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It has been shown that it is possible to generate egg yolk vesicles that could encapsulate protein. The egg yolk vesicles were in a size range that could be filtered by the oyster. That ingestion did indeed occur was demonstrated by the presence of fluorescence in the digestive tissue when oysters were fed egg yolk vesicles containing fluorescein isothiocyanate bovine serum albumin.

Uptake and metabolism of egg yolk vesicles was demonstrated by a radiotracer study involving addition of a radiolabeled lipid to egg yolk. Recovery of radioactivity in the tissues of oysters implied that the lipids from the egg yolk vesicles were also being taken up by the oyster and metabolized. Detailed analysis showed the radiolabel being transferred from the stomach into the other tissues. In addition, catabolism and subsequent resynthesis into non-lipid components was demonstrated based upon the recoveries of radioactivity in the insoluble and aqueous phases during extraction of lipid from the tissues. Transacylation of the radiolabeled fatty acid from phosphatidylcholine to other lipids requiring highly unsaturated fatty acids was believed to be the main factor responsible for the distribution of radioactivity

amongst the lipids in the stomach tissue. The factors for distribution of radioactivity in the lipids of the gill, mantle and muscle tissue were not elucidated.

During an investigation into the aspartate transcarbamylase (ATC) reaction in oyster tissue, the optimum assay conditions were determined. The optimum pH was 9.35 and the optimum temperature was 39.5°C.

Short term regulation of the ATC enzyme occurred while feeding oysters discontinuously. During an 8 hr period, significant differences were detected in the enzyme's activity.

By altering the amount of food (egg yolk-starch) fed to oysters, variations were noted in the activity of ATC in oyster tissue. The activity increased when oysters were presented with more food, however, the highest quantity of food led to decreased activity.

The composition of the oyster's diet also affected the levels of aspartate transcarbamylase activities in oyster tissues. Those oysters fed an egg yolk-starch diet contained significantly lower aspartate transcarbamylase activities than oysters fed an egg yolk-starch-salmon oil diet or a casein-starch-salmon oil diet. The aspartate transcarbamylase activities in oysters fed Phaeodactylum tricornutum or a starch diet were not significantly different from the activities in oysters fed the egg yolk-starch diet.

The Use of Chicken Egg Yolk Vesicles as a Potential Food System for Juvenile Pacific Oysters

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LITERATURE REVIEW

Anatomy of Bivalves

The central adductor muscle, attached firmly to each valve, is a prominent feature of the oyster and must be cut before the animal can be seen. The adductor muscle is divided into two parts. On the side nearest to the hinge is the quick muscle which is translucent in appearance. The remainder of the muscle, which is more opaque, is the catch muscle. The quick muscle is responsible for rapidly closing the valves when the animal is disturbed, whereas the catch muscle is able to maintain the valves in a closed position for long periods when conditions are unfavorable (1).

The mantle is a sheet of tissue that hangs on both sides of the body mass (stomach, intestine, heart and kidney). It lies against the shell where it is fixed at the border. The margin of the mantle is arranged into three folds, two of which are concerned with the secretion of the shell. The inner and largest fold acts as a curtain across the shell opening. The folds on the opposite sides of the mantle, by moving either together or apart, can control the flow of water into the mantle cavity (1).

The gills are four crescent-shaped plates that stretch from the mouth for about two-thirds of the distance round the body to a point where the opposite margins of the mantle fuse. This fusion divides the mantle cavity into a large inhalant chamber, containing the gills, and a much smaller exhalant chamber. Water is moved from the inhalant chamber

of the mantle cavity into the water tubes by the activity of numerous rows of whip-like cilia located on the filaments. These not only move the water, but also filter from it the small particles that make up the animal's food. The depleted water passes up the gill tubes into the exhalant chamber and out of the mantle cavity (1).

Filtration and Ingestion in Bivalves

Particles that are caught by the larger cilia of the gill are thrown on to the frontal surface of the gill filaments. This area bears many mucus-secreting cells and tracts of cilia. The mucus secretion traps the food particles. The arrangement of the tracts of cilia is such that the larger particles tend to move towards the free margin while the others move in the opposite direction. On their arrival at either of these two points, ciliary tracts will conduct the particle strings via the labial palps to the mouth (2).

Particle discrimination at the gills appears to be primarily a function of particle size. Haven and Morales-Alamo (3) found that oysters filtered naturally occurring particles in the 1.0 to 3.0 μm range with about one-third the efficiency as larger particles. From 3.0 to 12.0 μm there was no change in filtration efficiency.

Particles that are too large or too heavy to be held by the mucus string drop off to the mantle below. For the remaining particles, further particle selection takes place at the palps. The inner faces of the palps (of which there are a pair on each side of the mouth) are folded into numerous ridges. These palps are characterized by a complicated series of ciliary tracts that are used to separate food particles. Once separated, the smaller particles are carried to the mouth, and the larger particles to the edge of the palp where they drop

on to the mantle below. Rejected material from both gills and palps is moved along ciliary pathways on the mantle to an area close to the inhalant opening. Periodically, rejected material is discharged by a sudden and forceful closure of the shells that is sufficient to blow all the accumulated sediment from the mantle cavity. This rejected material is conveniently termed pseudofeces to distinguish it from the true feces formed from the waste material from the alimentary canal (1).

Pseudofeces may also occur as a result of excess particle concentration in the surrounding medium. Winter (4) found an increase in both ingestion rate and filtration rate as particle concentration increased. However, after some maximum particle concentration the ingestion rate remained constant while the filtration rate declined. Gerdes (5) found that the filtration rate of Crassostrea gigas decreased as the concentration of Isochrysis galbana was raised from 50 X 10^6 to 100 x 10^6 cells/liter. Consequently, the amount of algae removed was more or less constant at all three concentrations. Further increases in particle concentration led to decreased filtration and ingestion rates with concomitant pseudofeces production. The concentration at which this occurred has been termed the "critical cell density" (6), the value of which may increase with decreasing particle size (4).

Mechanisms of Digestion in Bivalves

Particle selectivity by the gills and palps appears to be minimal with regard to the nature of the filtered particles. When fed suspensions of unicellular algal cells and ferric hydroxide, Mytilus edulis did not discriminate in its filtering between the two types of particles (7). C. gigas did not discriminate between algae and bacteria during ingestion. The oysters ingested bacteria (Chromatium or

Chromobacterium) and algae (Chlorella or Tetraselma) equally well at loads less than 200 mg/liter. However, bacteria were digested much more readily than the algae, as the oysters produced feces containing few bacteria but much undigested material (2). This type of discrimination, occurring post-ingestion internally in the stomach, constitutes the second level of particle discrimination.

Particles passing into the stomach from the mouth encounter an elaborate structure. The stomach is comprised of two chambers with a variety of grooves and ridges and numerous openings leading to digestive tubules. The crystalline style, another structure present in the stomach, is a semi-transparent gelatinous rod lying freely in its sac. It protrudes across the cavity of the stomach and bears against the gastric shield, a hardened area of the stomach wall. A relatively short rectum terminates the digestive tract near the adductor muscle (1).

According to Barnes (8) the lower pH of the stomach along with mechanical activity of the crystalline style dislodges particles from the incoming mucous strings. These particles are directed against a portion of the stomach wall composed of a series of ciliated grooves. Coarse, heavy particles are segregated out and are directed into the intestine. Finer particles and products of partial digestion by enzymes of the style are retained by the cilia and directed into the caecal ducts of the digestive diverticula, via a non-ciliated inhalant portion. Waste material is conducted out of the gland along a ciliated exhalant portion of the main ducts. A typhlosole, or extended fold in the stomach prevents waste materials exiting the diverticula to mix with incoming food material.

A number of investigators (9, 10, 11 and 12) have shown that the dominant digestive process was phagocytosis of intact or partially digested food particles by the cells of the digestive diverticula. Extracellular digestion of whole cells assumed a secondary or supporting role.

The often demonstrated occurrence of live algal or bacterial cells in fecal material (13) attests to the weakness of the lytic power of the extracellular gastric fluids. Some cell types, however, may be more resistant than others to gastric enzymes. Dean (14) exposed various species of algae to style extracts from Crassostrea virginica. Cryptomonas disintegrated rapidly and Monoisochrysis was immobilized. Isochrysis was unaffected.

In large C. virginica, the time required for food to pass through the entire digestion tract was 90 to 150 minutes (15). Fecal matter (feces) was voided from the rectum as compact and slightly flattened ribbons which in many cases was characteristic of the species of bivalve (16).

Patterns of Digestion in Bivalves

Two patterns of digestion have been found in oysters. Morton (17) proposed a rhythmic digestion pattern that was imposed on C. gigas by the tidal cycle. According to Morton, the processes of extracellular digestion in the stomach and intracellular digestion in the digestive diverticula of intertidal bivalves were both discontinuous, alternate, and irrevocably interrelated since breakdown of the digestive cells of the digestive diverticula following intracellular digestion resulted in the dissolution of the crystalline style. Moreover, the cytological

structure of the digestive diverticula underwent a series of synchronized changes related to the tidal cycle.

Owen (18) proposed that digestive activity was under the influence of available food levels rather than an endogenous rhythm of digestion. This hypothesis was further supported by work conducted in the lab of Palmer (19). Hourly measurements of filtration rate and cell clearance rate for periods of 24 to 33 hours were made on C. virginica. Filtration behavior of C. virginica was generally characterized by alternating periods of high and low activity. Oysters filtered actively for 80% of all hourly periods in suspensions of Thalassiosira pseudonana and 91% in suspensions of Isochrysis galbana. Peaks of oyster filtration occurred two to three times per day and the period between peaks did not vary with experimental algal concentration (1.7-6.7 mg/liter). Local tidal sequence did not affect oyster filtration, therefore the most probable explanation given for variations in cell clearance rate was that they serve to regulate food levels in the stomach to permit a relatively constant level of intracellular digestion.

Feeding Studies in Bivalves

Feeding trials with marine bivalves can be divided into three groups: those utilizing cultured live food, those involving a synthetic or artificial food not normally considered part of the diet of such organisms, and those involving dissolved nutrients. Since the present study deals with juveniles and adult forms, some of the studies concerning these forms is reviewed here.

Algal Diets

It is fairly clear that wild oysters are deriving their energy input from some component of the phytoplankton introduced with the culture water. However, natural phytoplankton systems are extremely variable in quality and quantity of organisms in both time and space.

In an effort to rigorously examine the nutritional requirements of various bivalves, selected species of algae are often grown either as unialgal or axenic cultures, and then used as food in controlled quantities. Tenore and Dunstan (20) studied the effects of moderate and high concentrations of different species of phytoplankton on the feeding and biodeposition (feces and pseudofeces) rates of small and large oysters over a period of one week. T. pseudonana was consumed at the greatest rate followed by Skeletonema costatum, Nitzschia closterium and Dunaliella tertiolecta. The low feeding rate by oysters on D. tertiolecta indicated that pumping rate or filtration efficiency of the oyster must be depressed by this algae, possibly through some secretion of the algae. The average rates of biodeposition were greatest with S. costatum, followed by D. tertiolecta, N. closterium and T. pseudonana. These differences in response to feeding and biodeposition rates are important to our knowledge of food chain dynamics and may be used to optimize aquaculture systems.

Epifanio et al. (21) conducted several experiments to determine the nutritional value of several algal diets to six species of bivalves (C. virginica, C. gigas, Ostrea edulis, Mercenaria mercenaria, Tapes semidecussata and M. edulis). Shellfish were fed defined, non-axenic algal diets for periods ranging from 10 weeks to over 2 years in a recirculating seawater system. The diatom, Phaeodactylum tricornutum,

was shown to be a poor food for all species of bivalves tested while another diatom, T. pseudonana, proved to be a good food. T. pseudonana yielded growth comparable to that of a four part diet of Carteria chui, Croomonas salina, I. galbana and T. pseudonana when fed to C. virginica, C. gigas and T. semidecussata while the four part diet was superior with O. edulis and M. edulis.

C. chui appeared to be a better food for oysters than I. galbana (22). Algal diets consisting of P. tricornutum and C. chui (1:1 cell count ratio) supported faster growth of C. virginica than a diet of P. tricornutum and I. galbana (1:1 cell count ratio). Algal diets consisting of P. tricornutum, C. salina and C. chui (1:1:1 cell count ratio) supported faster growth of C. virginica than the algal diet consisting of P. tricornutum, C. salina and I. galbana (1:1:1 cell count ratio).

