treated.

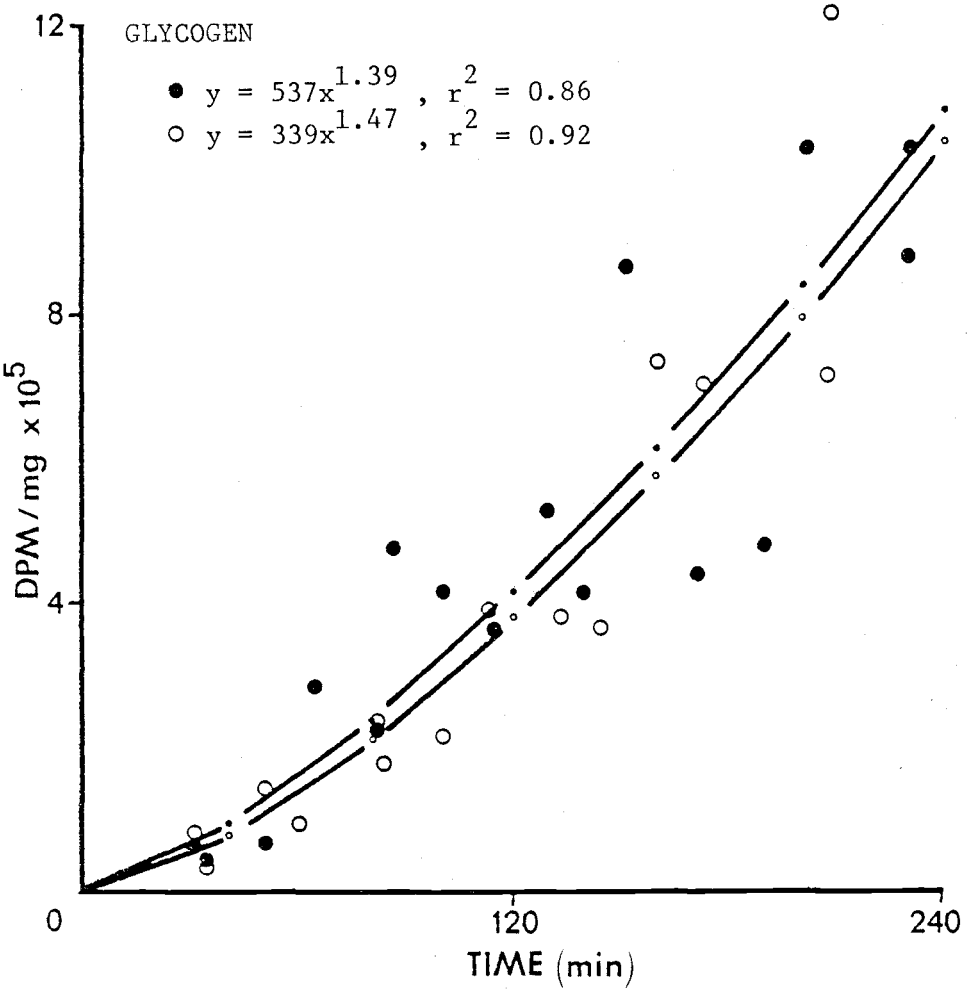

Figure 30. Specific activity-time curve for total ethanol insoluble polysaccharides (glycogen) expressed per mg dry wt in glucose equivalents. (○) control; (●) naphthalene-treated.

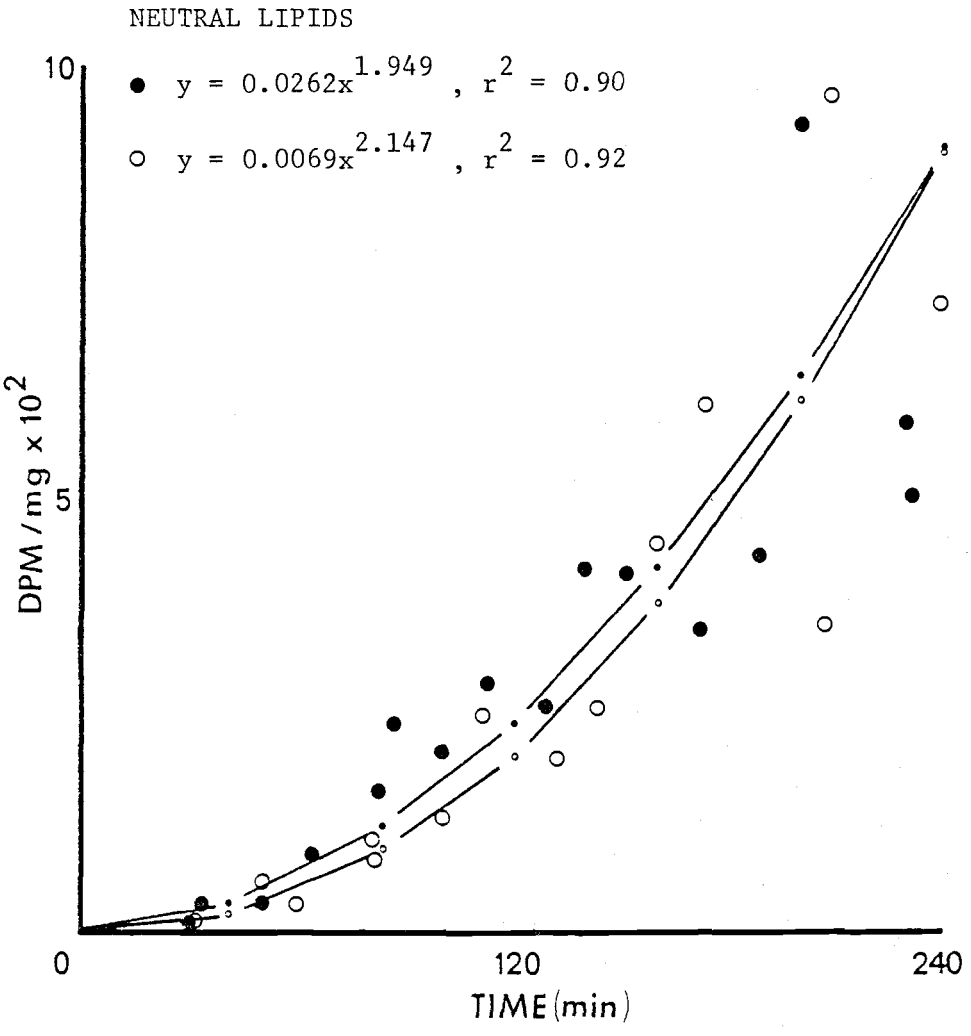

Figure 31. Specific activity-time curve for total neutral lipids expressed per mg dry wt in tripalmitin equivalents. (○) control; (●) naphthalene-treated.

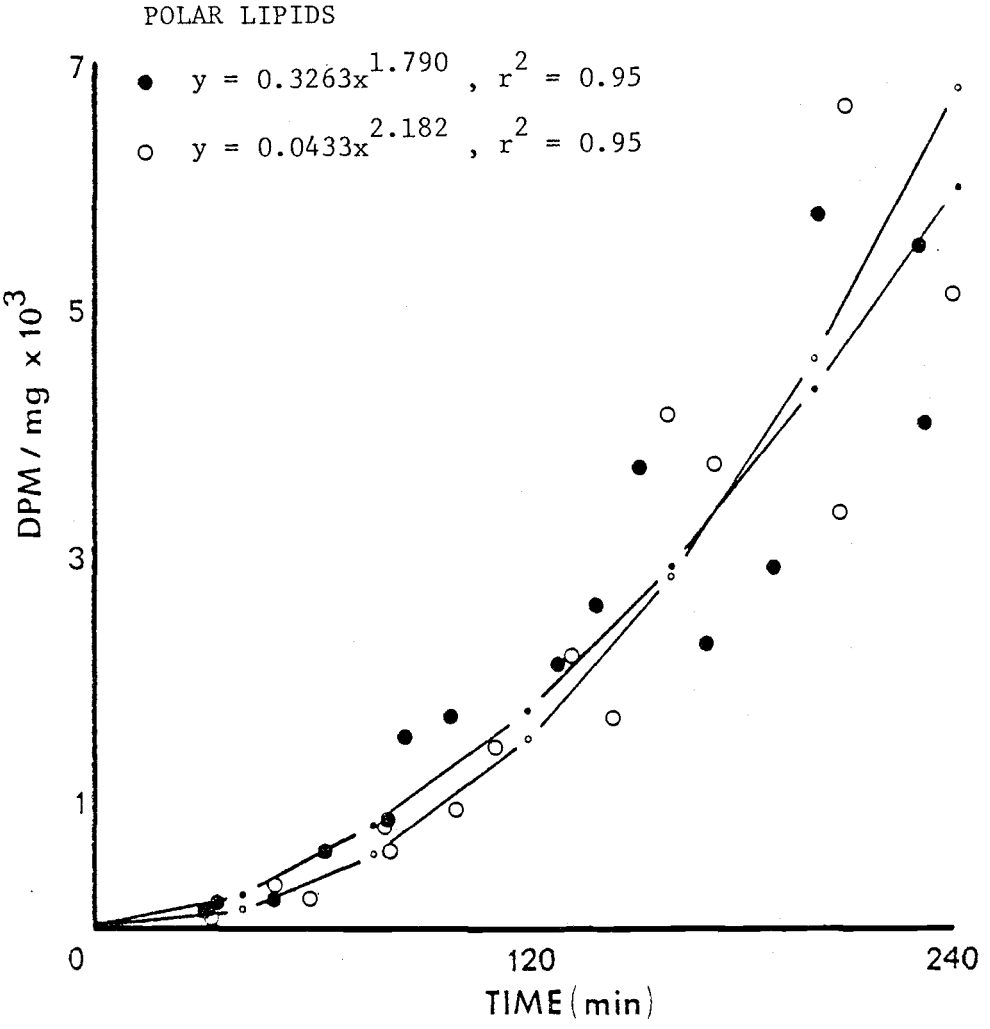

Figure 32. Specific activity-time curve for total polar lipids expressed per mg dry wt in tripalmitin equivalents. (○) control; (●) naphthalene-treated.

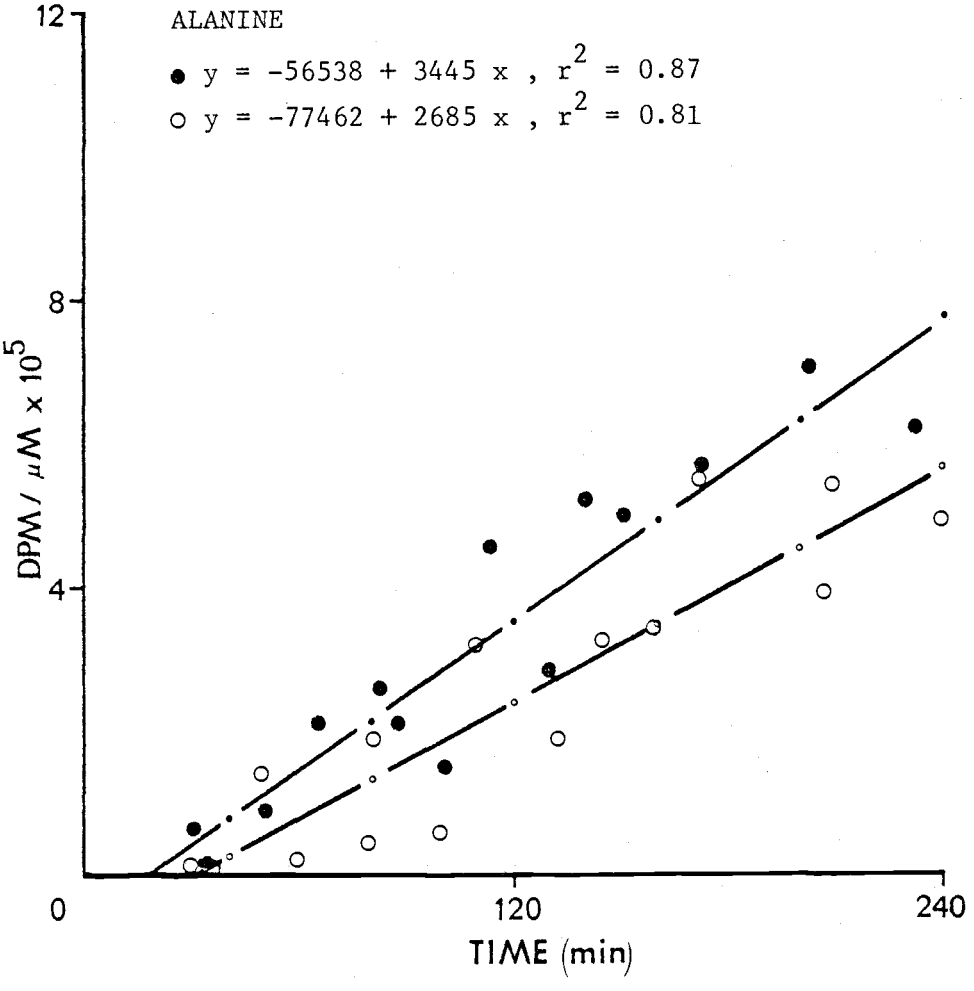

Figure 33. Specific activity-time curve for alanine expressed per μM alanine. (○) control; (●) naphthalene-treated.

