CHANGES IN MIDGUT GLAND MORPHOLOGY AND DIGESTIVE ENZYME ACTIVITIES ASSOCIATED WITH DEVELOPMENT IN EARLY STAGES OF THE AMERICAN LOBSTER HOMARUS AMERICANUS

by

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ABSTRACT

The development of the digestive system, specifically in relation to the midgut gland (hepatopancreas), in early life history stages of the American lobster Homarus americanus is examined in the present study by means of two separate but complementary approaches. A histological study on the light microscope level details the morphological changes which take place among the different cell types which form the midgut gland. Included in this portion of the study are scanning electron micrographs of the midgut gland of stage IV larva. Secondly, a biochemical study correlates changes in the activities of digestive protease, lipase, and amylase with the observed changes in the cells of the midgut gland. A sensitive method for detection of crustacean lipase was developed for this study. In order to optimize the assay conditions for measurement of digestive enzyme activities, a series of control tests was performed to determine the effects of several physical and chemical factors.

The developmental stages of the lobster which are examined in these studies include: well-advanced embryos at approximately three days prior to hatching, hatching prelarvae, newly hatched and intermolt stage I larvae, intermolt stage II, III, and IV larvae, and intermolt stage V and VI postlarvae. Particular attention is paid to specific transitional stages during development. These include: (1) the changeover from yolk metabolism by the embryos to the dependence on exogenous food by newly hatched larvae; (2) the alteration in body form which occurs at the molt from larval stage III to IV; and (3) the change in habitat from the plankton to the benthos which occurs at the molt from stage IV to V.

Results of the present study demonstrate that the R-cells (resorptive cells) of the earliest developmental stages, embryo through larval stage III, do not show the characteristic morphology classically ascribed to this cell type. Presumably because young lobsters do not begin to store excess lipids derived from the diet until stage IV, the R-cells do not contain large numbers of lipid vacuoles. By stage VI, however, the R-cells achieve the classic appearance. A previously unreported function for R-cells, that of storage of lipid derived from yolk metabolism in the embryo, is

described. F-cells (digestive enzyme synthesizing cells) are present in the midgut gland of embryos a few days prior to the hatch while B-cells (enzyme secreting cells) have developed by the time of hatching. Thus, morphologically the stage I lobster larva appears to possess digestive capabilities prior to ingestion of the first meal.

Changes in digestive enzyme activities during early development correlate well with the morphological changes observed in the midgut gland. Activities of protease, lipase, and amylase are very low in the well-advanced embryo and increase slightly by the hatching stage. Enzyme activities more than double by the time the stage I larva attains intermolt, regardless of whether the larva is fed or fasted. Digestive enzyme activities increase further by the time the stage II larva reaches intermolt; in general there is no significant difference in the levels of enzyme activities measured in the older stages (II through V).

The results of a more detailed time course examination of the levels of digestive enzyme activities in relation to first feeding by the stage I larvae are presented. Although there is a trend for slightly increased protease and amylase activities as the stage I larvae get older, the lipase activity is constant.

Lobster larvae normally hatch in late spring and early summer but larvae can be induced to hatch during the winter by maintaining the eggs at higher than ambient water temperatures. There is no consistent difference in digestive enzyme activities measured for larvae which hatch during the summer compared to those which hatch during the winter.

The influence of molt stage on the activity of digestive enzymes in wild caught stage IV larvae is also discussed. Only minor variations in lipase and amylase activity are detected during intermolt and premolt and in general there is no significant effect. Protease activity is significantly greater at D_0.

This work describes new findings on several aspects of digestion among early life history stages of a marine decapod crustacean and has added to our understanding of the functional morphology of the midgut gland during early development.