In contrast, another study by Epifanio (23) demonstrated that C. chui was a poor food source as growth was generally least in diets containing large proportions of C. chui. Platymonas suecica also proved to be a poor food source when utilized alone, but its food value increased when fed concomitantly with either I. galbana or T. pseudonana. Some growth promoting micronutrient found in relatively high quantities in I. galbana and T. pseudonana may be responsible for these nonadditive effects. An alternative explanation involves the relative digestibility of the various algal species. The cells of both C. chui and P. suecica are bounded by a nonrigid theca composed of a complex polysaccharide (not cellulose) and protein (24), and conceivably digestion of such materials is largely an extracellular process (25). High rations of C. chui and P. suecica might exceed the capacity of the

stomach to digest extracellularly the material present. However, when C. chui or P. suecica are fed in combination with a more digestible algae, the degree of extracellular digestion required is lessened and complete assimilation of the ration is increased (23).

To further test the extracellular digestion hypothesis, Romberger and Epifanio (26) measured efficiencies of assimilation and growth of juvenile oysters fed three species of algae, singly and in combination for 35 days. Dietary components were I. galbana (Diet I), P. suecica (Diet P), and T. pseudonana (Diet T). High (Diet TI), intermediate (Diets T, I, PT, PI), and low (Diet P) growth responses were observed. Intermediate and low growth responses were explained in terms of differing levels of ingestion and assimilation. The high growth response with Diet TI was not explained by measured values of ingestion and assimilation. There appeared to be no correlation between growth and chemical composition (protein, lipid, carbohydrate, or ash content) of the algal diets.

The poor food value of P. tricornutum could not be explained by Epifanio et al. (27). Juvenile oysters, C. virginica, were fed diets consisting of varying proportions of the diatoms P. tricornutum and T. pseudonana. Growth of the oysters over a 5 week period was inversely proportional to the amount of P. tricornutum in the diet. Analysis of the diatoms showed similar amounts of protein and lipid in the two species, while P. tricornutum was higher in carbohydrate and T. pseudonana higher in ash. P. tricornutum lacked the essential amino acid tryptophan, but this deficiency has also been reported for other diatom species that are good foods for bivalves. Other possible explanations for the poor food value of P. tricornutum involve exudation

of toxic metabolites, problems in digestion and assimilation of the cells or a deficiency in some trace constituent, such as vitamin or mineral.

When oysters were fed varying proportions of the yeast Candida utilis and T. pseudonana, the gross chemical or amino acid composition of the diets could not explain the relative food values of the different diets. Growth of soft tissue in C. virginica decreased with the amount of yeast in the diet (28).

By culturing T. pseudonana under light of different spectral distribution and by harvesting the algae from either exponential or stationary phases, Flaak and Epifanio (29) were able to change the composition of the algae. Subsequent feeding studies indicated that the nutritional value of T. pseudonana was directly related to the ratio of proteinaceous:nonproteinaceous material in the algal cell. Cells harvested from the stationary phase had higher C:N ratios than cells harvested from the exponential phase. C. virginica grew faster and had higher glycogen contents when fed diets with higher C:N ratios.

Artificial Diets

Nonliving sources of energy have also been tried as oyster food. These sources allow greater manipulation of the diet composition, but have met with only limited success to date.

One of the first studies designed to investigate artificial diets was undertaken by Gillespie et al. (30). Several series of experiments were conducted using adult American oysters to test the feeding value of soluble glucose, a combination of glucose and hydrolyzed corn protein, dried skimmed milk, wheat flour and cornmeal. The concentrations of feeds in the water were varied from 5 to 30 mg per liter throughout the

series. No significant growth responses were observed for the glucose and hydrolyzed corn protein diet, dried skimmed milk and wheat flour. A growth response was shown in adult oysters when finely ground cornmeal was added to the water surrounding them and maintained at a concentration range up to 20 mg per liter. These data suggested that soluble glucose can be utilized in small quantities by the oyster. However, as a practical feed, glucose in solution as the sole supplement appeared to have a very limited value.

Comparison of cornstarch and dextrose diets by Turgeon and Haven (31) showed that cornstarch was the better supplemental food. Glycogen content and tissue size were the major constituents affected by the presence of the cornstarch in the diet.

Castell and Trider (32) attempted to maintain juvenile oysters (C. virginica on a purified artificial diet for an extended period of time (12 weeks). The diets were mixtures of dissolved water-soluble nutrients and suspended particles ranging in size from less than 1 to about 50 μm in diameter with over 90% of the particles in the 5-15 μm diameter size range. Differences in growth rate, meat weights, and glycogen content corresponded with differences in dietary composition. High levels of dietary carbohydrate resulted in greater oyster glycogen production. Inclusion of cod liver oil also promoted glycogen deposition more so than corn oil. Increasing the dietary lipid from 5 to 18% of the dry weight resulted in greater meat production and earlier total weight gains. While this study showed that oysters could be maintained on an artificial diet, growth rates were only one-tenth those of oysters in the natural environment, indicating that the quality and/or quantity of nutrients in the artificial rations was lacking.

More recently Trider and Castell (33) undertook a study using artificial diets to indicate possible essential fatty acid requirements. Diets contained either ω6 series polyunsaturated fatty acids (as corn oil), ω3 polyunsaturated fatty acids (as cod liver oil) or saturated fatty acids (as hydrogenated coconut oil). Sterol-supplemented diets and sterol-deficient diets were also fed to determine sterol requirements. Data on growth and tissue composition implied that oysters have an essential fatty acid requirement for both linolenic (ω3) and linoleic (ω6) series fatty acids, with the former playing a more significant role. While the oysters were unable to synthesize sterols from ^{14}C-acetate, it was found that excessively high dietary sterol (1% of the dry weight of the diet) inhibited growth.

To test whether oysters will efficiently utilize dissolved protein, soluble whey protein concentrate in combination with wheat starch, cod liver oil and a vitamin premix were presented to Sydney rock oysters, Saccostrea commercialis (34). Bacterial protein ("Pruteen") was used as a potential particulate protein source for oysters. The weight gain data suggested that dissolved protein was not efficiently utilized by S. commercialis while weight gain was reported with bacterial protein fed oysters. The maximum weight gain was obtained when the oysters were fed 171 g "Pruteen"/kg dry diet. This value was in close agreement with the reported protein requirement for C. virginica (29).

Dissolved Nutrient Diets

Dissolved nutrients can also be utilized by oysters and other bivalves as attested by several investigators. The major categories of dissolved nutrients that have been investigated are glucose, amino acids and fatty acids.

Pequignat (35) provided evidence that the gills and mantle of M. edulis can concentrate several hundred times dissolved glucose and amino acids from very dilute solutions. Experimental evidence indicated that a carrier-mediated process was operating in the absorption of monosaccharide in the gill and probably also in the mantle of C. gigas (36). D-glucose was accumulated against the gradient in gill in substantially unaltered form, and kinetic analysis of glucose uptake confirmed the presence of a saturable component of absorption at low substrate concentrations, and entry by diffusion at high concentrations.

Swift et al. (37) and Riley (38) both showed that glucose absorbed by oysters was metabolized. The major recipient of glucose-carbon in "glucose-primed" oysters was primarily into glycogen while in fasted bivalves the disposal was primarily into amino acids and carboxylic acids.

In addition to meeting energy requirements, the uptake of dissolved glucose has the potential for contributing significantly to growth. In natural systems the significance is debatable since the concentration of easily metabolized substances is usually very low (38).

During a single passage of water through the mantle cavity of mussels, removal of naturally occurring specific free amino acids has been shown using high performance liquid chromatography. Removal proceeded unabated at ambient substrate concentrations as low as 38 nanomoles per liter (39).

Ferguson (40) confirmed that several molluscs including C. virginica exhibited influx of free amino acids at rates dependent on media levels and low, constant efflux. This influx could support at least 3-10% of their metabolism which is approximately sufficient to

provide for the needs of epidermal and associated tissues such as ciliary pumping over gills.

Dissolved free fatty acids were also removed from seawater by C. virginica at naturally occurring concentrations of 0.25 μM. The uptake was saturable for both palmitate and stearate but not oleate. The uptake of dissolved free fatty acids may serve an important physiological role by providing or supplementing essential fatty acids which may be found in limited amounts in the normal particulate diet (41).

Encapsulated Diets

The first study to look at the feasibility of using microcapsules as artificial food particles for marine bivalves was performed by Gabbott et al. (42). Two types of synthetic microcapsules were examined, one made from crosslinked nylon and protein, and the other from a glycopeptide fraction of ovomucoid. Digestion experiments using stomach juice from C. gigas showed that neither type of capsule was broken down extracellulary, although encapsulated starch did appear to be digested (based on disappearance of positive iodine staining). Short term feeding trials showed that both types of capsules would be ingested by mussels if in the size range of 10 to 100 microns. Larger particles were rejected in pseudofeces. On a preliminary growth experiment (21 days), microcapsules containing a diet of 10% hemoglobin, 7.5% rice starch, 2.5% soluble starch and 0.2% cholesterol were fed, once a day, at densities of 5-30 capsules/μl to oyster spat. Significant differences were found between starved controls and spat fed either of the microcapsules. Growth on the microencapsulated diet was clearly limited as shown by the greater growth when given an algal diet.

Growth rates as high as 73% that of algal-fed controls were obtained when feeding oysters a mixture of carboxymethyl cellulose microgel particles and lipid-walled microcapsules (43). The carboxymethyl cellulose microgel particles were used for the delivery of high molecular weight water-soluble nutrients. The lipid-walled microcapsules were used for the delivery of low molecular weight water-soluble nutrients.

The ingestion, uptake and metabolism of liposomes by juvenile C. gigas was studied by Parker (44) to assess their potential as encapsulating agents. Liposomes composed of egg phosphatidylcholine-cholesterol-stearylamine (7:1:2) formed readily and appeared stable in 20 parts per thousand (ppt) seawater. Evidence for intracellular uptake of liposomes was obtained with fluorescent microscopy after feeding oysters with liposomes containing bovine serum albumin conjugated with fluorescein isothiocyanate. The appearance of small fluorescent inclusions within the apical portions of many of the ducts and tubules of the digestive diverticula suggested phagocytosis of intact liposomes. Uptake was not observed in other parts of the alimentary canal. Additional support for uptake of liposomes came from radiotracer studies. Liposomes made with ^{14}C-radiolabeled cholesterol or phosphatidylcholine showed uptake of up to 40% of the dose in 24 hours, with the majority of uptake occurring in the visceral mass. Only slight amounts of radioactivity were observed in adductor muscle or mantle tissue. Radioactivity in lipid other than phosphatidylcholine, including triglyceride, phosphatidylethanolamine, and phosphonolipid, occurred due to breakdown of the ^{14}C-phosphatidylcholine via acyl transfer. Metabolism of liposome encapsulated substances was also

shown. The radiolabel from glucose appeared largely in a chloroform-methanol insoluble fraction, with little radioactivity recovered in the lipid or soluble aqueous fractions. The radiolabel from amino acids was recovered largely in trichloroacetic acid precipitable protein. The radiolabel from free glucose or amino acids entered the same fractions as the encapsulated radiolabel.

Enzyme Activity in Bivalves

Effect of Season on Enzyme Activities

The metabolism of bivalves in their natural habitat is seasonally variable. In M. edulis, changes have been observed for most major areas of metabolism, both in terms of molecular levels of glycogen, lipid, protein and amino acids (45) and in terms of rates of processes of nitrogen excretion (46), osmoregulation (47), pentose phosphate pathway activity and glucose utilization (48). The changes were regular and often large over a seasonal cycle and must have involved marked alterations in enzyme activity.

Seasonal changes of 17-β-hydroxysteroid dehydrogenase activity in C. gigas were followed histochemically by Mori et al. (49) to clarify the presence of the metabolism of steroid hormones associated with the sexual maturation. Oysters showed an increase in the enzyme activity in the epithelia of the nephridium and the digestive tracts as sexual maturation proceeded, and a decline after spawning.

Histochemistry also revealed seasonal distributions of some hydrolytic and mitochondrial enzymes (50). During the active feeding months it was noted that the digestive gland was especially rich in enzymes (alkaline phosphatase, acid phosphatase, non-specific esterase,

malate dehydrogenase, NAD diaphorase and cytochrome oxidase). As the epithelium of the digestive gland regressed during the winter hibernation period, activities of both classes of enzymes fell to low levels. The annual cycle of the gonad also showed marked fluctuations in levels for alkaline phosphatase and the mitochondrial enzymes.