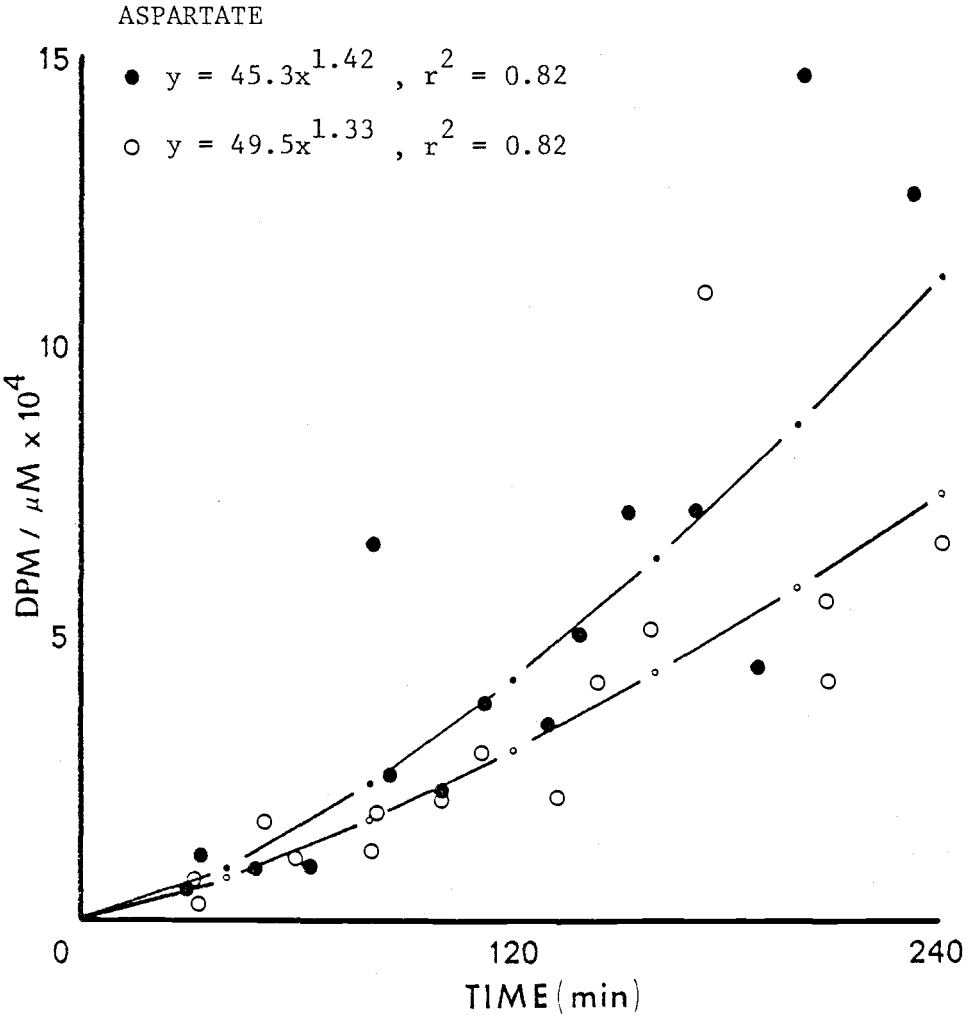

Figure 34. Specific activity-time curve for aspartate expressed per μM aspartate. (○) control; (●) naphthalene-treated.

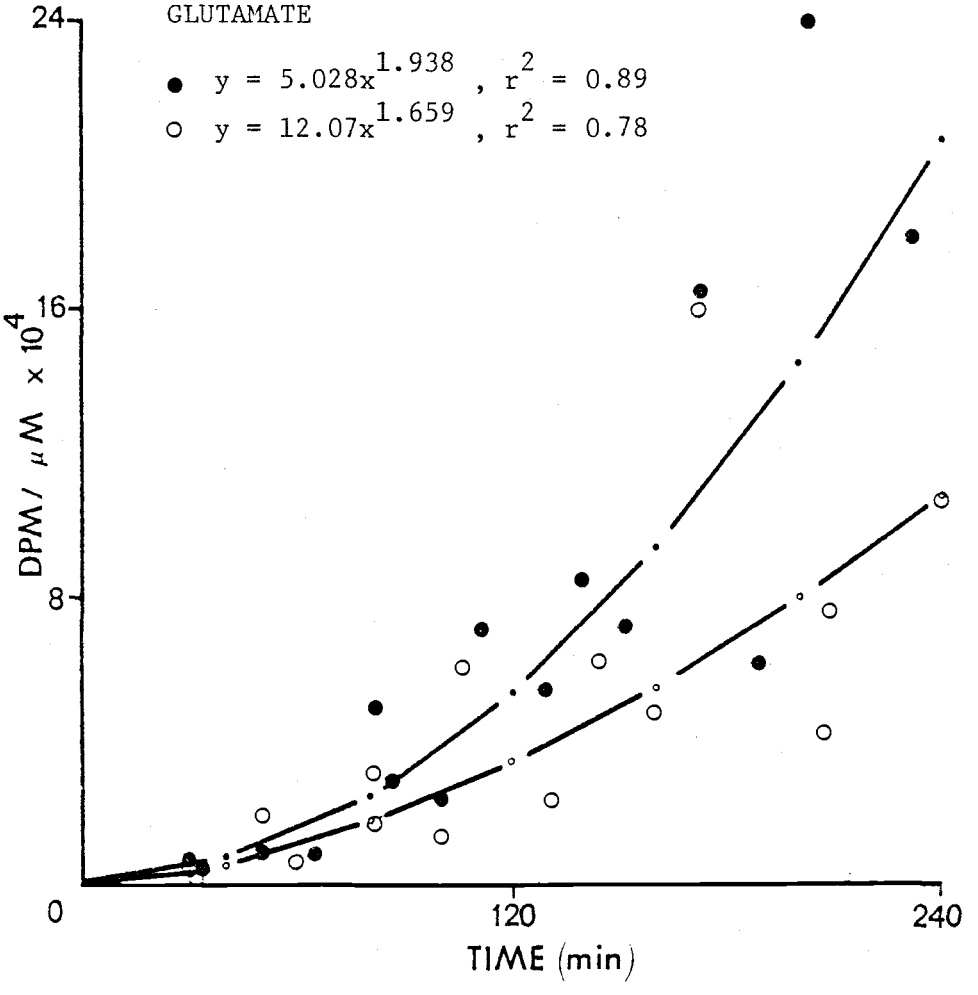

Figure 35. Specific activity-time curve for glutamate expressed per μM glutamate. (○) controls; (●) naphthalene-treated.

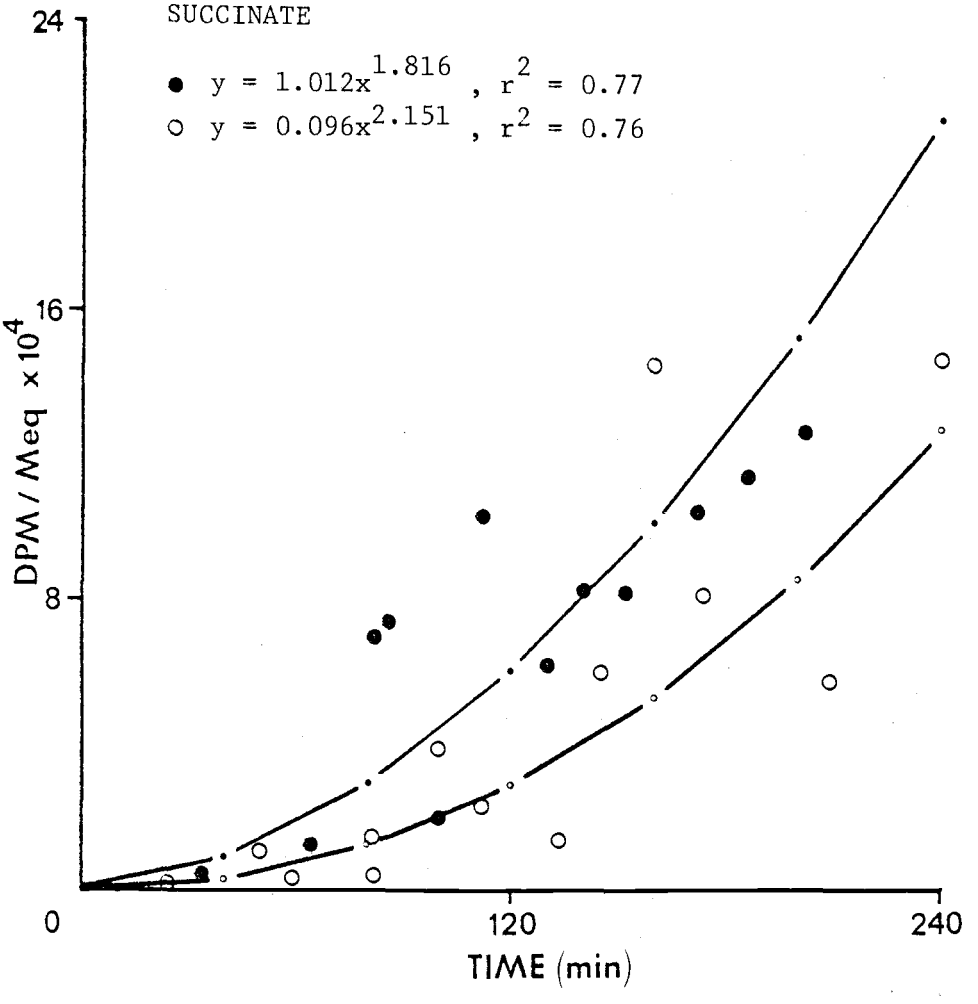

Figure 36. Radioactivity-time curve for succinate expressed per milli-equivalent of total acid in the Dow 1 eluate. (○) control; (●) naphthalene-treated.