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ABBREVIATIONS

A, absorbance

ANOVA, analysis of variance

A/T, amylase to trypsin ratio

BSA, bovine serum albumin

C:M, chloroform to methanol ratio

CPV, cardio-pyloric valve

1,2-DAG, 1,2-diacylglycerol

1,3-DAG, 1,3-diacylglycerol

EFA, essential fatty acid

ER, endoplasmic reticulum

FA, fatty acid

FFA, free fatty acid

FID, flame ionization detector

IA, indole acetate

LMW, low molecular weight

MAG, monoacylglycerol

NA, naphthyl acetate

OA, oleic acid

OAME, oleic acid methyl ester

O:N, oxygen to nitrogen ratio

PNR, point of no return

PRS, point of reserve saturation

PUFA, polyunsaturated fatty acid

RER, rough endoplasmic reticulum

S, salinity

SER, smooth endoplasmic reticulum

TAG, triacylglycerol

TLC, thin-layer chromatography

CHAPTER 1:
GENERAL INTRODUCTION

Successful development and metamorphosis of marine planktotrophic organisms depend upon the efficient utilization of metabolic resources. Thorsen (1950) stated that the most sensitive stages in the life cycle of benthic invertebrates are the early developmental ones: embryo and larva. The environmental and physiological requirements of the early life history stages are often much more narrowly defined than those of other stages in the life cycle. There are definite nutritional limitations on energetics and development of marine larvae, for example. The need for energy from dietary components can preclude their biosynthetic incorporation into tissues if the diet provides only sufficient energy to maintain basic metabolic processes and physical activity. In fact, if the diet provides less energy than is required for these energetic processes then tissue components will be catabolized. Thus, adequate nutrition is a factor which severely influences development of marine larvae. Survival of planktotrophic marine larvae depends upon the ability of the organisms to capture, ingest, and digest suitable food. Development of digestive functions by early life history stages is a prime consideration in the feeding ecology of decapod crustaceans. However, changes in the digestive processes and capabilities of this group are virtually unknown.

There are currently several catagories of ongoing research which deal with the process of digestion and the digestive enzymes of marine invertebrates. This work includes field studies in zooplankton

ecology which examine trophodynamic relationships among the plankton. The digestive enzymes of heterogeneous natural populations have been measured in an effort to relate seasonal feeding habits with the biochemical composition of the food particles (Bamstedt, 1984; Boucher and Samain, 1974 and 1975; Boucher et al., 1976; Gaudy and Boucher, 1983; Head et al., 1984; Mayzaud and Conover, 1976 and 1984; Mayzaud et al., 1984; and Stuart et al., 1985). Molecular and developmental biologists have examined the peaks of digestive enzyme production during early development of brine shrimp Artemia sp. in studies on the genetic expression of enzyme synthesis and its correlation with ecological factors (Burillo et al., 1982; Ezquieta and Vallejo, 1985; Garesse et al., 1980; Ollala et al., 1978; Osuna et al., 1977; Perona and Vallejo, 1985; and Sillero et al., 1980). In another area of research, the digestive enzymes of a few commercially important decapod crustaceans have been qualified and quantified with the intention that understanding the nutritional and digestive physiology of these species could optimize or at least increase their aquaculture potential (Lee et al., 1980 and 1984; Maugle et al., 1982b; Murthy, 1977; and Trellu, 1978). Recently a number of biochemical investigations have been conducted in an attempt to characterize the properties of crustacean digestive enzymes (Armstrong and DeVillez, 1978; DeVillez and Lau, 1970; Galgani et al., 1985; Gates and Travis, 1969 and 1973; Maugle et al., 1982b; Pfleiderer et al., 1967; Zwilling and Tomasek, 1970; and Zwilling et al., 1969). It is uncertain whether invertebrate digestive enzymes are homologous or analogous to mammalian enzymes.

Morphological studies of the decapod crustacean digestive system have been conducted for at least the past one hundred years. Physiological and biochemical aspects of digestion in this group have been examined for over fifty years. Thus, there exists an extensive body of work on digestion in the Decapoda. This work, however, is not unified and much remains to be known. Van Weel (1970) stated that "the physiology of digestion in crustacea has been studied extensively in only a few genera (Astacus, Procambarus, a few crabs, Homarus)." The studies to which he referred, however, have dealt almost exclusively with adult animals. Aspects of digestion in early developmental stages of decapod crustaceans have not been adequately addressed even in the few "well-studied" species, which include the American lobster Homarus americanus.

Smith (1873) and Bumpus (1891) described the embryonic development of H. americanus, beginning with the mature oocyte and continuing through to a "well advanced embryo". Herrick (1911) also described embryonic development of the lobster. From all accounts, the digestive tract of the embryo, particularly the midgut gland, is closely associated with the yolk mass during its entire development. In adult decapod crustaceans the midgut gland, also called the hepatopancreas or digestive gland, is responsible for synthesis and secretion of digestive enzymes, for resorption of digested nutrients, and for storage of energy reserves (cf. Gibson and Barker, 1979 for review) and thus plays a crucial role in digestion. As the midgut gland of the embryonic lobster differentiates, the yolk mass is enfolded within it. Thus, the midgut gland functions in digestion and

absorption of yolk in the lobster embryo although the precise mechanism is not known. Yolk is contained within the midgut of the older, developing embryo. This yolk is yellow and in the form of relatively large spheroids in contrast to the dark green, more granular nature of the bulk yolk. The midgut of the hatching prelarva also contains spherical globules of yolk along its length. A small amount of this yolk material remains within the enlarged anterior midgut caeca (Herrick, 1911; Hinton and Corey, 1979) and intercalated among the tubules on the outside of the midgut gland (Biesiot, 1983).