In maturing oysters, glucose-6-phosphate dehydrogenase (G6PDH) activity was observed in the epithelia of the nephridium, digestive diverticulum and intestine. Activity was also found in the visceral ganglion, cerebro-visceral connective, genital canal and gonoduct. A decline in G6PDH activity after spawning was observed in the nephridium, intestine, digestive diverticulum and cerebro-visceral connective. These observations strongly suggested that the level of activity of G6PDH was closely related to sexual maturation. Activation of the G6PDH enzyme led to increased levels of $NADPH_2$, a reductant important in steroid synthesis (51).

To investigate the mechanism of activation of G6PDH during maturation, several investigators have studied the regulatory mechanisms of G6PDH. Eggleston and Krebs (52) found total inhibition of G6PDH and 6-phosphogluconate dehydrogenase (6PGDH) when the $NADPH/NADP^+$ ratio approached 9. Since the free nucleotide ratio in rat liver cytoplasm was found to be on the order of 100 (53), Eggleston and Krebs (52) commenced a systematic investigation to find some factor capable of counteracting the inhibition of G6PDH by NADPH. Of the many cell constituents tested, only oxidized glutathione (GSSG) showed a marked effect on G6PDH at physiological concentrations. A highly unstable cofactor was shown to be required for GSSG to display an effect. After isolation and purification from mussel hepatopancreas, the cofactor was

demonstrated to be a polypeptide of molecular weight 15,000 daltons (54). The activity of the cofactor increased with increasing concentration, however, it displayed different effects on the enzymatic forms of G6PDH and 6PGDH from mussel hepatopancreas. These results suggested that the regulation of the two dehydrogenase activities *in vivo* constituted a complex system involving the deinhibition of the two dehydrogenases, the intracellular concentration of substrates and inorganic ions and the manufacture of the two dehydrogenases in varying proportions.

Further efforts were made to determine the nature of the mechanism(s) affecting the seasonal changes in activity of G6PDH. Livingstone (55) hypothesized that one level of control might reside in the altered synthesis of the regulatory enzyme as opposed to the production of isoenzymes. This author based his hypothesis on a study conducted with mussels. While the specific activity of the mantle enzyme varied, the apparent k_m values for glucose-6-phosphate and $NADP^+$ of the mantle enzyme changed little during a year.

Another enzyme that may supply NADPH for gonad development is isocitrate dehydrogenase. When the level of this enzyme was examined in the digestive gland of *C. gigas* and *M. edulis*, the level remained much more constant throughout the year than levels of G6PDH and 6PGDH (56, 57).

The seasonal cycle of storage and utilization of glycogen reserves in adult bivalves has also been shown to be related to the annual reproductive cycle and to the available food supply. In *O. edulis*, the glycogen content increased steadily from autumn to a peak in winter. Glycogen remained constant until February and declined slowly afterwards

(58). In contrast, the glycogen reserves in M. edulis accumulated in the summer and were then lost during the autumn and early winter. In both cases the pre-stored glycogen was utilized for gametogenesis (59). Since glycogen synthesis and breakdown took place on a seasonal basis, there must be mechanisms for both short-term and long-term control of the enzymes (glycogen synthase and glycogen phosphorylase) involved in glycogen metabolism.

The two forms, I (active, independent of glucose-6-phosphate) and D (inactive, dependent on glucose-6-phosphate), of glycogen synthase were identified in the mantle tissue of M. edulis (60). Under high physiological concentrations of P_i the D form mantle glycogen synthase was inactive. Accordingly, it was the specific activity and the conversion of the the D- to I- form of glycogen synthase that controlled the "switching on" of glycogen synthesis in the summer. The total activity (I and D) of glycogen synthase increased by a factor of 2-3 in the summer and the I-form, as a percentage of the total, increased from low values, 5-10% in the winter, to a summer level of 15-25% (61). The increase in the percentage I-form is believed to be the result of feeding activity and concomitant increase in tissue glucose concentration.

Maximal hexokinase activity in M. edulis coincided with the maximum observed rates of mantle glucose utilization. Molecular variants with lower K_m's for glucose may be responsible for handling the increased glucose loads during glycogen breakdown (62).

Relationship between Growth and Enzyme Activities

One of the first works that attempted to correlate growth with enzyme activity looked at shell growth. The enzyme carbonic anhydrase

was postulated to be involved with oyster shell formation by catalyzing the hydration of CO_2 and thus making carbonate more readily available for combination with calcium ion. The strongest supporting evidence for this view was the fact that low concentrations of acetazolamide and 2-benzothiazolesulfonamide, specific and strong carbonic anhydrase inhibitors, blocked oyster shell formation (63). More recent evidence, however, showed that the primary function of carbonic anhydrase was not shell formation. Tenfold higher activities of carbonic anhydrase were found in the blood rather than the tissues suggesting carbonic anhydrase may primarily be involved in respiration, maintenance of ionic strength of the blood or maintaining proper blood pH (64).

Acid phosphatase is another enzyme believed to be involved in shell deposition. Chambers et al. (65) found that acid phosphatase activity decreased moderately with increasing weight of oyster, probably reflecting a decrease in shell deposition as the organism ages.

The search for an index to estimate the instantaneous growth rate of marine animals led Bergeron and Alayse-Danet (66) to measure the specific activity of aspartate transcarbamylase (ATC) in scallop, Pecten maximus. ATC catalyzes the first specific enzymatic reaction for the biosynthesis in vivo of the pyrimidine bases, fundamental units of nucleic acids. Since any cellular multiplication taking place will require the production of nucleic acids, variations in levels of ATC activity may reflect variations in growth rate. In the scallop a correlation between the ATC specific activity and the relative growth rate was demonstrated.

The Uptake and Metabolism of Radiolabeled Phosphatidylcholine from an Egg Yolk-Starch Diet by Juvenile Pacific Oysters (*Crassostrea gigas*)

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ABSTRACT

Vesicles prepared from egg yolk were shown to encapsulate protein and to be in a size range that would be filtered by the oyster. A radiotracer study involving addition of radiolabeled phosphatidylcholine to egg yolk demonstrated that the egg yolk vesicles were taken up and metabolized by juvenile Crassostrea gigas. Catabolism of the radiolabeled lipid and subsequent resynthesis into non-lipid components occurred to a slight extent. The main factor responsible for the distribution of radioactivity amongst the lipids in the stomach tissue was believed to be transacylation.

INTRODUCTION

Although oysters have been cultured for centuries, rising levels of environmental contamination and a decrease in availability of natural growing areas has led to an increased interest in growing oysters in artificial culture systems. These systems have the potential to be very economical in terms of providing a source of animal protein since oysters as poikilotherms appeared to utilize more assimilated energy in growth than homiotherms (67).

Shellfish culture in artificial systems has generally depended upon supplies of unicellular algae as food sources for both larvae and juveniles. However, to maintain adequate supplies of algae, a considerable investment in trained manpower, equipment and space was required. In addition, it has not been possible to determine the nutritional requirements of oysters due to the unsuccessful attempts to correlate differences in oyster growth with the chemical composition of an algal species (23).

There have been many attempts to develop satisfactory artificial diets for marine bivalves. However, growth of the animals was considerably less than that of controls fed on algae or kept under natural conditions in the field (32). One of the main problems with artificial diets was developing a particulate food system that was both digestible and within a certain particle size range (2-100 μm in diameter) that could be filtered and ingested by the bivalves (42,43). The design of microencapsulated diets has attempted to alleviate this problem and allow more efficient uptake of dissolved nutrients.

Microcapsules formed from crosslinked nylon-protein or modified egg protein underwent only very slight digestion in the stomach of oysters and gave limited growth compared to oysters fed an algal diet (42). Langdon and Siegfried (43) had better success by feeding oysters a mixture of carboxymethyl cellulose microgel particles, used in the delivery of high molecular weight water-soluble nutrients, and lipid-walled microcapsules for the delivery of low molecular weight water-soluble nutrients. When the diet was continuously fed to oysters held in beakers, growth rates as high as 73% that of algal-fed controls were obtained. The lower growth rates may have been due to the use of sonication in the preparation of the microcapsules. High-energy sonication has been reported by Hauser (68) to cause oxidation and degradation of phospholipid. Damage to the solute molecules it was desired to encapsulate may also have occurred during sonication.

One microcapsule diet, liposomes, has been prepared without sonication (44). The successful demonstration of the encapsulation of dissolved nutrients as well as the uptake and metabolism of liposomes by oysters points to the potential for liposomes to be used in raising oysters. Since the costs of preparing liposomes from purified lipid may be too costly, egg yolk could prove to be a suitable alternative. With one of the key components in egg yolk being lipid, the idea was generated that vesicles could be made that would be similar to liposome vesicles. This paper describes the preparation of vesicles from egg yolk. The ingestion and metabolism of these egg yolk vesicles by Crassostrea gigas is demonstrated using fluorescent and radiotracer methods.

MATERIALS AND METHODS

Preparation of Fluorescent Liposomes

Fluorescent liposomes were prepared with fifteen μmoles of lipid, consisting of egg PC*/cholesterol/stearylamine in the molar proportion of 7:1:2 (P-L Biochemicals, Inc., Milwaukee, WI). The material was evaporated from chloroform to a thin film on the wall of a round bottom flask. Five ml of a solution of 27 ppt synthetic seawater, Instant Ocean (Aquarium Systems, Mentor, Ohio), containing 1 mg FITC-BSA and several glass beads were added and the flask swirled for one hour. Unentrapped FITC-BSA was removed by centrifuging the liposomes in an IEC International clinical centrifuge [315 x g] and replacing the supernatant with 27 ppt Instant Ocean. After several repetitions, no fluorescence was observed in the filtrate. The vesicles were diluted to 200 ml with 30 ppt Instant Ocean.

Preparation of Fluorescent Egg Yolk Vesicles

Fluorescent egg yolk vesicles were prepared by placing 0.2 gm of

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*Abbreviations used are: PC, phosphatidylcholine; ppt, parts per thousand; FITC-BSA, fluorescein isothiocyanate bovine serum albumin; ^{14}C-PAPC, phosphatidylcholine, L-α-1-palmitoyl-2-arachidonyl [arachidonyl-1-^{14}C]; TLC, thin layer chromatography; NL, neutral lipids; PPL, phosphonolipid; CP, choline phospholipids; EP, ethanolamine phospholipids; SP, serine phospholipids; ^{14}C-DPPC, di[1-^{14}C] palmitoyl phosphatidylcholine; CL, cardiolipin; AA, arachidonic acid; IP, inositol phospholipids; SPH, sphingomyelin.

fresh egg yolk in a 30 ml homogenizing tube and adding 20 ml of a solution of 30 ppt Instant Ocean containing FITC-BSA (10 mg), corn starch (96 mg), thiamin-HCl (3.9 μg), riboflavin (5.8 μg), niacin (39 μg), D-biotin (0.4 μg), Na-pantothenate (9.7 μg), pyridoxine (3.9μg), folic acid (1.9 μg), vitamin B_{12} (0.04 μg), myo-inositol (80 μg) and ascorbic acid (29μg). All chemicals were purchased from Sigma, St. Louis, MO. The materials were mixed with a high speed tissue homogenizer for 10 to 20 seconds. Unentrapped FITC-BSA was removed by centrifuging the egg yolk vesicles in an IEC International clinical centrifuge and replacing the supernatant with 30 ppt Instant Ocean. After several repetitions, no fluorescence was observed in the filtrate.

Preparation of Radiolabeled Egg Yolk Vesicles

Radiolabeled egg yolk vesicles were prepared by placing 0.21 gm of fresh egg yolk and 10 μC of ^{14}C-PAPC (New England Nuclear, Boston, MA) in a 30 ml homogenizing tube. After adding 21.0 ml of 30 ppt Instant Ocean containing 0.105 gm corn starch, the solution was mixed for 1 minute with a high speed tissue homogenizer. The solution was then diluted with 30 ppt Instant Ocean to a final volume of 210 ml.

Feeding and Rearing of Oysters

Juvenile Pacific oysters, C. gigas, weighing 1.5 to 2.0 gm, were obtained from Ted Kuiper in Eureka, CA. The oysters were placed on a screen mesh 2 inches above the bottom of a large bucket containing 20 liters of 30 ppt Instant Ocean at 19°C. Once a day for a month the oysters were fed unlabeled egg yolk vesicles.

For the fluorescent labeled study, six oysters were held in a 3 liter beaker containing 2 liters of 30 ppt Instant Ocean. The oysters were placed on a screen mesh one inch above the bottom of the beaker. Water, maintained at 19°C, was aerated and circulated using an airstone and a Whisper 800 airpump (Willinger Bros, Fort Lee, NJ). Twice a day fluorescent egg yolk vesicles were added to the beaker (2.5 ml/oyster).