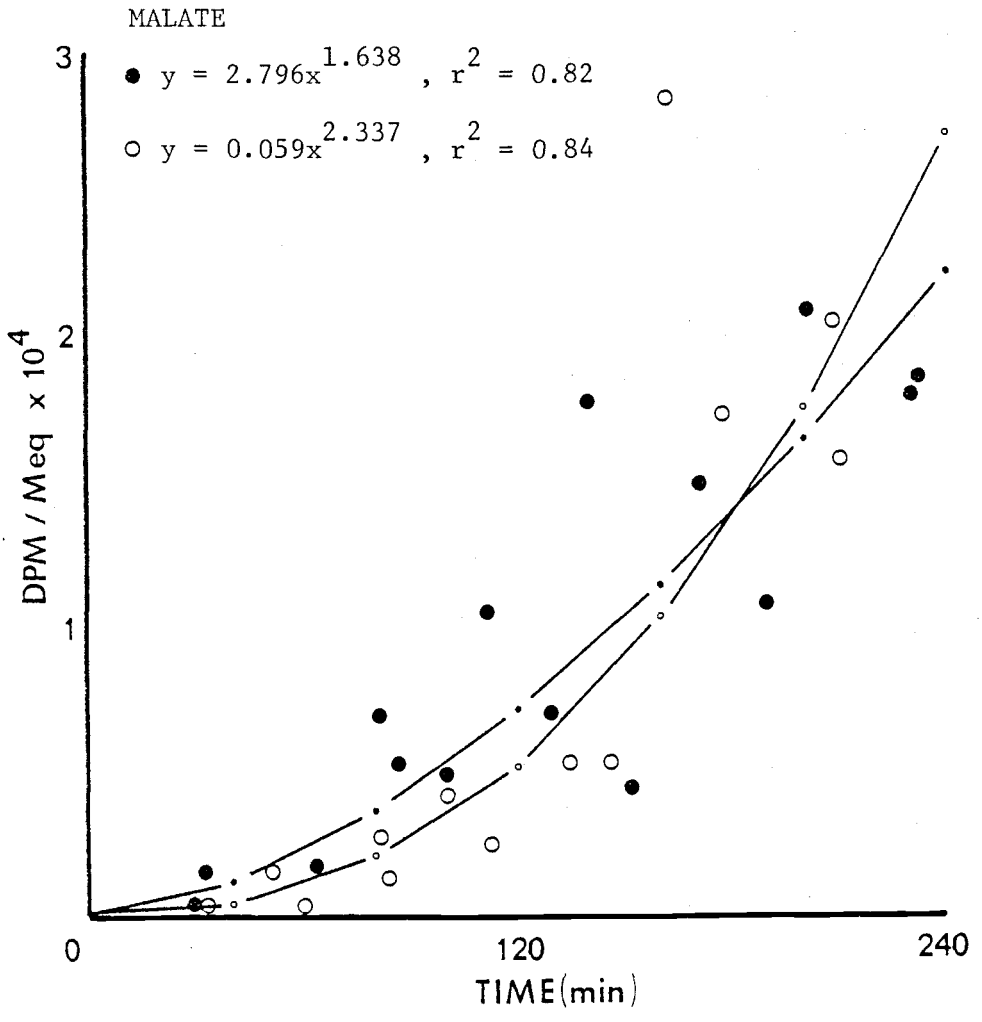

Figure 37. Radioactivity-time curve for malate expressed per milli-equivalent of total acid in the Dow 1 eluate. (○) control; (●) naphthalene-treated.

14C-label precursor into the observed pools, should follow a first-order model (Connett and Blum, 1971). However, the linear nature of this process can be observed easily only if samples are taken prior to extensive recycling and loss of the 14C-label (Campbell, 1975). The main purpose of this study was to compare the specific activity-time curves for the intermediates and end products of 14C-glucose metabolism for control and naphthalene-treated oysters while the incorporation of 14C-label was increasing with respect to time.

The specific activities of total protein, glycogen, neutral lipids, polar lipids, alanine, aspartate and glutamate, and the radioactivity per milli-equivalent of total acids for malate and succinate increased in an "intrinsically linear" fashion with respect to time until the final measurement. The specific activity-time curves were "intrinsically linear" (Neter and Wasserman, 1974) since they were usually best fitted to suitable transformations of the linear form. The final specific activity of intermediates and end products was often considerably less than expected for a linearly related process. The decrease was due primarily to the decrease in the 14C-label activity in the precursor pool coupled with catabolism of labeled products. Regression of the specific activity-time data ignored final sample values which would skew the models away from the linear or "intrinsically linear" condition. A rule was adopted for deciding whether or not the final datum point was to be ignored in the regression: if the final value was less than the value preceding the penultimate value, then the final

datum point was ignored in the regression.

Testing of Ct and Nt regressions for each specific activity-time curve was preceded by testing for the equality of the error variances. The treatments (Nt and Ct) corresponding to the two regression curves were tested to determine if they were identical (Neter and Wasserman, 1974). The test approach was:

1. C_1 : $B_{01} = B_{02}$ and $B_{11} = B_{12}$ (equality of intercepts and slopes)

   C_2 : Either $B_{01} \neq B_{02}$ or $B_{11} \neq B_{12}$ or both (inequality of either intercepts or slopes or both).

2. Fit the full, or unrestricted, model and obtain the error sum of squares (SSE(F)).

3. Obtain the reduced, or restricted, model under C_1, fit it, and determine the error sum of squares (SSE(R)) for the reduced model.

4. Calculate the F^* statistic, which involves the difference SSE(R) - SSE(F). The greater the difference, the more the data support C_2; the smaller the difference, the more the data support C_1.

The comparison of regression parameters was also done but in this case an indicator variable was used with a design matrix as follows:

Design Matrix

	B_{01}	B_{11}	B_{02}	B_{12}
Treatment 1	1	x_{1j}	0	0
Treatment 2	1	x_{2j}	1	x_{2j}

Treatment 1 $Y = B_{01} + B_{11}x$ = reduced regression

Treatment 2 $Y = B_{01} + B_{02} + (B_{11} + B_{12})x$ = Nt regression

The resulting coefficients were then selectively added or dropped from the two models and the entering and departing F^* values calculated.

With the exception of alanine, the best fits were obtained using an allometric model. For alanine, the best fit was obtained with a first-order model.

The Nt and Ct regression curves were significantly different in the cases of Ala ($P < .010$), succinate ($P < .025$) malate ($P < .050$), and polar lipids ($P < .05$) (Table 17).

The results of comparing regression parameters indicated that the relationship between the parameters, in those cases where the Nt and Ct regression lines were significantly different, were correlated in a manner that precluded confident interpretation about whether or not the difference in the regression lines was primarily a function of one parameter more than the other.

When sequentially dropping parameters (i.e., drop B_0 followed by drop B_1 or drop B_1 followed by drop B_0) without adding back the

Table 17. The results of comparing the two regression lines (Ct vs Nt) and the regression parameters of the specific activity-time curves for the intermediates and end products of glucose metabolism in the gill of the oyster.

Action	Probability[a]								
	Protein	Glycogen	Neutral lipids	Polar lipids	Ala	Asp	Glu	Succinate	Malate
Treatment 1[b] add, B_{02} and B_{12}	> .100	> .100	> .100	< .050	< .010	> .100	< .100	< .025	< .050
d.f.	2,26	2,28	2,26	2,26	2,25	2,26	2,26	2,21	2,24
Treatment 1 add, B_{02}	< .050	> .100	> .100	> .100	< .005	< .050	< .050	< .010	< .100
d.f.	1,27	1,29	1,27	1,27	1,26	1,27	1,27	1,22	1,25
Treatment 1 add, B_{12}	< .050	> .100	> .100	> .100	< .001	< .050	< .050	< .010	> .100
d.f.	1,27	1,29	1,27	1,27	1,26	1,27	1,27	1,22	1,25
Treatment 2 drop, B_{02}	> .100	> .100	> .100	< .025	> .100	> .100	> .100	> .100	< .050
d.f.	1,26	1,28	1,26	1,26	1,25	1,26	1,26	1,21	1,24
followed by drop, B_{12}	< .050	> .100	> .100	> .100	< .001	< .050	< .050	< .010	> .100
d.f.	1,27	1,29	1,27	1,27	1,26	1,27	1,27	1,22	1,25
Treatment 2 drop, B_{12}	> .100	> .100	> .100	< .050	> .100	> .100	> .100	> .100	< .100
d.f.	1,26	1,28	1,26	1,26	1,25	1,26	1,26	1,21	1,24
followed by crop, B_{02}	< .050	> .100	> .100	> .100	< .005	< .050	< .050	< .010	< .100
d.f.	1,27	1,29	1,27	1,27	1,26	1,27	1,27	1,22	1,25

[a] Probabilities are based on the entering and departing F^* values for the various actions as outlined in the first column.

[b] The values given in this row compare whether or not the two regression lines were different by testing the alternative conclusion C_1 and C_2 given in the text.

first parameter, the results indicated that the order in which parameters were dropped determined which parameter best explained the differences in the regression model. An example of this result can be found in comparing the Ala regression lines for Ct and Nt oysters. The overall regressions (add B_{02} and B_{12} to the reduced model) were significantly different ($P < .01$). When B_{02} was added to the reduced model, the results indicated that the Ct and Nt intercept values were significantly different ($P < .005$). When B_{12} was added to the reduced model the results indicated that the slopes were significantly different ($P < .001$). When B_{02} was dropped from the Nt regression followed by dropping B_{12}, the results indicated that only the slopes were significantly different ($P < .001$). When B_{12} was dropped followed by dropping B_{02}, the results indicated that only the intercepts were significantly different ($P < .005$). These ambigious results suggest that the relationships between the parameters is complex and that they are interdependent. No explanation of the regressions based on only one parameter would be sufficient to explain the differences in the regression lines.

For total protein, aspartate and glutamate, the Nt and Ct regression lines were not significantly different but comparison of the regression parameters indicated that it was possible differences did exist. Visual inspection of these regressions would seem to support this conclusion (Figures 29, 34, and 35).

Comparison of the Nt and Ct regression curves and also the regression parameters for polysaccharides and neutral lipids

indicated no significant differences at the $\alpha < .05$ significance level.

The flow of 14C-label into neutral compounds and taurine (Figure 38a and b) did not follow linear patterns. The scatter plots of specific activity against time were too variable to permit meaningful regression. There was no discernable effect of naphthalene treatment in either case. With the exception of R-3 control oysters, the specific activity of the neutral compounds peaked early and then decreased through the rest of the run. Taurine followed a similar pattern but seemed to reach a plateau quickly and then decreased. The control oysters from R-2 showed very high specific activities of taurine.

In order to assess the route of entry of 14C-label into amino acids, the activities associated with Ala, Asp, and Glu before and after development with ninhydrin were compared (Table 18). The low recovery of Ala was expected considering that the resultant product after development (acetalaldehyde) was very volatile at room temperature. Assuming that glutamate and aspartate were formed via transamination reactions with α-ketoglutarate and oxaloacelate respectively, then the recovery of activity (assuming no volatilization) was dependent on the route of entry of 14C-label into these compounds. If the only route of entry into α-ketoglutarate was via the Krebs cycle and the entry of 14C-label into the cycle was by way of acetyl CoA, then at equilibrium, the labeling pattern for α-ketoglutarate should have been random and α-decarboxylation of glutamate should have resulted in a 20% decrease of 14C-activity.