For the radiotracer study, 80 oysters starved for 24 hrs, were placed on a plastic tray (1.0 mm mesh) suspended one inch above the bottom of a dish pan (10 in X 14 in) containing 7 liters of 30± 1 ppt Instant Ocean. The water was maintained at 19°C in a temperature controlled room. An airlift pump and 3 airstones attached to a Whisper 800 airpump were used to aerate and circulate the water. Oysters were fed the radiolabeled egg yolk vesicles twice a day for 3 days. After transfer to fresh Instant Ocean (30 ppt), the oysters were fed unlabeled egg yolk vesicles, twice a day, for 5 days.

Analysis of Fluorescent Tissue and Vesicles

Oysters fed the fluorescent egg yolk vesicles were examined at intervals of 1, 2 and 3 days. The digestive tissue was dissected from the oyster and fixed for 48 hrs in Davidson's fixative (see Appendix I). Tissues were dehydrated in ethanol and xylene and embedded in paraffin using an automated Tissue Tek II processor (Sakura Finetechnical, Tokyo, Japan). Sections were cut at 6 μm through the tissue, placed on a slide and covered with a coverslip using a glycerol (pH 8.5) wet mount.

Fluorescent microscopy was performed on the liposomes, egg yolk vesicles and tissue sections using a Zeiss Universal microscope.

Photographs were taken using Ektachrome 400 ASA/27 Din film. An ocular micrometer was used to measure the size of liposomes and vesicles.

Analysis of Radiolabeled Tissue and Egg Yolk

Analysis of oysters after 0, 1, 3 and 8 days consisted of removing 24 oysters from the container and dividing them into two groups. Assuming equal access of oysters to the radiolabeled food, each oyster from day 1 had been exposed to 1.53×10^5 dpms and each oyster from days 3 and 8 had been exposed to 3.71×10^5 dpms. After each oyster was weighed, the oyster was dissected. The mantle, muscle and gills of each group were pooled and weighed. In addition, the digestive tissues of each group were pooled and weighed. Moisture content was determined by lyophilization of the pooled tissues.

Lipid extraction of the lyophilized tissue was performed according to the method of Folch (69). Lipid was also extracted from radiolabeled egg yolk vesicles. Material insoluble in the chloroform-methanol phase was collected on a glass wool packed column, digested in NCS solubilizer (Amersham, Arlington Heights, IL), and counted for radioactivity using Bray's scintillation solvent system (70). Samples of both aqueous phase and lipid extract were also measured for radioactivity using the liquid scintillation medium 3a70B (Research Products International Corp., Mount Prospect, IL).

Two dimensional TLC was used to separate individual phospholipids on Silica Gel H plates (0.25 mm thickness). The first dimension solvent system consisted of chloroform:methanol:ammonia (65:25:4, v/v/v). The second dimension solvent system consisted of

chloroform:methanol:ammonia (100:50:12, v/v/v). Exposure to iodine revealed the location of each phospholipid as well as NL. All phospholipids with the exception of PPL were identified by comparing to purified phospholipid standards subjected to the same solvent systems. The PPL spot was identified by the procedure of Stillway and Harmon (71).

Quantitation of individual phospholipids from the day 0 samples was accomplished by the phosphorus assay of Bartlett's (72). Individual phospholipids from radiolabeled lipid samples were scraped into vials, hydrated with water and counted for radioactivity in 3a70B.

Neutral lipids from radiolabeled samples were separated on Silica Gel H plates (0.25 mm thickness) by one dimensional TLC. The solvent system employed was hexane:ethyl ether:glacial acetic acid (80:20:1, v/v/v). Bands 0.5 cm in width were scraped into vials, hydrated with water, and counted for radioactivity in 3a70B.

Quantitation of triacylglycerols from the 0 day samples was accomplished by applying each lipid sample to Silica Gel H plates prepared with 0.04% sodium fluorescein. After running the plate in the neutral solvent system described above, the triacylglycerol spot was detected under fluorescent light and scraped into a test tube. After eluting the triacylglycerols from the silica gel with chloroform, the eluates were evaporated to dryness and then quantified according to the procedure of Van Handel and Zilversmit (73).

Total lipid from the mantle, gill and muscle tissue and total lipid from the digestive tissues were analyzed for distribution of

radioactivity between the acyl portion and the glycerol-base backbone. A portion of each lipid sample was hydrolyzed with 4% H_2SO_4 and the solution extracted with hexane. Samples of both the aqueous and organic phases were taken for liquid scintillation counting.

The fatty acid compositions of NL and of each phospholipid from the digestive tissues and from the gill, mantle and muscle tissues were determined for day 0 oysters. The lipids were subjected to methanolysis for 90 minutes in 4% H_2SO_4 in methanol (90°C). The methyl esters formed were extracted with hexane, dried over anhydrous sodium sulfate and purified by development in benzene on thin layer plates (Silica Gel H).

Gas liquid chromatography of the esterified fatty acids was done with a Varian Aerograph Model 1200 gas chromatograph equipped with a Hewlett-Packard 3380A computing integrator. The column was packed with 10% SP-2330 on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, PA). Chromatographic peaks were identified by comparison of their relative retention times with those of standards subjected to the same conditions.

RESULTS AND DISCUSSION

Encapsulation and Ingestion of Fluorescent Egg Yolk Vesicles

FITC-BSA-containing liposomes shown in Figure 1 resembled those of liposomes prepared by Parker (44). It was demonstrated (Figure 2) that egg yolk vesicles prepared with FITC-BSA were similar to liposomes in shape and size. Most of the fluorescence appeared to be intravesicular indicating that the protein was encapsulated or trapped within the internal aqueous space of the vesicle. Absorption of protein may have occurred to a slight extent to the outside of the vesicle. However, the high ionic strength of the synthetic seawater should have reduced the absorption of protein (74).

Ingestion of the fluorescent egg yolk vesicles by oysters is shown in Figure 3. It was not possible to distinguish whether or not this material had been absorbed by the digestive tissue. Alternatively, the material could have been in the process of being rejected as feces, as was the fate for kaolin (43).

Analysis of Lipids from Oyster Tissue

Initial analysis of oyster tissues revealed that approximately 81% of the weight was water (Table 1). These values corresponded to those obtained with pre-starved C. gigas (75). The recovery of lipid from total oyster agreed closely to recoveries obtained by Watanabe and Ackman (76) for Crassostrea virginica and Ostrea edulis.

Less than 10% of the lipid from total oyster was found in the triacylglycerol fraction. In contrast, C. virginica and O. edulis have been reported to contain at least 20% of their lipid as triacylglycerol (76). As oysters showed decreased triacylglycerol

levels when under physiological stress (77), the low content of triacylglycerol found in the present study would suggest that the nutritional state of the oysters was poor. Species differences, on the other hand, may have been responsible for the altered lipid distribution.

The phospholipid distribution in lipids of oyster tissues is shown in Table 2. The major phospholipids were CP and EP followed by SP. The percentage of PPL in total oyster was less than the PPL content measured for C. virginica (78). The findings by Swift suggested that the PPL content in tissues would increase when oysters were starved. If this were the case for C. gigas, the data indicated that these oysters were not starved.

The major fatty acids found in the lipid of oyster tissue is detailed in Table 3. Compared to the fatty acid composition found for C. gigas by Langdon and Waldock (79), the data agreed fairly well for C16 and C22 fatty acids. The main discrepancy appeared in the percentage of C18 fatty acids. The large amounts of C18 fatty acids found in this study may be due to the high amounts of these acids in egg yolk (80). Oysters had been maintained on an egg yolk diet for one month. After this amount of time, the lipid components in the oyster tissue could have been modified. More specifically, the triacylglycerol components would have been modified. The fatty acid composition of this lipid has been shown to reflect that of the diet (79).

Incorporation of Radioactivity from Egg Yolk Vesicles

Lipid from egg yolk vesicles prepared with ^{14}C-PAPC was subjected to two dimensional TLC to establish the distribution of the radiolabeled lipid into egg yolk vesicles. Greater than 97% of the radioactivity was found in CP indicating no degradation of the radiolabel during the preparation of vesicles.

Oysters sacrificed after one day recovered 12.7% of the dpms from the radiolabeled food compared to a recovery of 22.8% by oysters fed ^{14}C-DPPC-containing liposomes for one day (44). A slightly greater recovery of radiolabel was shown after 3 days when oysters were fed the ^{14}C-PAPC containing egg yolk vesicles (12.8%), than when they had been fed ^{14}C-DPPC-containing liposomes (10.6%). Oysters from day 8 were found to contain 16% of the exposed dose.

Seventy-seven percent of the label fed to oysters was unaccounted for in oyster tissue or the culture vessel water. This unaccounted label could have been located in expired $^{14}CO_2$ or in settled matter. Inadequate suspension of the egg yolk vesicles could have contributed to large amounts of label in settled matter. Langdon and Siegfried (43) reported that aeration was not always satisfactory as a means of agitation because the turbulence produced tended to be localized around the bubble source and food settlement occurred in less disturbed areas of the culture vessel. In addition, particle clumping would have led to increased amounts of label in settled matter. Previous to feeding, egg yolk vesicles were found to be 5-30μm in diameter. While these particle diameters were capable of being filtered and ingested, clumping of particles may have prevented their ingestion. Enhancement of bacterial activity by

addition of foods to culture systems could have resulted in the production of bacterial films which would have increased particle clumping and adherence of particles to surfaces (81).

The radiolabel was distributed in approximately equal portions between the stomach tissue and that of the gill, mantle and muscle tissue. Transport of the radiolabel from the stomach to the other tissues probably occurred via the blood. Allen and Conley (82) found that after ingestion of 1-^{14}C-palmitate, the specific activities of plasma and hemocyte lipids rose over a period of 10 hours and then maintained roughly steady levels. In addition, they found radioactivity in several lipid classes having only blood contact with the digestive tract, thus supporting the premise of transport of lipid through the hemolymph.

Metabolism of Radiolabeled Phosphatidylcholine

The distribution of radioactivity from oyster tissues after lipid extraction is shown in Table 4. The majority of radioactivity was found in the lipid phase. However, recovery of radioactivity in the aqueous and insoluble phase implied that metabolism of the ^{14}C-PAPC had occurred and the metabolites were shunted into non-lipid components. This type of activity appeared to have occurred to a much greater extent in the gill, mantle and muscle tissue than in the stomach tissue.

Analysis of Lipids from Radiolabeled Oyster Tissue

After separation by two dimensional TLC, the distribution of radioactivity among the phospholipids was determined (Tables 5 and 6). The results were first expressed in terms of specific activity,

dpms/μg lipid. The lipid in stomach tissue having the highest selectivity for the radiolabel was SP, followed by triacylglycerol, EP and CL. It was not possible in the present study to evaluate the degree of selection taken by CP in stomach tissue due to the flooding of its pool with ingested labeled PC. In gill, mantle and muscle tissue, CL was shown to have the highest selectivity for the radiolabel, followed by CP for days 1 and 3. The higher specific activity of SP than CP after 8 days may reflect the smaller pool size of SP. Degradation of similar quantities of cold CP and SP would raise the specific activity of SP to a higher degree than CP.

By considering the total amount of radiolabel present in the individual lipids, it was shown that after one day, only 27% of the radiolabel remained in CP in the stomach tissue. In this tissue, SP, EP and triacylglycerol were the other lipids with a major portion of the radiolabel. In gill, mantle and muscle tissue, 40-50% of the radiolabel remained in CP. EP and SP were the other lipids with a large percentage of the radioactivity.

Analysis of Radiolabeled Transesterified Lipid

To provide information about the routes by which radioactivity was incorporated into oyster lipids, the total lipid in each tissue was examined for intramolecular distribution of the label. In the stomach tissue, 60-75% of the radiolabel was found in the hexane soluble portion or fatty acid moiety (Table 7) compared to 100% found when oysters were fed ^{14}C-DPPC-containing liposomes (44). Transacylation could have been the main process used to transfer the radiolabeled fatty acid from PC to the other lipids in stomach tissue.

As the radiolabel was originally in AA, it was possible that the acyltransferases were acting to distribute this fatty acid to those lipids which later would release the fatty acid for conversion into prostaglandins. Substances resembling prostaglandins have been found in tissues of scallops (83). In addition, binding sites for prostaglandins have been observed in the gill tissue of the bivalve, Modiolus demissus (84). Therefore, one could hypothesize that the specific activity of a lipid would reflect its content of AA and its ability to serve as a precursor for prostaglandins. However, the content of AA (20:4) in the phospholipids did not explain the specific activities found for the individual lipids (Table 8). Rather the degree of unsaturation (sum polyunsaturated) of the phospholipids reflected the specific activities found (Figure 4). A regression coefficient of 0.953 was found between the polyunsaturated content and the specific activity of the phospholipids in stomach tissue from oysters analyzed after 8 days. It is suggested that the fluidity of the tissue membranes regulated the activity of the acyltransferases in their transfer of radiolabeled fatty acids (85).