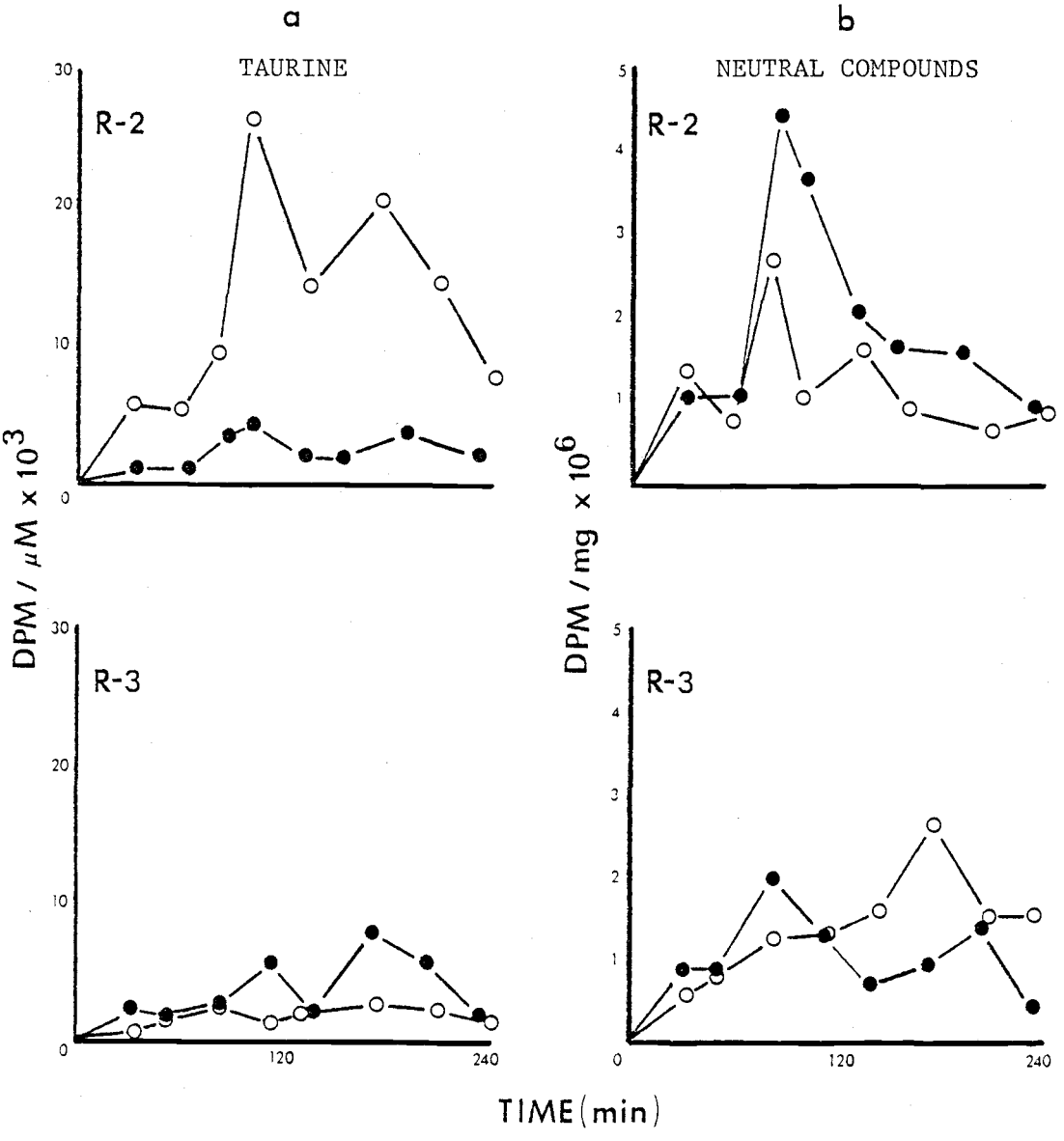

Figure 38. Changes in the specific activity in taurine (a) and neutral compounds (b) for runs R-2 and R-3. Taurine expressed per μM taurine and neutral compounds per mg glucose equivalent. (○) control; (●) naphthalene-treated.

Table 18. The percent radioactivity remaining in alanine, aspartate, and glutamate after development with ninhydrin.

	Ala	Asp	Glu
$\bar{x}$	16.9	64.9	80.3
95% C.I.	±2.4	±4.3	±3.9
n	8	17	17

That was precisely what was observed. Aspartate on the other hand should have been reduced 25%. It was in fact reduced considerably more, suggesting that aspartate was not randomly labeled but was labeled more in the α-carboxyl group than the other carbons.

IV. DISCUSSION

Oysters were chosen as the experimental animal because of their sedentary nature, ubiquitous distribution and economic importance. Too, there is a substantial amount of information available on the uptake and effects of numerous chemical pollutants (Malins, 1977) on oysters, and on their physiology and metabolism (Galtsoff, 1964; Hammen, 1969; Joyce, 1972). Mussels, especially M. edulis, are probably the only bivalves for which there is more metabolic information presently available (Gabbott, 1976).

Clutchless O. edulis were selected because they had very uniform shell morphology; the valves were considerably thinner and of a more uniform thickness than in other species. These two factors made the removal of the shell material over the gills and cloaca relatively simple and also reduced the chance of injuring the underlying tissues. The clutchless spat, identical in age, were obtained from a hatchery where small numbers of brood stock were spawned, thereby reducing the amount of diverse genetically-derived metabolic differences. Mussels were not used as test animals because removal of the shell material over the gills invariably injured the underlying mantle tissue. Also, cultured mussel spat was not readily available from local suppliers.

Ostrea species are extremely hardy and can be easily maintained in recirculating synthetic seawater aquaria without any apparent source of nutrition. Ostrea lurida have been held in this laboratory for more than two years with a mortality rate of less

than 1% per year. A final reason for choosing O. edulis was their small size; even at the age of 10 months the overall length was only 2.5-4.0 cm. This permitted the convenience of using smaller incubation vessels and the economy of utilizing lesser amounts of reagents and 14C-labeled compounds than if a larger species had been used.

It is a common strategy of bivalves to close their valves for long periods of time when even mildly disturbed. When shell closure occurs, ventilation ceases, intracellular oxygen is rapidly depleted, the pH decreases due to accumulating organic acids, and the metabolism shifts from a typically aerobic pattern to an anaerobic one (de Zwaan and Wijsman, 1976). Since the purpose of this study was to study the effects of a chemical pollutant on aerobic glucose metabolism it was essential that the intracellular oxygen concentration remain high.

During metabolic studies, when numerous animals are sampled for making measurements of specific metabolically active compounds, it is important that the metabolic rates of the animals be standardized to reduce the variability in the results. Under conditions of constant temperature and activity, the metabolic rate of an animal is to a large extent dependent on its physiological condition and nutritional state. It has long been known that prolonged starvation results in reduced oxygen consumption (Brand, Nolan, and Mann, 1948). Long-term starvation of M. edulis results in the reduction of oxygen consumption to a "standard level" defined as the "minimum metabolic rate compatible with the

maintenance of a functionally integrated organism" (Bayne, 1973b). The standard metabolic rate is a fasting rate which is determined by the catabolic rate of stored energy reserves. This catabolic rate approaches a steady-state asymptotic value after a certain period of starvation. The length of this period is a function of the amount of stored energy reserves (primarily glycogen), and the extent to which gametogenesis is occurring. When active gametogenesis occurs in M. edulis, the decline to the standard rate requires 25-30 days in the absence of food (Bayne, 1973a). In the Pacific oyster, C. gigas, the overall catabolism of the major stored energy reserves (proteins, lipids, and carbohydrates) does not reach a minimum value until between 50 to 125 days after the starvation period commences in spring (Riley, 1976).

The oysters used in the present study were placed in recirculating synthetic seawater aquaria in mid-March (spring) and starved for 67 to 105 days before each run. It was assumed that the oysters were metabolizing at the steady-state "standard level" prior to each run. Because the intent of this study was to compare the flux of glucose carbon into the various intermediates and end products of glucose metabolism, it was necessary to standardize the metabolic rates of the oysters. Glucose was added to the seawater medium to provide a common energy source in an attempt to ensure that the metabolic pathways were also standardized.

The removal of the shell material was an attempt to ensure that the tissues were constantly bathed in naphthalene-containing seawater, that aerobic conditions were maintained intracellularly,

and that the oysters had a common "metabolic objective"; namely, to provide energy for replacement of the removed shell material. As mentioned previously in the Materials and Methods section, oysters which did not show significant new shell growth within 9 days after removing the shell material were not used in the experiments.

The gill was chosen as the tissue for analysis because: (i) it is metabolically the most active (Bass, 1977); (ii) it is the first and primary site of absorption of dissolved organics (Pequignat, 1973); and (iii) it has been shown to be the least affected oyster tissue during starvational stress, in terms of dry wt loss (Riley, 1976).

In previous studies the role of microbes in the tissues and in the experimental systems have been largely ignored. This is generally true for studies concerning the accumulation and metabolism of pollutants and in physiological and metabolic studies on bivalves. In the absence of any precautionary measures, the effect of microbial oxidation of naphthalene (Figure 11) and glucose (Table 2) and the uptake of dissolved oxygen (Table 6) in the flow-through system, were considerable. Even in the presence of streptomycin, microbial activity was evident, although considerably reduced. The ideal experiment would have been conducted in the absence of all microbial activity; however, the only possible way to accomplish this feat would have been to administer massive doses of antibiotics (Bunting, 1978). That was not considered desirable because of the unknown effects of such massive antibiotic doses on

the intermediary metabolism of oysters. Excluding massive antibiotic treatment, the best alternative was to maintain the microbial population at a reduced level. That would allow for the accumulation and maintenance of relatively high levels of unoxidized naphthalene and glucose while contributing only insignificantly to oxygen consumption and to ammonia-nitrogen levels in the flow-through system for each 72-hr run.

The fact that microbial oxidation of naphthalene in static assays occurred only after considerable lag times (Figure 12a), suggests that induction of protein synthesis was a prerequisite to extensive microbial naphthalene oxidation. For this reason, streptomycin, an antibiotic which interferes with bacterial protein synthesis, was chosen to inhibit the bacterial oxidation of naphthalene in the flow-through system. It was evident that streptomycin was effective in reducing the bacterial oxidation of naphthalene both in static assays (Figure 14) and, to a lesser degree, in the flow-through system (Figure 16). However, streptomycin did not eliminate bacteria from the flow-through system (Figure 15). The presence of 1 mM glucose and excreted organics in the seawater made the enriched seawater in the flow-through system an attractive growth medium for microbes.

In static assays, the presence of 1 mM glucose had an inhibitory effect on the microbial oxidation of naphthalene (Figure 12a and b). The percent of the total oxidation of naphthalene to carbon dioxide was reduced (Figure 13a) and the rate of formation of non-carbon dioxide polar metabolites increased

(Figure 13b). A possible explanation for the reduced oxidation was that glucose, being a more easily oxidized substrate, was preferentially utilized. There is no evidence available that would suggest high concentrations of easily oxidized dissolved organics inhibit the microbial oxidation of aromatic hydrocarbons. However, there is considerable evidence supporting the conclusion that bacterial systems preferentially utilize organic compounds based on their ease of oxidation (termed catabolite repression) (Paigen and Williams, 1970).

Microbial oxidation of glucose and naphthalene, and the associated consumption of dissolved oxygen, were reduced in the presence of streptomycin during run R-S_w. Both with and without streptomycin (run R-S), glucose oxidation and dissolved oxygen consumption generally occurred before the passage of seawater through the incubation vessels. However, this was much less evident when streptomycin was present. The dissolved oxygen concentration in the common aerated seawater reservoir for system #1 and #2 (R-S_w) at the final sample interval, was 5.12 ml O_2/l while for system #3 (R-S_o), it was the same as that in the water from the inlet sample port, 1.41 ml/l. The dissolved oxygen concentration in the common seawater reservoir for systems #1 and #2 was higher than the seawater entering the incubation vessels #1 and #2. This observation indicated that microbial activity in the metering burets was probably responsible for the oxygen consumption observed prior to the passage of seawater through the incubation vessels, and that the microbial activity in buret #2

was greater than in #1. The cloudiness of the seawater in metering buret #2 provided further support for this conclusion. These two observations suggested that: (i) the oyster shells were not the sole source of microbial contamination; (ii) the entire system was somehow contaminated by either backflushing from the incubation vessels or when refilling the metering burets; and (iii) since the extent of microbial contamination in buret #2 was much greater than in #1 ($R\text{-}S_w$), perhaps naphthalene enhanced the microbial oxidation of glucose, thus resulting in a larger microbial population. The last suggestion does not fit well with current theories concerning the effects of naphthalenes on microbial glucose oxidation. Lee and Anderson (1977) have recently found that the inhibition of glucose oxidation in natural seawater by oils is a function of the concentration of naphthalenes in the oil. Concentrations of only 40 ppb naphthalenes caused an inhibition of glucose oxidation in excess of 50%. The naphthalene concentration in metering buret #2 was 196 ppb for run R-S.

Both oysters and bacteria contributed to the total glucose oxidized and oxygen consumed in the system. In comparing runs with (R-0, R-1, R-2, R-3) and without ($R\text{-}S_w$) oysters present, the following differences were evident: (i) with oysters present at the final sample interval, naphthalene accumulated at a rate greater than or equal to that predicted by the quantitative model (Figure 19), whereas in run $R\text{-}S_w$, the accumulation of naphthalene and the quantitative model were diverging during the final sample interval (Figure 16); (ii) the $^{14}CO_2$ concentration with oysters

present decreased during the final sample interval (Figure 19), whereas in run R-S_w, the $14CO_2$ concentration increased (Figure 16); (iii) cloudiness was never observed in the incubation vessels with oysters present, whereas a slight cloudiness was noted in the incubation vessels during run R-S_w. Also, during run R-3 the apparent bacterial population decreased during the final sample interval (Figure 15). Streptomycin prevented microbial blooms and reduced naphthalene and glucose oxidation and the consumption of dissolved oxygen when only shells were present. The presence of oysters and streptomycin further inhibited microbial growth resulting in reduced naphthalene oxidation, presumably microbial glucose oxidation, and dissolved oxygen consumption. It would have been difficult if not impossible to determine the relative amounts of glucose oxidized and oxygen consumed by oysters and bacteria when both were present in the system.

The microbial oxidation of naphthalene in synthetic seawater was not simply a laboratory artifact. Natural seawaters contain microbes capable of completely oxidizing naphthalene to carbon dioxide and water (Lee and Anderson, 1977). During the oxidation of aromatic hydrocarbons, many intermediates are formed (Gibson, 1976). Some of these intermediates may be excreted or incorporated into the microbial cell; in those cases where the intermediates are known toxicants, they may be of considerable environmental interest (Karrick, 1977).

Besides $14CO_2$, the only other naphthalene metabolites considered in this study were the total saponifiable metabolites

and monohydroxy-derivatives, 1-naphthol and 2-naphtol. The monohydroxy-derivitives were selected because they were extractable into very non-polar solvents, thus yielding extremely clean extracts for TLC. Also, the monohydroxy-derivitives are one of the major non-conjugated metabolites that have been identified in marine organisms (Varanasi and Malins, 1977). No 1-naphthol or 2-naphthol was detected; however, detectable quantities of other metabolites were detected by thin layer chromatography in the hexane extract of seawater from run $R\text{-}S_w$. Two areas of radioactivity were detected; one near the origin which represented very polar metabolites and a second area that was intermediate to the areas corresponding to the known standards of 1-naphthol and 2-naphthol. The mobility relative to 1-naphthol was 0.83, a value very close to that of one of the contaminants found in the unpurified [1-14C] naphthalene obtained from Amersham Co. The [1-14C] naphthalene used in run R-S was pre-purified by TLC immediately before the run. Since the runs were conducted under reduced lighting conditions, the possibility of photo-oxidation products was slight. This particular metabolite (based on mobility) was not observed in other runs and therefore it was unlikely that it was an extraction artifact. Recently however, it has been demonstrated that derivatives of benzo[a]pyrene may be formed as a result of the extraction technique (Bunting, 1978). The derivatives found in the present study may have been of microbial origin, either as excreted metabolites or extractable microbial cellular components. Since the water was not filtered prior to extraction, it was not

possible to differentiate between cellular and excreted metabolites.

It is apparent that microbial activity in the flow-through system, even in the presence of antibiotics was considerable. The presence of oysters in addition to the antibiotics seems to have had a negative effect on the microbial population but microbial activity was still significant. When both oysters and bacteria were present in the same system it was not possible to distinguish oyster from bacterial glucose oxidation and oxygen consumption. The fact the oxidation of glucose ceased once oysters were removed from the glucose incubation vessles, indicated that oysters and their shells and associated bacteria were the sole sinks for glucose loss and the source of all $14CO_2$ production while in the glucose incubation vessels. When interpreting the results of studies such as this, it is imperative to understand that the microbial contribution cannot be easily distinguished from the animal contribution.

The acute toxicity of oils to marine organisms is closely related to the di- and tri-cyclic hydrocarbon content (Neff et al., 1976). With the exception of the mono-cyclic hydrocarbons (benzene, toluene, etc.), the naphthalenes—mono-, di- and trimethyl analogues and unsubstituted naphthalene—are usually the most concentrated aromatic hydrocarbons in the water soluble fraction and in oil in water dispersions of oils (Anderson et al., 1974; Parker, Winters, Van Baalen, Batterton, and Scalan, 1976). Oysters (C. virginica) exposed to oil in water dispersions of No. 2 fuel oil accumulated the naphthalenes to the greatest extent (Neff, 1975). This is also generally true of other invertebrates

(Neff, 1975; Rossi, Anderson, and Ward, 1976). Refined oils are generally more toxic to marine invertebrates than crude oils (see Table 2 in Rice et al., 1976). The greater toxicity of refined oils is partially explained by the increased concentration of aromatic hydrocarbons (Neff et al., 1976). Neff and his co-workers found that the relative acute toxicity of an aromatic hydrocarbon is a function of the rates at which it is accumulated and depurated. Aromatics that are rapidly accumulated and slowly depurated are the most acutely toxic. On this basis, phenanthrenes are considerably more toxic than naphthalenes. Alkylation of the aromatic nucleus seems to increase the acute toxicity of the parent compound (Neff et al., 1976). The most toxic hydrocarbon evaluated by Neff et al., (1976) was 1-methylphenanthrene.

Although the methylnaphthalenes and phenanthrenes are considered to be more acutely toxic than unsubstituted naphthalene, the latter was chosen for use in this study because it was readily available as a radiolabeled isotope.

During each 72-hr run, approximately 150 µg of naphthalene entered the incubation vessel; of this only about 5.0% was recovered in oyster tissues. Of that 5.0%, about 5.0% was in the form of non-CO_2 saponifiable metabolites. The mode of naphthalene accumulation being primarily a function of the lipid/water partition coefficient (Neff, 1975). Accumulated naphthalenes in oysters are rapidly depurated when the animals are placed in hydrocarbon-free seawater environments (Neff, 1975).

Lee, Sauerheber, and Benson (1972) proposed that the hepatopanceas of the mussel (M. edulis) was probably the main storage site for accumulated hydrocarbons. The data presented in Table 3 and Figure 17 provide further confirmation of Lee's conclusion. Lee et al. (1972) also proposed that hydrocarbons were accumulated initially by the gills and subsequently transported to other tissues; this proposal is consistent with the fact that the bivalve gill is the primary site of uptake of dissolved organics (Pequignat, 1973). The data presented in Figure 17 suggests that the body always contains more naphthalene than the gills. However, the sample times were such that measurement of the initial rates of accumulation, necessary to ascertain which tissue first took up naphthalene, was not possible.

During preliminary studies to assess the effectiveness of various extraction methods, a static assay was conducted in which four oysters were placed in separate beakers containing 0.68 ppm 14C-naphthlaene in sterile seawater at 15°C. The shell material over the gill and cloaca had been removed. Oysters were removed at 16, 33, 70, and 145 min and the gills and body plus adductor muscle were dissected out, rinsed, weighed, and the total cyclohexane extractable radioactivity determined (Figure 39). The rapid increase of 14C-label in the gills relative to the rest of the tissues suggested that the initial accumulation occurred via the gills. Since uptake is thought to be a passive process, the results could reflect the fact that the gills are always the first tissues (including the mantle edge) that comes in contact with

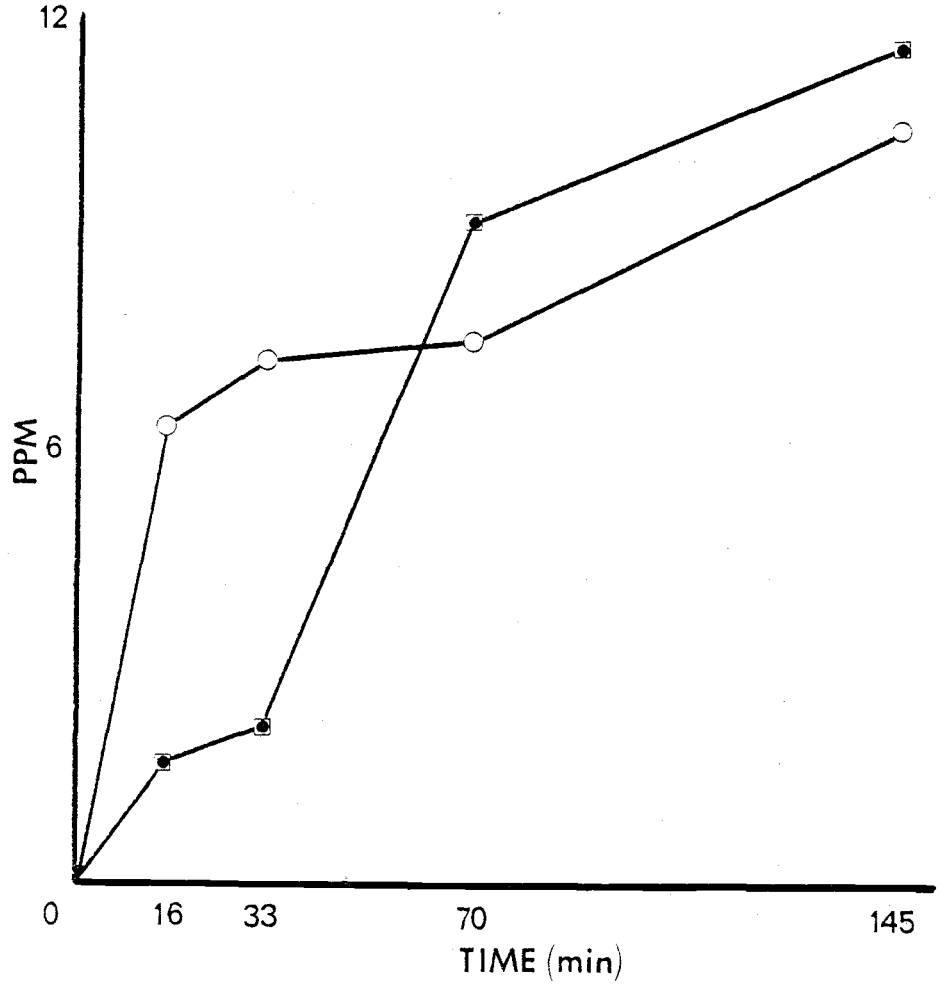

Figure 39. The initial accumulation of naphthalene in the gills (○) and in the body and adductor muscle (◙).

organics in solution.

The rapid accumulation by the body plus adductor muscle was preceded by a substantial lag period (Figure 39) which may reflect a very rapid internal transport between tissue compartments or may simply reflect the fact that the body plus adductor muscle are always processing water that has previously been processed by the gill tissue. It may follow then, that only after the storage sites in the gill are saturated does the rest of the tissue come in contact with substantial naphthalene concentrations. An experiment to resolve whether or not rapid internal transport occurs would require that the two compartments be somehow isolated, perhaps by following the initial accumulation with and without the gill tissue and mantle edge excised. Also, since the shell is the primary obstruction to uniform mixing around all the tissues, the complete removal of the shell would eliminate the lag period if internal transport was not the reason for the lag period.