For the gill, mantle and muscle tissue, 47 to 54% of the radioactivity was found in the fatty acid moiety (Table 7). Since a large portion of the radioactivity was found in the glycerol backbone, it was not possible to correlate the specific activities found in these tissues with the polyunsaturated fatty acid content of the lipid (Table 9).

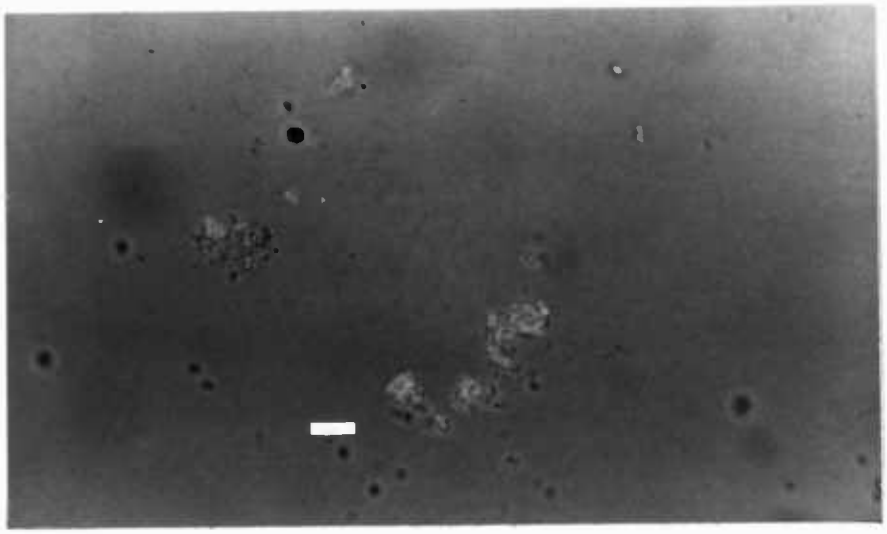

Figure 1. Optical characteristics of liposomes containing FITC-BSA as viewed with fluorescent microscopy. Bar equals 10μm.

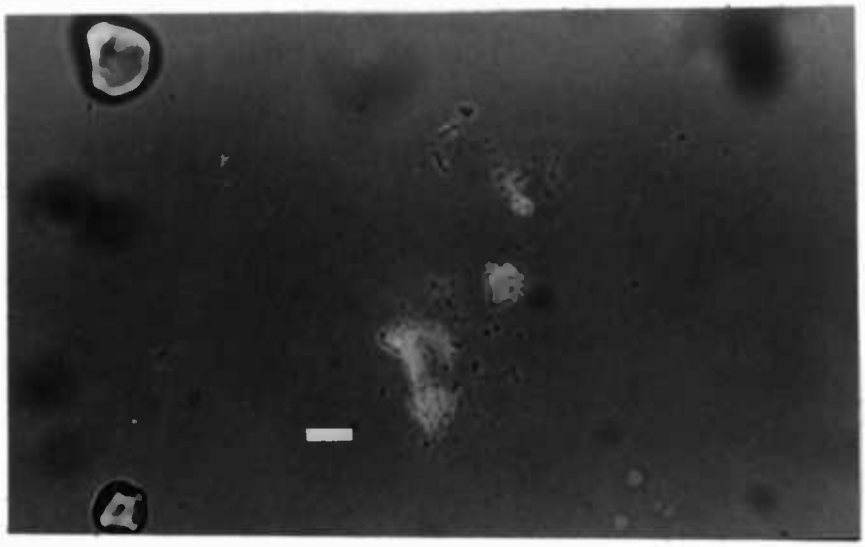

Figure 2. Optical characteristics of egg yolk vesicles containing FITC-BSA as viewed with fluorescent microscopy. Bar equals 10μm.

Figure 3. Paraffin-embedded section of digestive tissue from oysters fed FITC-BSA-containing egg yolk vesicles as viewed with fluorescent microscopy.

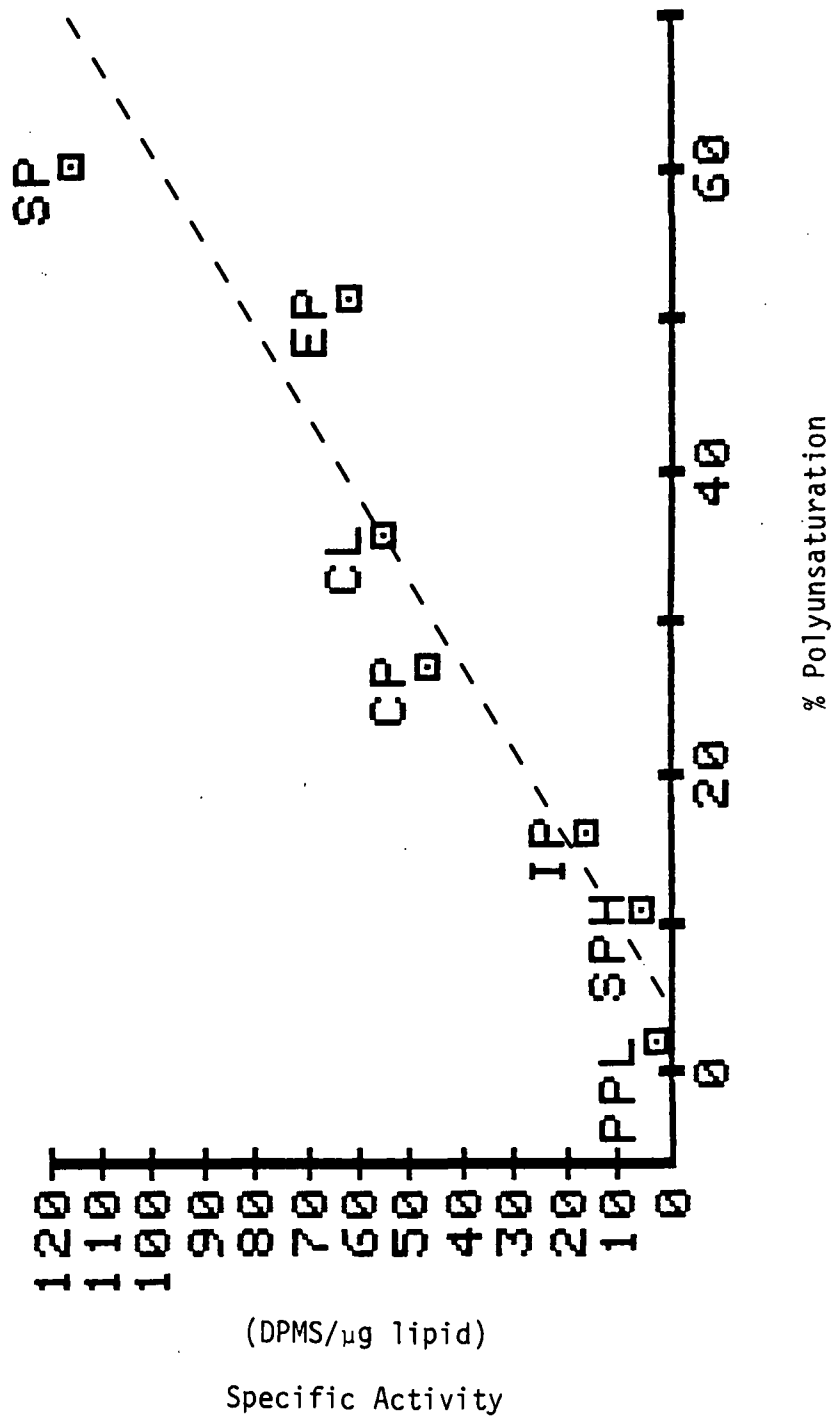

Figure 4. Relationship between % polyunsaturated fatty acids and the specific activity in individual phospholipids.

TABLE 1

Moisture and Lipid Content of Oysters[a]

	Tissue		
	Total Oyster	Stomach	Gill, Mantle, Muscle[b]
Moisture (%)	81.9	80.3	81.7
Lipid (% of wet wgt)	1.6	2.7	1.4
Triacylglycerols (% of total lipid)	9.2	16.5	7.8
Polar Lipids (% of total lipid)	68.0	55.1	71.4

[a]Each value is an average of 2 groups with 12 oysters in each group.

[b]Tissues were pooled before analysis.

TABLE 2

Distribution of Phospholipids in Oysters

	Tissue		
	Total Oyster	Stomach	Gill, Mantle, Muscle[a]
	% of total phospholipids		
Inositol phospholipids	2.8	3.2	2.9
Serine phospholipids	14.3	14.1	14.2
Sphingomyelin	6.2	4.8	6.5
Phosphonolipid	15.4	13.7	15.8
Choline phospholipids	29.4	32.9	28.4
Ethanolamine phospholipids	30.0	29.0	30.4
Cardiolipin	1.9	2.3	1.8

[a]Tissues were pooled before analysis.

TABLE 3

Major Constituent Fatty Acids of the Total Lipid From Juvenile Pacific Oyster (*C. gigas*)

Fatty Acid[a]	Wt. %
16:0	20.40
16:1	3.12
18:0	14.79
18:1	26.50
18:2	4.26
18:3	10.96
20:4	6.49
20:5	3.23
22:4	0.89
22:5	1.17
22:6	8.19

[a]Fatty acid identified by number of carbon atoms and double bonds.

TABLE 4

Distribution of Label in Various Tissues from Oysters Fed Egg Yolk Vesicles Containing Phosphatidylcholine, L-α-1-Palmitoyl-2-Arachidonyl [Arachidonyl-1-^{14}C][a]

	Day		
	1	3	8
	% of total dpms in tissue		
	Stomach		
Lipid phase	96.1	94.6	95.2
Aqueous phase	1.2	2.1	1.3
Insoluble phase	2.7	3.3	3.5
	Gill, Mantle, Muscle[b]		
Lipid phase	85.9	82.7	87.9
Aqueous phase	3.0	3.2	3.3
Insoluble phase	11.1	14.1	8.8

[a]Data from 12 oysters per group, two groups analyzed.

[b]Tissues were pooled before analysis.

TABLE 5

Specific Activities of Lipids Isolated from the Stomach Tissue of Oysters Fed Egg Yolk Vesicles Containing Phosphatidylcholine, L-α -1-Palmitoyl-2-Arachidonyl [Arachidonyl-1-^{14}C]

	Oay		
	1	3	8
	Opms/ g		
Inositol phospholipids	12.3 (1.3)[a]	24.1 (0.9)	16.0 (0.7)
Serine phospholipids	43.8 (20.7)	120.7 (19.8)	115.3 (22.8)
Sphingomyelin	2.4 (0.4)	6.0 (0.3)	5.6 (0.4)
Phosphonolipid	1.3 (0.6)	2.9 (0.5)	2.0 (0.4)
Choline phospholipids	24.4 (27.1)	83.5 (32.2)	46.4 (21.6)
Ethanolamine phospholipids	22.6 (22.1)	68.3 (23.2)	61.7 (25.2)
Cardiolipin	21.7 (1.7)	48.7 (1.3)	55.2 (1.8)
Triacylglycerol	27.0 (25.1)	72.8 (20.8)	68.6 (26.0)

[a]Numbers in parenthesis represent the % of radioactivity from total lipid extract present in the individual lipids.

TABLE 6

Specific Activities of Lipids Isolated from the Gill, Mantle and Muscle Tissue[a] of Oysters Fed Egg Yolk Vesicles Containing Phosphatidylcholine, L-α -1-Palmitoyl-2-Arachidonyl [Arachidonyl-1-^{14}C]

	Day		
	1	3	8
	Dpms/ g		
Inositol phospholipids	3.2 (1.4)[b]	5.1 (0.8)	8.8 (1.1)
Serine phospholipids	9.5 (19.4)	26.2 (19.8)	41.6 (24.2)
Sphingomyelin	0.5 (0.5)	1.2 (0.4)	2.3 (0.6)
Phosphonolipid	0.3 (0.7)	0.6 (0.5)	1.0 (0.6)
Choline phospholipids	11.6 (47.3)	32.8 (49.6)	35.0 (40.9)
Ethanolamine phospholipids	3.6 (15.7)	9.7 (15.7)	14.6 (18.3)
Cardiolipin	16.6 (4.3)	51.2 (4.9)	79.8 (5.9)
Triacylglycerol	3.9 (8.2)	8.8 (5.8)	11.3 (5.8)

[a]Tissues were pooled before analysis.