Stegeman and Teal (1973) found that in oysters (C. virginica) in a flow-through system, a direct correlation existed between the accumulation of petroleum hydrocarbons and the total lipid content of the oysters. This relationship was not apparent until after the first 2 days of accumulation; during the first 2 days, the accumulation was more closely related to the wet weight. In the present study the bioaccumulation factors for the gills of O. edulis consistently increased with the length of starvation (Table 3). The total percent lipids (sum of neutral and polar lipids, Table 11) in the Nt gills (vessel #2) also consistently increased with the

length of starvation, although significantly only between runs 1 and 3 ($P < .025$). The bioaccumulation factor for R-3 was 26% greater than R-1 while the percent total lipid was only 8% greater. The increased concentration of naphthalene in the gill was not fully explained by the differences in total lipids.

The differences in the bioaccumulation factors between the other tissues were also not fully explained by the differences in the total lipid concentrations. Analysis of the total lipids in the Nt gills, body and adductor muscle from run R-3 vessel #2 revealed that the percent total lipids was 9.91% ± 0.52, 8.86% ± 0.93 and 4.21% ± 0.53 (± 95% C.I., n = 8) respectively. For this run (Figure 17), the adductor muscle had the least lipids and the lowest bioaccumulation factor. The lipid concentration in the body was less than the gills but the bioaccumulation factor was greater in the body. The mean wet weights of the gill, body and adductor muscle for the oysters from R-3, vessel #3 were 0.096 g, 0.073g, and 0.040 g respectively. These results suggest that although the lipid concentration and wet weight (and dry wt) of the tissues are directly related, the bioaccumulation factors were not clearly related to either, at least between the body and gills. The body accumulated naphthalene to an extent greater than expected, based on either its relative weight or lipid content. There are numerous possible explanations for these results.
(i) The naphthalene which accumulates in the body was actually not related to the lipid content of the tissue but to some other variable such as adsorption to mucus strands which originated on

on the gills and was eventually deposited and accumulate in the hepatic ceaca (Pequignat, 1973). (ii) The lipid composition (lipid classes) of the body differs from that of the gills in such a manner that the retention of naphthalene was considerably more favored in the body. (iii) Naphthalene uptake was a passive process with the concentration of naphthalene in a tissue determined by the ratio of influx and efflux which was in part a function of the tissue/water partition and in part a function of the ratio of the external surface area to tissue mass. The gill tissue has a high external surface to mass ratio and also a very high rate of influx and efflux. The body on the other hand has a low external surface to mass ratio (internal surface area is probably very high) so the influx and efflux were relatively low. The flux of water through the gill may have been high and therefore the naphthalene spent less time in the tissue and the probability of being removed from solution was reduced. The water flux through the body was low and most of the naphthalene which entered was removed from solution. If the ratio of influx to efflux was greater in the body than the gills, then regardless of the initial rate of accumulation, the body would eventually accumulate naphthalene to a greater extent. (iv) A final possibility is that when the tissues were rinsed prior to digestion, the efflux of naphthalene from the gills was much greater than that from the body due to the greater external surface area and resulting greater water flux through the gills (the naphthalene was more easily washed out).

Variations in the bioaccumulation factors in the adductor muscle (Table 3) do not lend themselves to a logical interpretation. It can be postulated that the variations were correlated with variations in lipid content, but this is highly unlikely considering that during run R-1 and R-2, the bioaccumulation factors in the adductor muscle actually exceeded those in the gill. In C. gigas, the total lipid concentration in the adductor muscle was always around one-third of the lipid concentration in the gills and palps, even during prolonged starvation (Riley, 1976). O. edulis contains more lipids than C. gigas (Galtsoff, 1964). The fact that the bioaccumulation factors in the adductor muscle decreased (Figure 17) with time suggests that the number of sites for the storage of naphthalene in the adductor muscle are limited. This correlates well with the low lipid content of this tissue.

In addition to demonstrating a correlation between lipid concentration and naphthalene accumulation, Stegeman and Teal (1973) demonstrated that the accumulation of naphthalene in oyster tissue was directly correlated to the naphthalene concentration in the flow-through system, up to some maximum (900 μg/l) when the oysters simply closed their valves. The results of the present study confirm that tissue accumulation was in proportion to the naphthalene concentration in the seawater, at least in the gills and body, but the correlation was not as evident for the adductor muscle (Figure 17).

Lee et al. (1972) exposed mussels (M. edulis) to 14C-naphthalene for 4 hr in a static system with an original

14C-naphthalene concentration of 32 ppb. Their results were expressed as ppm based on the dry tissue weights. Utilizing the data from Lee et al. (1972) and assuming that the water content of the tissues was 80% and ignoring the fact that naphthalene readily evaporates from open seawater systems (Figure 8), the calculated bioaccumulation factors for gills, body and adductor muscle were 56, 44, and 38 respectively. These values are surprisingly close to those found in this study (Figure 17) although the relative values for the specific tissues are quite different. This is not surprising considering that the study was done with unstarved animals of a different taxon in a static assay that lasted for only 4 hrs.

Until recently there was little evidence that bivalve mollusks are capable of metabolizing aromatic hydrocarbons. Lee et al. (1972) reported that neither muscles (Mytilus edulis) nor the normal bacterial flora associated with mussels had the capability to metabolize aromatic hydrocarbons. Bend, James, and Dansette (1977) demonstrated, in vitro, the presence of significant levels of epoxide hydrase activity in the hepatopancreas of soft-shell clams, Mya arenaria, and low but detectable levels of the same enzyme in M. edulis. Anderson (1978) reported that in vitro formation of monohydroxylated benzo[a]pyrene derivatives in homogenates of the oyster (C. virginica) digestive gland. Five different benzo[a]pyrene metabolites were found in organic solvent extracts; however, unidentified water soluble metabolites constituted the major metabolite fraction. Anderson's (1978)

study was the first published report providing indirect evidence for the presence of aryl hydrocarbon hydroxylase activity (AHH) in an oyster. Induction of the responsible enzyme systems was demonstrated by utilizing a commercial polychlorinated biphenyl mixture. In the present study, the naphthalene metabolites from the 72-hr formic acid digests were presumed to be free of $^{14}CO_2$. The increase in the total non-CO_2 saponifiable metabolites (Table 3) in each tissue as the length of starvation increased, suggests that starvational stress induced the synthesis of enzymes responsible for the formation of these metabolites.

It is possible to theorize about how starvational stress could induce the formation of those enzymes responsible for aromatic hydrocarbon metabolism. It must be assumed the enzymes are similar to those associated with the major detoxification pathways demonstrated in vertebrates and that AHH in bivalves is not substrate specific. Initially, starvation of oysters results in a decrease of all stored reserves. After prolonged starvation, when all stored energy reserves are depleted, the remaining lipid molecules are those with the greatest structural or metabolic importance. These would include the lipids associated with the structural integrity of the plasma-membrane, and the intracellular membranes essential to metabolic integrity (e.g. cholesterol). The major function of lipid metabolism during prolonged starvation, after lipid energy reserves (triglycerides) have been depleted, should likely be the synthesis of those lipid molecules that are essential for the maintenance of the structural and metabolic

integrity of the oyster. During prolonged starvation, the concentration of enzymes important for energy metabolism and for synthesis of storage products should be at a minimum and those enzymes necessary for synthesis of essential molecules, needed for the maintenance of structural and metabolic integrity, at a maximum. Cholesterol, being a major structural component of membranes, should be a highly conserved molecular species during prolonged starvation. This would be reflected by increased concentrations of those enzymes responsible for cholesterol synthesis. An important step in cholesterol synthesis from acetate or mevalonate is the formation from squalene of the squalene 2, 3 epoxide by a mixed-function oxidase. Neither cytochrome P-448 nor P-450 have been found in bivalve mollusks. At present, the evidence on whether or not oysters can synthesize cholesterol from acetate is conflicting. Voogt (1972) found distinct labeling of sterols in O. edulis after injection of [1-14C] acetate every other day for 7 days but did not find any labeling of sterols after a single injection of [1-14C] acetate in an experiment lasting just 6.5 hr. If the mixed-function oxidase whose existence is implied by sterol synthesis from acetate was the same as that utilized for detoxifying aromatic hydrocarbons, then the increased levels of naphthalene metabolites during starvational stress could be attributed to higher intracellular concentrations of the mixed-function oxidase resulting from increased sterol synthesis due to prolonged starvation. It is of interest to note that exposing flatfish (Fundulus heteroclitus and Stenotomus versicolor) to low

levels of petroleum hydrocarbons increases the rates of sterol synthesis from 14C-acetate (Sabo and Stegeman, 1976). The apparent effects of petroleum on hepatic lipid synthesis were similar to those observed in fish starved for a week or more; also, chronic exposure of fish to petroleum resulted in increased levels of AHH activity (Stegeman and Sabo, 1976).

It has been suggested that the digestive gland is the major site of aromatic hydrocarbon metabolism in C. virginica (Anderson, 1978). The body of O. edulis consistently had the highest concentrations of non-CO_2 saponifiable metabolites (Table 3, Figure 18). This suggests that the body (which includes the digestive gland) was the major site of metabolite production. However, the increased level of metabolites may simply reflect the fact that the body concentrated naphthalene to the greatest extent and does not indicate that the tissue is somehow specialized for the metabolism of naphthalene. The ratio of metabolites to unmodified naphthalene (Table 19) indicates that this was precisely the case. The concentration of naphthalene metabolites was an apparent function of the concentration of unmodified naphthalene in the tissue. Anderson (1978) found that the digestive gland was the only tissue in which AHH levels were high. That conclusion was apparently based primarily on indirect evidence (isolation of benzo[a]pyrene derivatives from the tissue) since no direct supporting data were included.

The major benzo[a]pyrene metabolites demonstrated by Anderson (1978) in C. virginica were water soluble metabolites.

Table 19. Ratio of non-CO_2 saponifiable naphthalene metabolites to unmodified naphthalene in the gills (G), body (B), and adductor muscle (A) for runs R-1, R-2, and R-3.

	G	B	A
RUN			
R-1	.046	.036	.040
R-2	.067	.060	.036
R-3	.092	.089	.115

Of the non-polar saponifiable metabolites demonstrated by high pressure liquid chromatography, monohydroxylated derivatives were the most abundant. In this study the monohydroxylated naphthalene derivatives were the most commonly observed hexane extractable metabolites (Table 4). In runs R-0 and R-1 14C-naphthalene containing several contaminants was used. This may explain why considerably more 1-naphthol was recovered from the oyster tissues after these runs. The contaminants were not 1-naphthol or 2-naphthol but could have been transformed in the tissue to 2-naphthol. One-naphthol was not found until run R-2. In run R-3, the level of monohydroxylated derivatives seemed to be inversely related to the level of very polar metabolites at the origin. Only a fraction of the activity in the total non-CO_2 saponifiable metabolites was recovered in the monohydroxylated derivatives. This fraction was much greater for runs R-0 and R-1, and thus does not support the contention that starvation induces AHH. The supporting evidence that bivalves cannot totally or partially oxidize aromatic hydrocarbons comes primarily from studies utilizing autoradiography of thin layer separations of 2:1 chloroform:methanol extracts of mussels incubated with 14C-naphthalene (Lee et al., 1972), studies utilizing assays involving the quantifying of known metabolites utilizing assay conditions optimized for vertebrate systems (Carlson, 1972; Vandermeulen, Keizer, and Penrose, 1977), or studies indicating that after initial depuration a small percentage of unmodified aromatics were retained in a "stable" pool for long periods of time (Lee, 1976;

see panel discussion). The lack of metabolites in the "stable" pool was considered as evidence that metabolism had not occurred. It should be noted however, that autoradiography and strip scanning may not be sensitive enough to detect metabolites when very low levels of activity are present, and use of enzyme assays optimized for vertebrate systems may preclude the detection of metabolites even when radiolabeled substrates are used since the conditions optimum for vertebrates may be inhibitory to mollusks. Anderson (1978) found that molluscan AHH is effectively inhibited by NADPH concentrations commonly used in mammalian MFO studies. Finally, the absence of metabolites in the "stable" pool does not take into account the possibility that metabolites may be rapidly excreted and thus detectable levels are not present. Also, these "stable" pools may not be accessible to the enzyme systems responsible for aromatic hydrocarbon metabolism.

Of all the studies that have been conducted concerning the accumulation of radiolabeled aromatic hydrocarbons by marine bivalves, none has considered the possibility that soluble hydrocarbon metabolites may be excreted into the seawater medium. The major naphthalene metabolite in seawater from the flow system (vessel #3) was $^{14}CO_2$. It was assumed that the only source of this $^{14}CO_2$ was microbial. There is evidence that n-alkanes are considerably modified by oysters in a manner closely resembling microbial degradation (Stegeman and Teal, 1973). In this study considerable levels of monohydroxylated derivatives were detected in the seawater whenever oysters were present (Table 5). Such

metabolites may have been excreted from the oysters or they may have been of microbial origin. In the latter case, they may have resulted from the incomplete oxidation and excretion from microbes or from cell-free enzyme systems. The possibility that there was a flux of metabolites from the seawater into the oysters cannot be excluded.

The observation that $^{14}CO_2$ concentrations in the seawater (vessel #3) were 10-30 times less when oysters were present, indicated that the oysters could have been the sink for $^{14}CO_2$. Carbon dioxide fixation by oysters occurs both in the secretion of shell carbonate (Wilbur, 1972) and in intermediary energy metabolism (Hammen, 1966; Simpson and Awapara, 1964). Much of the activity in non-CO_2 saponifiable metabolites could be from metabolites resulting from $^{14}CO_2$ fixation by the oysters. The fact that neither of the two monohydroxylated derivatives were recovered from the seawater in run R-S, suggests that the presence of the oysters was essential for the production of these two metabolites, and that they were not extraction artifacts; however, that possibility cannot be completely excluded (Bunting, 1978).

Oxygen uptake by oysters is independent of the concentration in water as long as the concentration is above some critical value (Galtsoff, 1964). Levels lower than the "critical oxygen tension" for *O. edulis* is 4.0 ml oyxgen/l seawater at 22°C (Gaarder and Eliassen, 1955). The dissolved oxygen measured in all vessels during runs with oysters present fell below this critical value by the first sample interval (Table 6). The QO_2 during the final

sample period were consistently lower for naphthalene-treated oysters (Table 7). It is not clear whether or not hydrocarbon pollution universally stimulates or inhibits respiration rates in bivalves. M. edulis, M. demissus and M. arenaria all exhibited increased respiratory rates in the presence of low levels of crude oils (Gilfillan, 1975; Gilfillan et al., 1976). However, B. variabilis and D. trunculus exhibited reduced respiratory rates when exposed to light Arabian crude oil (Avolizi and Nuwayhid, 1974), and M. edulis showed reduced respiratory rates when exposed to lubricating oil (Dunning and Major, 1974). Kitteredge, Takahashi, and Sarinana (1974) found that 1 ppm naphthalene decreased the activity of the gill cilia which pump water through the gills in C. virginica. Recently it has been shown that in M. arenaria exposed to low oil concentrations, respiration was stimulated, while at high concentrations it was inhibited (Stainken, 1978). Obviously, the respiratory responses of different bivalve species to hydrocarbon pollution are quite variable.

In the present study, inadequate control of microbial respiration and the fact that the oxygen tension fell quickly below the critical value, precluded a confident interpretation of the respiratory data. However, the naphthalene treatment did seem to inhibit respiration. From data presented in Galtsoff (1964), consumption in O. edulis is 141 to 176 µl/g/hr at 25°C. Assuming a Q_{10} of 1.5 (Gaarder and Eliassen, 1955) between 10° and 25°C, the estimated 15°C QO_2 would be 94 to 117 µl/g/hr. That is

approximately 2-4 times greater than the QO_2 measured in this study (Table 7). Bayne (1973a) indicated that the routine rate of oxygen consumption in mussels, M. edulis, was approximately twice the standard rate. The results of the present study suggests that even in the presence of 1 mM glucose in the seawater media, the oysters were metabolizing at the standard rate.

Ammonia-nitrogen excretion has been shown to be a sensitive indication of protein catabolism in bivalves (Bayne, 1973b). It was apparent in this study that ammonia-nitrogen excretion steadily decreased with time (Table 8). This decrease could have been due to increased utilization of the intracellular ammonia pool, a response to decreasing oxygen tension in the incubation vessels. Bayne (1973b) found a similar decrease in ammonia-nitrogen over a 25-hr period in static assays with M. edulis . Hammen (1968) reported similar decreases with the stout razor clam, Tagelus plebius. Utilizing the atomic ratio of oxygen consumed to nitrogen excreted as a metabolic index of temperature and nutritive stress, Bayne (1973b) found that during winter months when carbohydrate and lipid reserves were low, starvation or temperature stress resulted in extensive protein catabolism as indicated by low O:N ratios.

Since few measurements were made of ammonia-nitrogen excretion and QO_2 in the present studies, it was not possible to compare the O:N ratio for either starvation effects or effects due to naphthalene treatment. During run R-0, it was evident that the ammonia-nitrogen excretion peaked very early (Table 8). Assuming

that the accumulation of ammonia in the incubation vessels was linear for the first 5 hr, then for run R-0 the ammonia excretion rate for vessel #1, #2, and #3 was 5.61, 3.62, and 5.94 μg/g/day respectively. Those are considerably lower than the values published for C. virginica of 25 to 35 μg/g wet wt/day (Hammen, Miller, and Geer, 1966), but they are relatively close to the values from a later study (Hammen, 1968) of 4.77 to 15.65 μg/g wet wt/day. The differences were probably due to the fact that the former experiment was conducted in late spring and the latter in mid-summer. Bayne (1973b) showed that in mussels (M. edulis), ammonia-nitrogen excretion was greatest in the winter when carbohydrate reserves were depleted and lowest in the summer when carbohydrate reserves were high. In the spring a greater proportion of protein is catabolized than carbohydrates or lipids. The oxygen to nitrogen ratio is a measure of the significance of protein catabolism in this balance. A O:N ratio of greater than 100 would indicate that carbohydrates and lipids account for 90% or more of the energy metabolism (Bayne, 1973b). For run R-0, the ratios of O:N for vessels #1, #2, and #3, using the values calculated above and from Table 7, were 196, 261, and 158, respectively. These ratios represent minimum values since the rate of oxygen consumption during the first time interval was probably much greater than the last two because the oxygen tension had dropped below the critical value. Therefore, lipid and carbohydrates were the major energy substrates which was not surprising since the oyster metabolic system was primed with

glucose.

The uptake of dissolved amino acids and glucose from seawater may play an important role in bivalve nutrition (Pequignat, 1973). The accumulation of amino acids from solution by the bivalve molluscan gill has been found to conform to Michaelis-Menten type kinetics (Wright, Johnson, and Crowe, 1975); the transport of amino acids may involve a sodium coupled cotransport mechanism (Wright and Stephens, 1977). Whether or not accumulation of glucose by the gill is an active process is not certain. It was apparent that the uptake of glucose from solution was affected significantly by exposure to naphthalene (Figure 20). The uptake of glucose by the gill tissue may be a "mediated" process which requires a specific transport protein. It has been suggested that the accumulation of petroleum hydrocarbons may effect the fluidity of membranes (Sabo and Stegeman, 1976). If that is true then membrane-bound enzymes which are embedded in the lipid bilayer may undergo spatial changes which would affect the kinetic characteristics of membrane-bound enzymes (Harold, 1970).

In this study, the accumulation of 14C-label was not significantly different in any of the tissues examined (gills, body or adductor muscle). The finding that the 14C-label from glucose was rapidly and evenly mixed between the various tissues (Figure 21) does not correlate well with the findings of Pequignat (1973) for M. edulis. Pequignat found that the gill had the greatest accumulation of 14C-label derived from glucose, and the digestive gland the least. The difference was evident over the

entire period (> 18 hr) in which the 14C-label accumulation increased linearly in the tissues. The delayed accumulation of the 14C-label in the digestive gland was due to the movement of gill produced mucus, to which 14C-glucose was absorbed, into the hepatic caeca. Autoradiographic data incidated that the gill was labeled quickly but the foot and hepatic caeca were not significantly labeled for 5-10 hr and even after 24 hr, the muscle tissues were only sparsely labeled. It is apparent from the results of the present study (Figure 21) that under the conditions of uptake, O. edulis did not conform to the pattern established by Pequignat (1973) for M. edulis.

Under normal aerobic conditions in animal cells, the major end product of carbohydrate catabolism is CO_2. The fact that $14CO_2$ recovery was very low (Table 10, Figure 22) probably reflected the fact that most of the $14CO_2$ produced was quickly bound up in shell carbonates. It is unfortunate that the new shell material was not counted. The incorporation of 14C-label into total end products and intermediates of glucose metabolism (Figure 23) did not include 14C-label that ended up in the carbonate pool. After $14CO_2$ had been driven off by acidification (Figure 20), the loss of 14C-label from the glucose incubation seawater included that lost to all sinks including the carbonate pool. If the shell carbonate pool had been measured, the statistical differences evident in the loss of 14C-label from the glucose incubation medium, may also have been evident in the accumulation of 14C-label in the total end products (including 14C-label in the new shell) and

intermediates of glucose metabolism. If the $14CO_2$ production had been greater in Nt oysters than Ct oysters, then the $14CO_2$ would have to be derived from sources other than those directly linked to oxidative energy metabolism. It is also possible that the apparent inhibition of oxygen consumption in naphthalene-treated oysters was erroneous, or perhaps the linkage between the Krebs cycle and electron transport system was somehow severed allowing an initial increased carbon flux through the Krebs cycle without creating a disturbed redox balance.

One of the most basic effects of stress in bivalves involves changes in the concentrations of specific chemicals. For example, prolonged starvation and temperature stress cause an increase in protein catabolism (Bayne 1973b) and salinity stress results in changes in the free amino acid pools (Lynch and Wood, 1966). A reduction in the dissolved oxygen concentration causes an increase in the levels of certain amino acids and organic acids (de Zwaan and Wijsman, 1976). In the present study, there were three types of effects evident in the total pool sizes: effects attributable to starvation, effects attributable to either the reduced dissolved oxygen concentrations in the flow-through system or the difference in the glucose concentrations in the flow-through system and glucose incubation vessels, and effects due to naphthalene treatment.

The increased percent total lipids (Table 11), decreased total amino acids (Table 12), and decreased total organic acids (Table 14) were all effects attributable to starvation.