[b]Numbers in parenthesis represent the % of radioactivity from total lipid extract present in the individual lipids.

TABLE 7

Distribution of Label Between the Hexane and Water Soluble Fractions after Transesterification of Lipids from Various Tissues of Oysters Fed Egg Yolk Vesicles Containing Phosphatidylcholine, L-α-1-Palmitoyl-2-Arachidonyl [Arachidonyl-1-^{14}C]

	Day		
	1	3	8
	% of total dpms in transesterified lipid		
	Stomach		
Hexane soluble	61.1	nd[a]	76.3
Water soluble	38.9	nd	23.7
	Gill, Mantle, Muscle[b]		
Hexane soluble	47.6	51.1	54.4
Water soluble	52.4	48.9	45.6

[a]Not determined.

[b]Tissues were pooled before analysis.

TABLE 8

Constituent Fatty Acids [Weight %] of Various Lipids from the Stomach Tissue of Juvenile Pacific Oyster (*C. gigas*)

Fatty Acid[a]	EP	CP	SP	IP	CL	PPL	SPH	NL
14:0	-	-	-	11.50	1.24	6.95	18.10	2.02
16:0	9.62	22.75	3.61	17.27	12.68	46.34	16.01	9.81
16:1	2.49	3.30	-	-	-	6.00	-	-
17:0	-	-	-	-	-	2.09	-	-
18:0	18.25	8.12	23.55	29.39	15.73	24.35	25.95	12.20
18:1	18.01	38.82	12.43	25.65	34.61	11.78	28.86	53.16
18:2	3.81	7.84	1.54	3.03	2.13	1.25	5.46	10.41
18:3	7.24	2.68	27.43	6.91	4.02	-	2.24	3.23
20:4	6.17	5.66	16.08	3.59	10.99	1.25	3.37	3.89
20:5	9.36	4.55	9.63	-	4.30	-	-	3.04
22:4	2.39	-	-	2.65	-	-	-	-
22:5ω6	3.33	-	1.16	-	-	-	-	-
22:5ω3	2.69	-	-	-	-	-	-	-
22:6	16.64	6.27	4.58	-	14.31	-	-	2.25
Sum Polyunsaturated	51.63	27.00	60.42	16.18	35.75	2.50	11.07	22.82

[a]Fatty acid identified by number of carbon atoms and double bonds.

TABLE 9

Constituent Fatty Acids [Weight %] of Various Lipids from the Gill, Mantle and Muscle Tissue[a] of Juvenile Pacific Oyster (C. gigas)

Fatty Acid[a]	EP	CP	SP	IP	CL	PPL	SPH	NL
14:0	-	-	-	13.74	13.20	3.88	17.81	5.24
16:0	6.78	19.80	3.80	7.31	12.91	68.56	13.55	14.42
16:1	2.80	3.91	1.06	-	1.38	6.81	-	2.52
17:0	-	-	-	-	-	2.35	-	-
18:0	18.28	6.21	28.22	30.01	16.60	12.13	32.05	19.90
18:1	16.79	30.78	10.08	23.59	25.48	6.28	27.93	33.29
18:2	2.75	7.92	-	2.08	4.22	-	2.87	7.24
18:3	12.49	4.15	34.99	20.97	-	-	4.57	5.33
20:4	5.49	7.76	13.32	2.29	-	-	1.22	6.75
20:5	5.02	9.20	6.36	-	-	-	-	2.18
22:4	2.36	-	-	-	1.56	-	-	-
22:5ω6	4.12	-	-	-	-	-	-	-
22:5ω3	2.14	-	-	-	1.13	-	-	-
22:6	20.97	10.26	2.18	-	23.52	-	-	3.13
Sum Polyunsaturated	55.34	39.29	56.85	25.34	30.43	0.00	8.66	24.63

[a]Tissues were pooled before analysis.

[b]Fatty acid identified by number of carbon atoms and double bonds.

A Comparison of Aspartate Transcarbamylase Activity in Juvenile Pacific Oysters (*Crassostrea gigas*) When Fed Various Diets

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ABSTRACT

The optimum pH for measurement of aspartate transcarbamylase activity in oyster tissue was determined to be 9.35 while the optimum temperature was 39.5°C. Aspartate transcarbamylase activity varied significantly over short periods of time (hrs) possibly due to fluctuations in the amount of food digested. The composition of the oyster's diet also affected the levels of aspartate transcarbamylase activities in oyster tissues. Those oysters fed an egg yolk-starch diet contained significantly lower aspartate transcarbamylase activities than oysters fed an egg yolk-starch-salmon oil diet or a casein-starch-salmon oil diet. The aspartate transcarbamylase activities in oysters fed Phaeodactylum tricornutum or a starch diet were not significantly different from the activities in oysters fed the egg yolk-starch diet.

INTRODUCTION

The metabolism of bivalves in their natural habitat is seasonally variable. In Mytilus edulis, changes have been observed for most major areas of metabolism, both in terms of molecular levels of glycogen, lipid, protein and amino acids (45) and in terms of rates of processes of nitrogen excretion (46), osmoregulation (47), pentose phosphate pathway activity and glucose utilization (48). The changes were regular and often large over a seasonal cycle and must have involved marked alterations in enzyme activity.

Seasonal changes in enzyme activity of bivalves have occurred in response to the reproductive cycle (49, 51), and to the available food supply (61). The total activity (I and D) of glycogen synthase increased by a factor of 2-3 in the summer and the I-form, as a percentage of the total, increased from low values, 5-10% in the winter, to a summer level of 15-25%. The increase in the percentage I-form was believed to be the result of feeding activity and concomitant increase in tissue glucose concentration.

As the first step in the biosynthesis of the pyrimidine bases, aspartate transcarbamylase (ATC) has been found to be particularly active in the tissues of rapid growth (86-88). Moreover, Bergeron and Alayse-Danet (66) found a correlation between the ATC specific activity and the relative growth rate of mantle tissue in scallops. Since growth is a reflection of the food digested and assimilated, the present study was conducted to examine the response of the ATC enzyme in oysters to diets that were quantitatively and qualitatively different.

MATERIALS AND METHODS

Preparation of the Diets

A vitamin mix was prepared containing 20 mg thiamin, 30 mg riboflavin, 200 mg niacin, 2 mg D-biotin, 50 mg Na-pantothenate, 20 mg pyridoxine, 10 mg folic acid, 200 µg vitamin B_{12}, 400 mg myo-inositol and 150 mg ascorbic acid. Twenty-three mg of this vitamin mix was added to 2.5 liters of 30 parts per thousand (ppt) Instant Ocean. This solution was designated Soln. "A".

An egg yolk-starch diet was prepared by placing 0.20 gm of fresh egg yolk in a 30 ml homogenizing tube and adding 20 ml of Soln. "A" containing 104 mg corn starch. The materials were mixed with a high speed tissue homogenizer for 10 to 20 seconds, then frozen.

An egg yolk-starch diet supplemented with salmon oil was prepared by first adding 20 mg salmon oil to 1 gm egg yolk. Then 0.20 gm of the egg yolk-oil mixture was placed in a 30 ml homogenizing tube along with 20 ml of Soln. "A" containing 97.6 mg corn starch. The materials in the tube were mixed with a high speed tissue homogenizer for 10 to 20 seconds, then frozen.

A diet consisting of casein, starch and salmon oil was prepared by placing 73 mg salmon oil in a 30 ml homogenizing tube and adding 20 ml of Soln. "A" containing 104.4 mg corn starch and 36.4 mg of a casein hydrolysate (Sigma, St. Louis, MO). These materials were mixed with a high speed tissue homogenizer for 10 to 20 seconds, then frozen.

A starch diet was prepared by adding 2.60 gm corn starch to 500 ml Soln. "A". The diet was kept at 4°C until used.

The algae, Phaeodactylum tricornutum, was cultured in F/2 medium (89) in 1 liter Erlenmeyer flasks at 20°C under white fluorescent light. Algae used in the diet were from the stationary phase of culture. Before feeding the oysters with P. tricornutum, the algae were concentrated to 2.35 x 10^8 cells/ml by centrifugation. This algae concentrate was kept at 4°C throughout the experiment except when aliquots were withdrawn to feed to the oysters.

Analysis of Diets

Percent dry weight of egg yolk was determined by lyophilization of aliquots (0.50 to 0.65 gm) of fresh egg yolk. The dry weight of P. tricornutum (pg/cell) was determined by filtering a volume of algal culture of known concentration through a preweighed filter capable of removing particles greater than 0.40 μm. The filters were washed with 300 ml of 0.5 M ammonium formate, dried at 110°C for 2 hr, and then weighed.

Egg yolk and algae were both subjected to lipid extraction by the method of Folch et al (69). These extracted lipids in addition to salmon oil were transesterified to determine their fatty acid composition. Transesterification consisted of heating aliquots of the lipid for 90 minutes at 90°C in the presence of 4% H_2SO_4 in methanol. The methyl esters formed were extracted with hexane, dried over anhydrous sodium sulfate, and purified by development in benzene on thin layer plates (Silica Gel H). Gas liquid chromatography of the esterified fatty acids was done with a Varian Aerograph Model 1200 gas chromatograph equipped with a Hewlett-Packard 3380A computing integrator. The column was packed with 10% SP-2330 on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, PA).

Chromatographic peaks were identified by comparison of their relative retention times with those of standards subjected to the same conditions.

Analysis of the protein content of egg yolk and algae consisted first of weighing 0.15 gm of fresh egg yolk and homogenizing the material in 8 ml 0.1 N NaOH. Triplicate aliquots (25 µl) of this egg yolk mixture and triplicate aliquots (50 µl) of the concentrated algae diet were analyzed for protein by the Lowry procedure (90, 91).

Experimental Design Used in Feeding Diets to Oysters

Juvenile Pacific oysters, Crassostrea gigas, weighing 0.85 to 2.40 gm, were obtained from Ted Kuiper in Eureka, CA. For each diet analyzed, five oysters were placed on a mesh screen one inch above the bottom of a 3 liter beaker. Two liters of 30 ppt Instant Ocean, filtered to remove particles greater than 0.2 µm, were added to the beaker. Water was aerated and circulated using an airstone and a Whisper 800 airpump (Willinger Bros., Fort Lee, NJ). The temperature of the water was maintained at 20°C by placement of the beakers in a temperature controlled room.

Upon arrival to the laboratory, the oysters were cleaned with a brush, placed in beakers and starved for 3 to 5 days before the start of an experiment. Oysters were then fed three times per day. With the exception of the second experiment, daily rations for oysters were predicted from a modification of the equation used by Epifanio (28): $Q_R = 0.005W^{-0.33}$ where Q_R is gm dry weight of ration/gm live weight of bivalve and W is gm live weight of bivalve.

Three groups of oysters were placed on an egg yolk-starch diet for 3 days in the first experiment. The variations in ATC activity

from one feeding period to the next were examined by sacrificing and analyzing one group 2 hr after, one group 4.5 hr after and the last group 7 hr after the food was given to the oyster.

The second experiment consisted of placing 4 groups of oysters on an egg yolk-starch diet. Daily rations varied for each group. Group one was fed 20%, group two 35%, group three 50% and group four 65% of the ration predicted by the equation: $Q_R = .01W^{-0.33}$ (28). After 5 days, the oysters from each group were sacrificed and their tissues weighed and analyzed.

The last experiment involved 6 groups of oysters. One group was starved while the remaining groups were fed one of the following diets: egg yolk-starch; starch; P. tricornutum; egg yolk-starch-salmon oil; or casein-starch-salmon oil. After 5 days, each group was sacrificed and analyzed.

Analysis of the ATC Activity and Protein Content in Oyster Tissue

The enzymatic activity of ATC was determined using a modification of the procedure of Bergeron and Alayse-Danet (66). The assay system consisted of L-aspartic acid (9.18 µmoles), L-(U-^{14}C) aspartic acid (0.85 nmoles, 017 µC), carbamyl phosphate (6.4 µmoles), borate buffer (12.0 µmoles), pH 9.35, mercaptoethanol (0.31 µmoles) and 0.2 ml of homogenized tissue extract in a total volume of 0.5 ml. The incubation was conducted at 39.5°C for 90 minutes unless otherwise indicated. The reaction was stopped by the addition of 0.1 ml of 1N HCl refrigerated at 4°C. Phenol red was introduced as an internal indicator and 1N NaOH added to pH 7.5. After centrifugation on the Beckman Microfuge B (Beckman Instruments, Palo Alto, CA) 0.55 ml of the supernatant fraction was added to a column (0.6 cm x 5 cm)

of Dowex 50 (x8; H^+ form; 200-400 mesh). The complete elution of carbamylaspartate (CA) was obtained after washing with 3.0 ml of 0.1 M Tris buffer (pH 8.0). The eluate (1.0 ml) was placed in a counting vial with 5 ml of 3a70B scintillation medium (Research Products International Corp., Mount Prospect, IL) and counted for radioactivity. The volume of supernatant remaining in the reaction mixture was measured in order to determine the total amount of CA formed. The specific activity of ATC was expressed as pmoles of CA formed per minute per mg tissue.

Protein concentration of the homogenized tissue extract was determined by the Lowry procedure (90, 91). The total protein content of the tissue was calculated on the basis of total ml of tissue extract.

One way analyses of variance of the ATC activities were carried out and if the differences between the treatments were significant ($p<0.05$) group means were compared using the Student-Newmann-Kuel's (SNK) test ($p<0.05$). In cases where the variances were found to be heterogeneous, treatments were compared using the Kruskall-Wallis test.

RESULTS AND DISCUSSION

Optimal Conditions for the ATC Assay

Substrate concentrations have been reported to influence the pH optimum of the ATC enzyme (92). At concentrations of aspartate less than 5 mM, maximum activity for the Escherichia coli enzyme was shown at pH 6.8 while at concentrations greater than 25 mM, a pH optimum of 8.2 was noted.

In the present study, using concentrations of 18 mM aspartate and 12.6 mM carbamyl phosphate, the optimal pH for measurement of ATC activity in oyster tissue was 9.35 (Figure 5). Using similar substrate concentrations for measurement of activities in scallop tissue, Bergeron and Alayse-Danet (66) found pH 9.0 to be the optimum.

With oyster tissue, greater enzymatic activities were recorded at 39.5°C (Figure 6). The highest activities of ATC in scallop tissue were measured at 35°C (66).

The rate of the reaction after 90 minutes was found to be proportional to the quantity of protein present in the assay medium. As the rate of the reaction was also shown to be linear over a 120 minute incubation period with the highest quantity of enzymatic extract present, the enzyme was judged to be stable under the assay conditions.

Effect of Feeding Regime on ATC Activities

Using a discontinuous feeding regime where food was presented every 8 hrs, the level of food digested by the oysters could vary with time. In previous studies, oysters have been shown to exhibit

periods of high filtering activity and periods of relative quiescence (93). When sufficient quantities of food were available, the oysters ingested some maximum quantity of food and then decreased their filtration rate considerably while digesting the material.

The Student-Newmann-Kuel's test revealed significant differences in activities from oysters sacrificed 4.5 hrs after feeding versus the activities in oysters sacrificed either 2.0 or 7.0 hrs after feeding (Figure 7). These differences in activities could have been in response to differences in quantities of food digested. If such were the case, short term regulation of the ATC enzyme must have taken place.

Effect of Ration Size on ATC Activities

When oysters were fed a diet consisting of egg yolk vesicles and starch granules, the ration size was found by the Kruskal-Wallis test ($p<.01$) to affect the ATC activity. The highest ATC activity was found in the group fed quantities predicted from the equation $Q_R = 0.005W^{-0.33}$ (Figure 8). With oysters fed smaller or larger rations, the enzymatic activities were less. A similar response by mussel growth to ration size was shown by Thompson and Bayne (94). Growth rates in mussels increased with increasing ration size to a maximum and then decreased. The maximum growth rate was achieved when the oysters were able to ingest the greatest quantity of food with the least amount of energy expended. Due to the similar responses to ration size, it could be possible that the ATC activities parallel the growth rates.

Effect of the Diet on the ATC Activity

Various diets were fed to oysters for five days. For Diets A, B and D, slight changes were noted in the gross composition (Table 10) while the fatty acid compositions were markedly altered (Table 11) by the inclusion of salmon oil in the diet. Salmon oil was responsible for providing the polyunsaturated fatty acids, 20:5ω3 and 22:6ω3. Data on growth and tissue composition have implied that oysters have an essential fatty acid requirement for the ω3 series fatty acids (33). While the oyster had some ability to elongate and desaturate ω3 fatty acids to produce 20:5ω3 and 22:6ω3, this activity was not believed to be sufficient to sustain optimal growth (95). This statement was further supported by the work of Langdon and Waldock (79) who demonstrated an improvement in oyster growth with the addition of 20:5ω3 and 22:6ω3 in the diet. A similar response was displayed by ATC activity in the present study (Figure 9). Oysters fed diets containing salmon oil (Diets B and D) had significantly higher ATC activities than oysters that had been fed an egg yolk-starch diet (Diet A). Phaeodactylum tricornutum (Diet E), however, contained a large quantity of 20:5ω3 fatty acids (Table 11). The activities in oysters fed Diet E were not significantly different from activities in oysters fed Diet A. These data indicate that 22:6ω3 was responsible for the increases in ATC activity in oysters fed Diets B and D.

High levels of ATC activity were also found in oysters that had been starved for 5 days. These high activities may have resulted from an enhancement of the oysters essential metabolic processes. Such an enhancement would help to maintain cellular homeostasis to

the detriment of less essential functions such as systemic growth. Increases in ornithine decarboxylase activity during prolonged malnutrition were also believed to be a result of enhanced essential metabolic processes (96).

Effect of the Diet on the Protein Content

Oysters fed Diet D were found to contain a significantly greater percentage of their wet weight as protein than oysters that had been starved (Diet F) or oysters fed Diet C or Diet A. Two explanations could account for the higher protein content in oysters fed Diet D than oysters fed Diet A. The amino acids that are considered essential by the oyster could have been supplied to a greater degree by casein than by egg yolk. Consequently a casein diet could have led to higher rates of protein synthesis. Alternatively, the routes by which casein and egg yolk were taken up could have affected the rate of protein synthesis. Ingestion of the egg yolk protein would have occurred in the digestive diverticula since this protein was a constituent in the particulate vesicle (97). On the other hand, the casein hydrolysate consisted of peptides and amino acids which would have been in the dissolved state. Uptake of dissolved amino acids and peptides have been demonstrated in the gill of the bivalve, Mytilus edulis (39, 98, 99). Those materials taken up by the gill could have been shunted directly into protein synthesis while protein absorbed in the digestive diverticula could have been metabolized preferentially for energy.

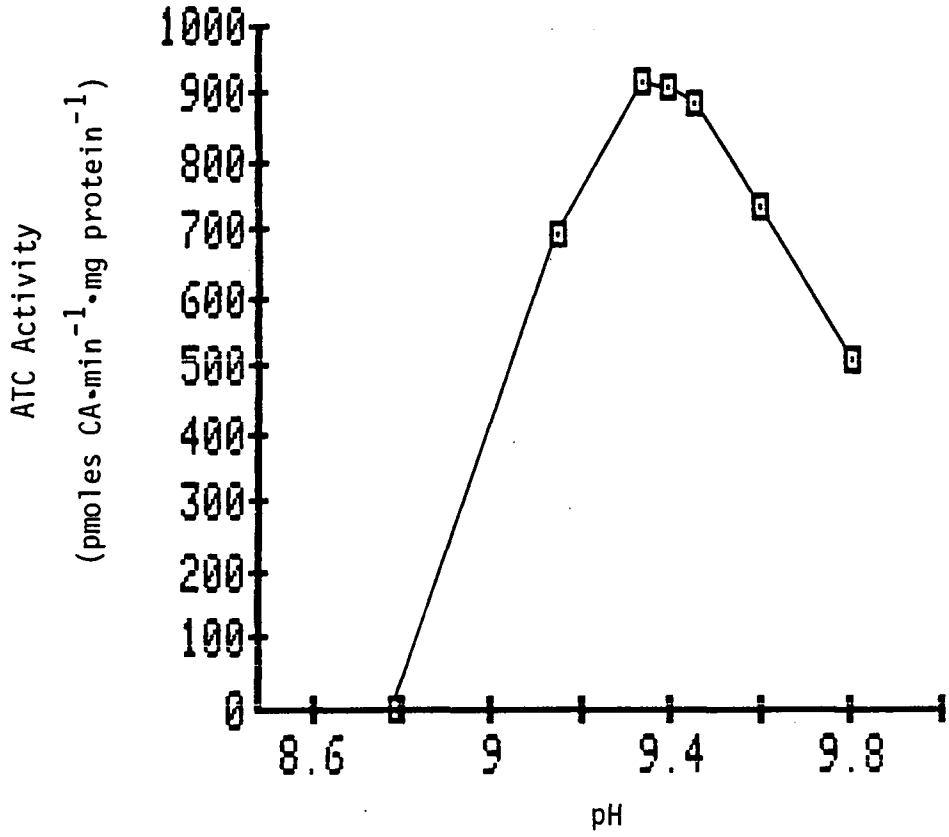

Figure 5. Effect of pH on the rate of the aspartate transcarbamylase reaction.

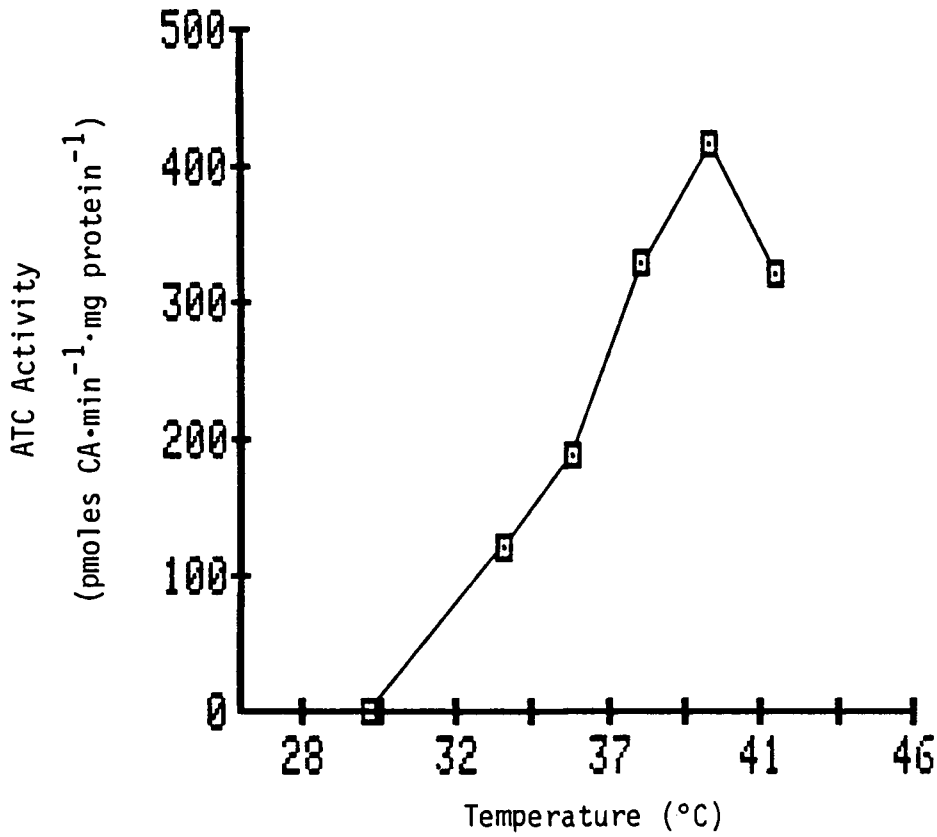

Figure 6. Effect of temperature on the rate of the aspartate transcarbamylase reaction.