The decreasing Ala:Glu/Asp:Glu ratio (Figure 24) may have been a result of the fact that oysters were moved from seawater where the dissolved oxygen concentration was below the critical value (the flow-through system) to seawater which was saturated with dissolved oxygen (the glucose incubation vessels). The decrease may have involved a metabolic transition from anaerobic pathways to aerobic pathways of energy metabolism. Alanine has been shown to be a common end product of anaerobic metabolism in many bivalves (de Zwaan and Wijsman, 1976) and accumulates rapidly after the onset of anoxia (Kluytmans, de Bont, Janus, and Wijsman, 1977). Alanine concentrations are probably reduced rapidly when oxygen becomes available. The increased alanine concentration was expected during periods of reduced oxygen concentration but the concomitant decrease in aspartate concentration was not expected based on the work by Kluytmans et al. (1977). Assuming that the rate of protein catabolism and aspartate excretion were not affected by reduced oxygen concentration, then the reduction in aspartate concentration may have been due to changes in the synthesis of aspartate from oxaloacetate via glutamate-oxaloacetate transaminase. Du Paul and Webb (1970) found a high correlation between decreases in aspartate and increases in alanine in the adductor muscle of M. arenaria during salinity stress. Wickes and Morgan (1976) speculated that this relationship indicated a direct pathway in the synthesis of alanine from the decarboxylation of aspartate. Cripps and Reish (1973) also noted that increased alanine concentrations in the polychaeta worm, Neanthes

arenaceodentata, due to reduced oxygen concentrations, were correlated with reduced aspartate concentrations. They speculated that this reduction was the result of an increased utilization of oxaloacetate by malate dehydrogenase. It should be noted that control of the aerobic-anaerobic transition is probably not regulated by a simple "on/of" mechanism and it is quite probable that both pathways operate simultaneously in bivalves (Gabbott, 1976). The flow of carbon through the phosphoenolpyruvate branch point is determined by the degree of tissue hypoxia and the extent to which one pathway will dominate over the other depends on the particular conditions within the body tissue. When, in mussels (M. edulis), the acid end products of anaerobic metabolism are expelled from the tissues and the pH of the intracellular fluids does not drop, alanine accumulates but succinate, the other end product of anaerobic metabolism in bivalves, does not (Kluytmans et al., 1977). That would explain why in this study the total organic acids did not decrease during the glucose incubation period, as did alanine, when the dissolved oxygen concentration was increasing. A second possible explanation for the decreasing Ala:Glu/Asp:Glu ratio assumes that the transport of glucose across the gill epithelia was via a phosphotransferase enzyme system which utilized PEP as the energy source for sugar transport, similar to those systems described for bacteria (Roseman, 1971). The transported end products would be the phosphorylated glucose derivative and pyruvate. Assuming the glucose concentration was high when the conversion of pyruvate back to PEP was low, then the build-up

of pyruvate could be prevented by rapid transamination of pyruvate to alanine. When the oysters were transferred to the 14C-glucose incubation vessels the external glucose concentration was reduced by a factor of 10^3. The PEP requirement would then also be reduced and likewise the alanine concentration.

The decreased total protein and polar lipid concentrations (Table 11), increased total free amino acids (Table 12), and increased organic acids (Table 14) relative to controls were all possible effects of naphthalene treatment. The fact that total protein and total polar lipids were significantly reduced in the Nt oysters suggests that naphthalene stimulated the catabolism of these compounds. Heintz et al. (1974) demonstrated increased activity of leucine aminopeptidase in C. gigas exposed to petroleum hydrocarbons. It is possible that in the present study the increased mobilization of polar lipids was a response to the increased carbon flux through glycolysis and intermediary metabolism into amino acids and organic acids; the point of entry would probably have been at the level of triose phosphate. Gabbott (1976) suggested that glycerol from lipids may be a source for generating pyruvate or phosphoenolpyruvate (PEP) when these glycolytic intermediates need to be replenished. The presence of abundant free glucose in the cells would seem to preclude the need for replenishing PEP by this route. However, if the entry of glucose into the oyster involved a PEP:glucose phosphotransferase system similar to that involved in the transport of sugars across the bacterial cell membranes (Roseman, 1971), then the

replenishment of PEP from sources other than glucose would be very important, especially if most of the glucose was being directed into storage products (i.e. glycogen) as was in this study. Then, the accumulation of alanine, discussed previously, would not be a result of the reduced dissolved oxygen concentration but rather, would be associated with the increased pyruvate concentration as a result of the pivotal role of PEP in the translocation of sugars by the phosphotransferase system. Pyruvate accumulation would be prevented by transamination to alanine. The reduction in alanine resulting from the transfer of the oysters from the flow-through system containing 1 mM glucose, to the glucose incubation vessels which contained only labeled glucose (< .001 mM) may have been the result of a reduced production of pyruvate due to the fact that large quantities of Pep were no longer required for glucose transport. It must be emphasized that a PEP:glucose phosphotransferase system has never been demonstrated in a bivalve and that the above discussion is purely speculative.

The increased levels of amino acids and organic acids could have reflected either a disturbed protein metabolism or an increased dependence on anaerobic pathways. Besides leucine aminopeptidase, two other enzymes were significantly affected by exposure to petroleum hydrocarbons in the study by Heintz et al. (1974): glutamate-oxaloacetate transaminase and malate dehydrogenase. In the present study the increased protein catabolism may have reflected an increased requirement of amino acids for transamination of pyruvate to alanine. The resulting increased

intracellular concentration of amino acids and organic acids in naphthalene-treated oysters would then have been a result of the increased levels of amino acids due to protein catabolism and the organic acids resulting from transamination.

It is possible that the flow of carbon into PEP from polar lipids may have been directed into aspartate synthesis via phosphoenol-pyruvate carboxykinase. The observation that aspartate was labeled more in the α-carboxyl position than would be expected if all the label into oxaloacetate and eventually into aspartate, originated from uniformly labeled glucose supports this conclusion (Table 18). The PEP from polar lipids may be functionally compartmentalized relative to PEP from glucose.

The very high carbon flux into glycogen (Figure 30) relative to other end products and intermediates may have reflected the low glycogen content of the tissue as a result of prolonged starvation. In O. edulis glycogen is a negative feedback inhibitor which regulates its own synthesis (L-Fando, Garcia-Fernandez, and R-Candela, 1972).

The differences between the specific activity-time curves for total proteins, total polar lipids, alanine, glutamate, aspartate, and the radioactivity-time curves for malate and succinate, of control and naphthalene-treated oysters were effects associated with naphthalene treatment (Figures 29, 32, 33, 34, 35, 36, 37, and Table 17). In general, the specific activity-time curves for Ala, Asp and Glu and the radioactivity-time curves for malate and succinate suggested that the carbon flux through the Krebs cycle and associated amino acids was stimulated by

naphthalene. Gilfillan (1975) found that petroleum hydrocarbons increased carbon respired but decreased carbon assimilated. In this study, the naphthalene-treated oysters generally showed increased levels of 14C-label incorporation into the various pools when expressed as specific activities. The 14C-flux through the metabolites more commonly associated with energy metabolism (Ala, Asp, Glu, malate and succinate) were generally greater for Nt oysters. However, the true carbon fluxes could not be calculated since the actual size of the precursor pool (glucose) was not known for each sample interval. It can be reasoned that since the absolute pool size of amino acids and organic acids were greater for Nt oysters, then the higher specific activities for Nt oysters was attributable to a greater 14C-flux into the amino acid and organic acid pool. The opposite argument can be used to show that the increased specific activities for proteins and to a lesser extent for polar lipids, were a result of the reduced concentrations of these compounds in their respective pools. There were no significant differences between Nt and Ct oysters in either the pool sizes or specific activity-time curves for glycogen or neutral lipids. The finding that 14C-flux through the intermediates increased while 14C-flux into end products may not have increased, suggests that the efficiency of assimilation into end products had been reduced by naphthalene treatment.

Based on the "stress syndrome" characterized for fish by Gronow (1974), Bayne (1973a) proposed four general responses in M. edulis which characterized the "stress syndrome" of the mussel

during starvation. These were: (i) reduced oxygen consumption; (ii) disturbed protein metabolism; (iii) the utilization of body reserves; and (iv) an increased anaerobic metabolism. The results of this study indicate that the responses of the general "stress syndrome" suggested by Bayne (1973a) may describe the "stress syndrome" induced by naphthalene in the oyster O. edulis.

V. SUMMARY AND CONCLUSIONS

The purpose of this study was to evaluate the potential of utilizing an in vivo kinetic analysis of glucose metabolism as an approach for assessing the effects of chemical pollutants on bivalve mollusks.

Starved oysters were stressed in the presence of naphthalene in an open flow-through system that modeled the entry of the pollutant as if from a point source with the ambient pollutant concentration being zero at time zero and the eventual steady-state concentration approaching 90 ppb at the end of 72 hr.

Each 72-hr run consisted of exposing three separate groups of oysters to three different treatments. The first group, the "control treated" (Ct) oysters, was never exposed to naphthalene; the second group was "naphthalene-treated" (Nt) and was exposed to unlabeled naphthalene dissolved in seawater; the third group was exposed to [1-14C] naphthalene dissolved in seawater. Oysters in the former two groups were utilized for measuring the pool sizes of the major precursors, intermediates, and end products of glucose metabolism and for the in vivo kinetic analysis of glucose metabolism, and oysters in the latter group were used for measuring the naphthalene and naphthalene metabolite concentrations in the various tissues of the oysters.

The in vivo kinetic analysis involved tracing the carbon flow from D-[U-14C] glucose into the intermediates and end products of glucose metabolism in oysters, maintained in unstressed (control)

and naphthalene-stressed environments. Specific radioactivity-time curves for ethanol-insoluble polysaccharides (primarily glycogen), total protein, total polar lipids, total neutral lipids, neutral compounds (primarily glucose, free alanine, aspartate and glutamate, taurine, and total organic acids were determined for control and naphthalene-stressed oysters. Radioactivity-time curves for malate and succinate were also determined.

The water from the flow-through system was analyzed for dissolved oxygen, ammonia-nitrogen, and for the build-up of [1-14C] naphthalene from the initial zero concentration at time zero. The extent of bacterial metabolism of naphthalene, and the effects of the bacterial population on the dissolved oxygen concentration, and ammonia-nitrogen were also evaluated.

The results of this study indicated that there were three types of effects evident: effects attributable to starvation, effects attributable to either reduced oxygen concentrations in the flow-through system or difference in the glucose concentrations in the flow-through system and glucose incubation vessels, and effects due to the naphthalene treatment.

In each run approximately 150 μg of naphthalene entered the flow-through system containing the naphthalene-treated oysters during the 72-hr run. Of the naphthalene that entered only about 5.0% was recovered in oyster tissues. Of this 5.0%, about 5.0% was in the form of non-CO_2 saponifiable metabolites. Monohydroxylated naphthalene derivatives were the most commonly observed hexane extractable metabolites based on thin layer chromatographic

procedures.

Increased catabolism of proteins and polar lipids, increased levels of amino acids and organic acids, increased initial rate of glucose uptake, and significant differences in the specific activity-time curves for alanine, aspartate, glutamate, protein, and polar lipids and radioactivity-time curves for malate and succinate, were all effects attributed to naphthalene treatment.

The fact that total protein and total polar lipids were significantly reduced in the naphthalene-treated oysters suggested that naphthalene treatment stimulated the catabolism of these compounds. The increased levels of amino acids and organic acids in naphthalene-treated oysters could have reflected either a disturbed protein metabolism or an increased dependence on anaerobic pathways.

In general, the specific activity-time curves for Ala, Asp and Glu and the radioactivity-time curves for malate and succinate suggested that the carbon flux through the Krebs cycle and associated amino acids was stimulated by naphthalene treatment. The fact that ^{14}C-flux through the intermediates increased while ^{14}C-flux into end products may not have increased, suggested that the efficiency of assimilation into end products had been reduced by naphthalene treatment.

The purpose of this study was to evaluate the potential of utilizing an in vivo kinetic analysis of glucose metabolism as an approach for assessing the effects of chemical pollutants on bivalve mollusks. The results of the present study suggest that

this approach could be a valuable tool for evaluating the low level effects of chemical perturbants on marine organisms.

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