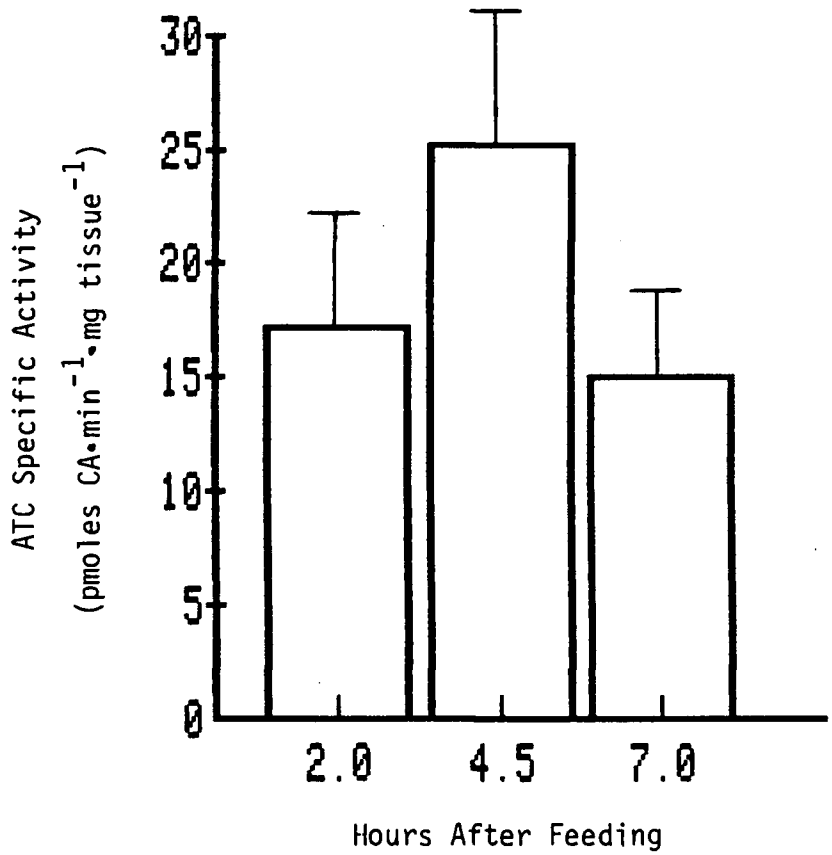

Figure 7. Aspartate transcarbamylase activity in oyster tissues at various postfeeding intervals. Statistics: (T) indicates 95% confidence limits of the mean.

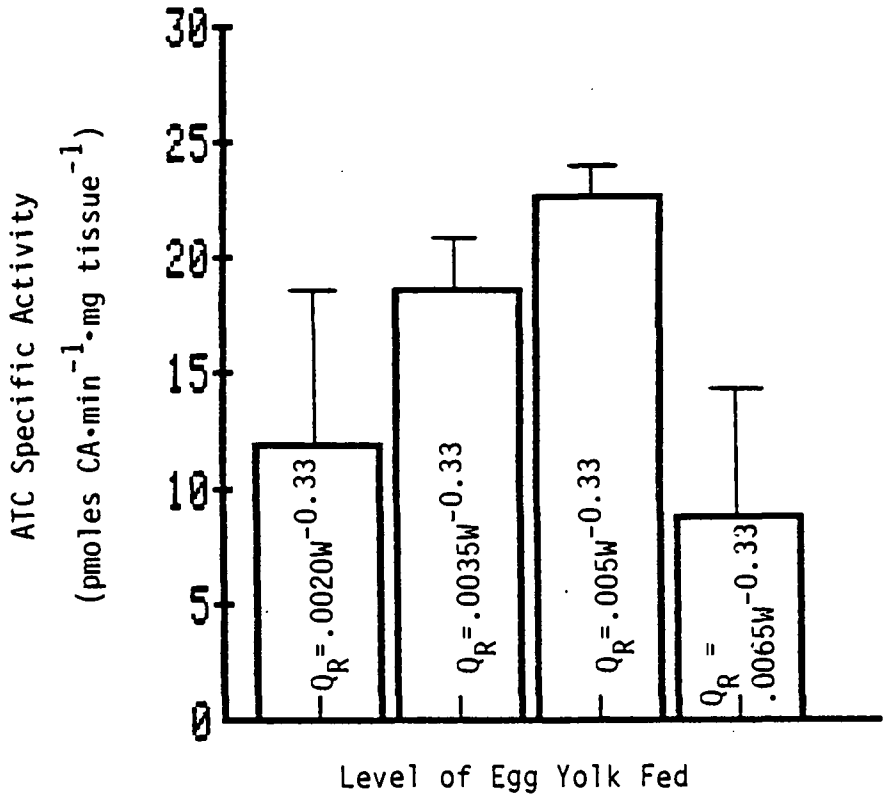

Figure 8. Aspartate transcarbamylase activity in oysters fed various levels of egg yolk diet. Q_R is gm dry weight of ration/gm live weight of bivalve and W is gm live weight of bivalve. Statistics: (T) Indicates 95% confidence limits of the mean.

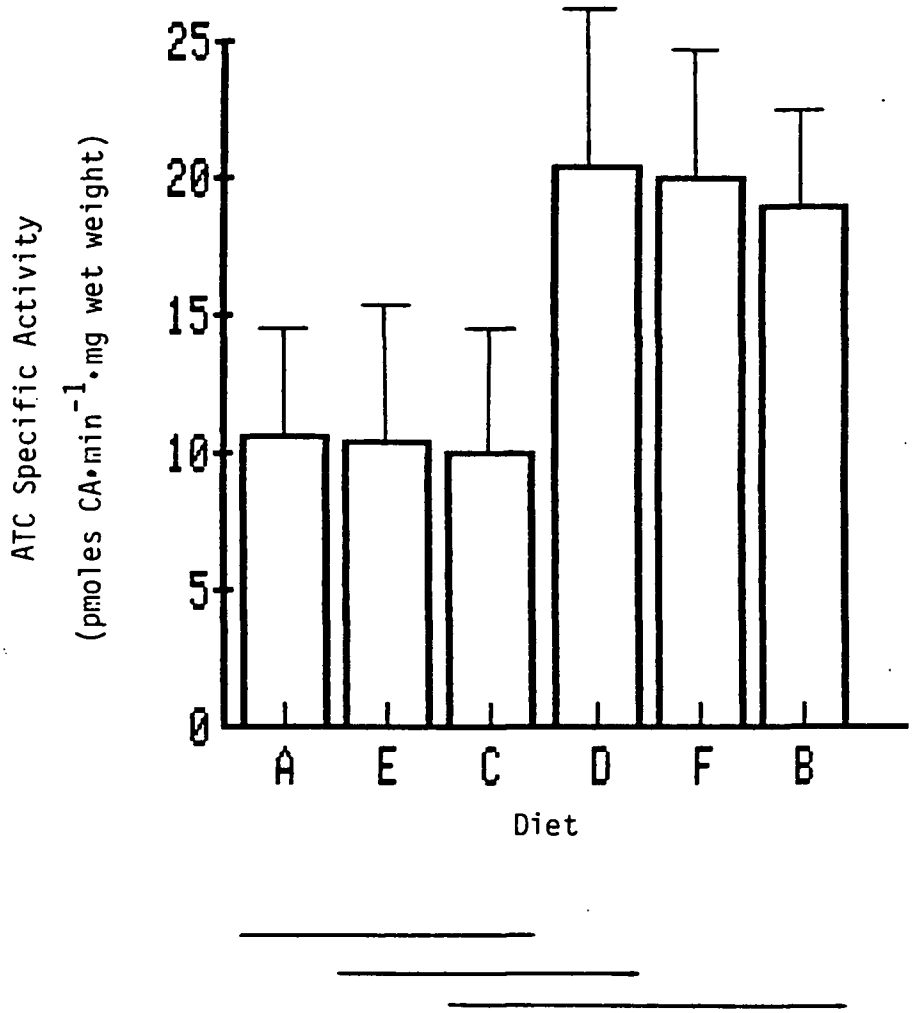

Figure 9. Aspartate transcarbamylase activity in oysters fed various diets for 5 days. Amount of diet fed to oysters determined by equation, $Q_R = .005W^{-0.33}$, where Q_R is gm dry weight of ration/gm live weight of bivalve and W is gm live weight of bivalve. Diet A = egg yolk-starch; Diet B = egg yolk-starch-salmon oil; Diet C = corn starch; Diet D = casein-starch-salmon oil; Diet E = Phaeodactylum tricornutum; Diet F = no food fed. Statistics : (T) Indicates 95% confidence limits of the mean. (——) Signifies statistically similar groups (Student-Newmann-Kuel's test, $p<.05$).

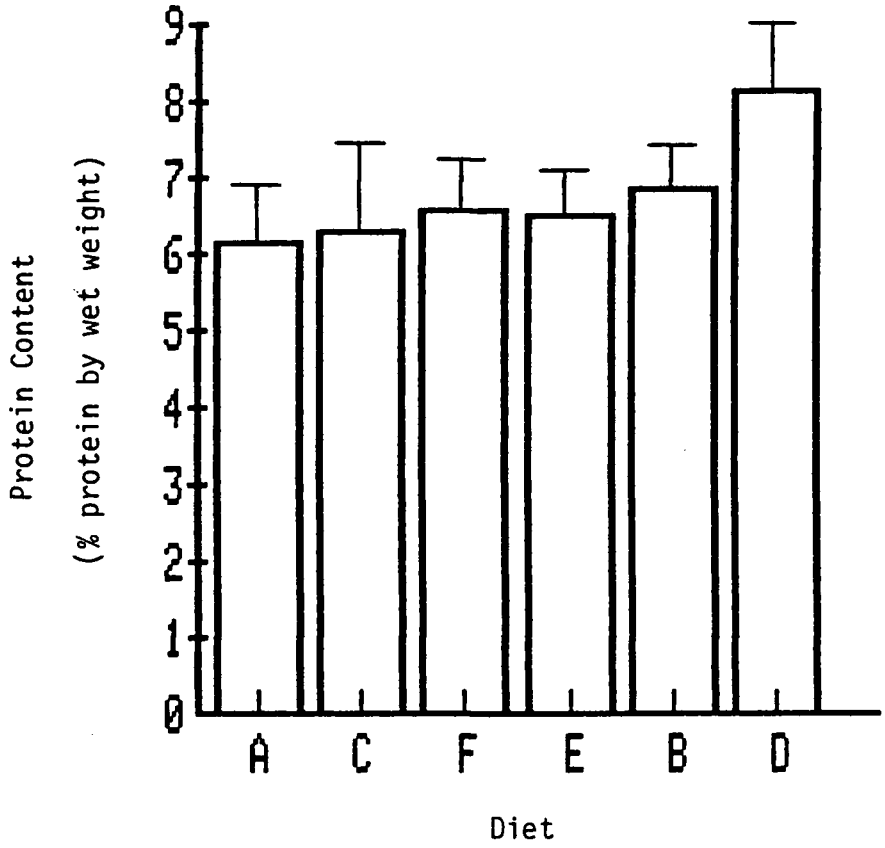

Figure 10. Protein content in oysters fed various diets for 5 days. Amount of diet fed to oysters determined by equation, $Q_R = .005W^{-0.33}$, where Q_R is gm dry weight of ration/gm live weight of bivalve and W is gm live weight of bivalve. Diet A = egg yolk-starch; Diet B = egg yolk-starch-salmon oil; Diet C = corn starch; Diet D = casein-starch-salmon oil; Diet E = Phaeodactylum tricornutum; Diet F = no food fed. Statistics: (T) Indicates 95% confidence limits of the mean. (——) Signifies statistically similar groups (Student-Newmann-Kuel's test, $p<.05$).

TABLE 10

Composition [% of dry weight] of Diets Fed to Juvenile Pacific Oysters (*C. gigas*)

Diet[a]	Protein	Lipid	Carbohydrate[b]
A	16.6	32.5	50.9
B	16.6	34.5	48.9
C	-	-	100.0
D	17.0	34.2	48.8
E	22.1	11.5	66.4[c]

[a]Diet A = egg yolk-starch; Diet B = egg yolk-starch-salmon oil; Diet C = corn starch; Diet D = casein-starch-salmon oil; Diet E = *Phaeodactylum tricornutum*.

[b]Calculated by difference.

[c]Includes ash content.

TABLE 11

Fatty Acid Composition [Weight %] of the Diets Fed to Juvenile Pacific Oysters (C. gigas)

Fatty Acid	Diet[a]			
	A	B	D	E
14:0	0.44	0.72	5.58	2.66
16:0	27.68	27.25	19.97	22.98
16:1	5.20	5.42	9.20	35.41
18:0	9.36	9.08	4.46	1.86
18:1	44.17	43.12	25.53	15.95
18:2ω6	10.94	10.43	1.78	1.31
18:3ω3	0.62	0.58	-	0.12
20:1	-	0.68	11.09	-
20:4ω6	1.60	1.51	-	0.41
?	-	0.09	1.66	-
22:1	-	0.57	10.14	-
?	-	-	-	2.60
20:5ω3	-	0.41	7.33	11.38
22:4ω6(?)	-	-	-	4.95
24:1	-	-	-	0.33
22:6ω3	-	0.18	3.23	0.05

[a]Diet A - egg yolk-starch; Diet B = egg yolk-starch-salmon oil; Diet D = casein-starch-salmon oil; Diet E = Phaeodactylum tricornutum

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APPENDIX

APPENDIX I

Recipe for Davidson's Fixative

Stock

10 ml glycerol

20 ml 37% formaldehyde

30 ml 95% ethanol

30 ml filtered Instant Ocean

Working Solution

90 ml stock

10 ml glacial acetic acid