Although the yolk remnants in the midgut are utilized by the stage I lobster larva, these reserves are not normally sufficient to fulfill the metabolic requirements for successful growth and molting beyond this stage. Therefore, the newly hatched lobster larva must commence feeding in order to obtain nutrients for energy and biosynthesis. Complex nutrient molecules, protein, lipid, and carbohydrate, are degraded by all heterotrophic organisms. However, development of digestive functions by lobster larvae has not been previously studied.

Studies concerning digestion in larval and juvenile American lobsters have thus far been limited to histological examinations of the developing digestive tract. Both Smith (1873) and Bumpus (1891) described changes which occur in the digestive tract during embryonic development. Factor (1979, 1981) produced a detailed account of the development of the digestive system of stage I through IV larvae and the morphological changes that occur at metamorphosis. In a similar study, Hinton and Corey (1979) also described the development of the digestive system and mouthparts of larval lobsters. These studies,

however, focused primarily on development of the foregut with only cursory mention of changes in the midgut gland and they made no attempt to elucidate or even to speculate upon the function of the different cell types during development.

SCOPE OF THESIS RESEARCH

The present research was undertaken to investigate aspects of digestion in early developmental stages of the American lobster H. americanus, with special reference to the midgut gland. A histological study was conducted in order to demonstrate morphological changes during development. A subsequent study was performed to correlate changes in digestive protease, lipase, and amylase activities with the changes observed in midgut gland morphology. Both facets of this work focused on key periods in early lobster development including: (1) the transition between metabolism of stored yolk reserves by the embryo and the initiation of exogenous feeding by the newly hatched larva; (2) the dramatic change in body form which occurs at the molt between the stage III and stage IV larva; and (3) the change in habitat from the plankton to the benthos which occurs at the molt to stage V postlarva.

A comprehensive study of the developing digestive system in early stages of H. americanus is appropriate in several contexts. The American lobster is a good model system for investigating basic biological questions among the decapod crustaceans. Berried females can be maintained in the laboratory throughout the period of embyrogenesis so that sample collection at various points during embryonic development is feasible. It is also possible to rear the

larval and juvenile stages in the laboratory. Young lobsters of known age and reared with a controlled nutritional history can be obtained. Since the embryos and larvae are quite large compared to those of other crustaceans, fewer individuals are needed for assays, thus providing a potential measure of the variability among individuals of a population. The molt stage of the larval lobsters can be accurately and conveniently determined (Sasaki, 1984), an important consideration since a number of behavioral and physiological processes are affected by the molt cycle (cf. Skinner, 1985 for review).

Detailed knowledge of the levels of digestive hydrolases (protease, carbohydrase, and lipase) in an individual species such as the lobster may be valuable in interpreting the effects of seasonal and developmental changes in feeding habits of a population of zooplankton. There have been a number of zooplankton trophodynamic studies reported in the literature but lipase levels have not been measured in any of them, primarily because of difficulties encountered in detection and measurement of this enzyme. There is a nutritional requirement for lipid in marine organisms and it is an important energy source for crustaceans. An ecophysiological study which measures protease and carbohydrase but ignores lipase is incomplete and may result in misleading conclusions. Mayzaud and Conover (1976), for example, surveyed the biochemical composition (protein, carbohydrate, and lipid) of the particulate matter throughout one year in the Bedford Basin (Nova Scotia, Canada) and tried to relate the activity of several digestive enzymes of the neretic zooplankton to the changes observed. The authors reported a significant correlation

of activities of carbohydrases and proteases with the seasonal variations in particulate matter. They did not detect lipase activity even though lipid was present in the particulate matter. Unless crustacean lipase is characterized so that assays can be conducted at optimal conditions, the apparent absence of measureable lipase activity in these zooplankton populations cannot be considered evidence that the enzyme does not occur. Berner and Hammond (1970) assayed for digestive lipase specificity using a number of marine invertebrate species representing several phyla. They demonstrated true lipase activity in about half of the invertebrates, whereas esterase activity was demonstrated for all species.

Attempts to formulate a crustacean larval feed for aquaculture have thus far been unsuccessful in part because of a lack of knowledge of the nutritional requirements but also because the larval digestive process is not well known. If the process of digestion and the levels of digestive enzyme activities in relation to developmental variables can be well defined in the laboratory for early stages of *H. americanus*, the results may be applicable to other less easily studied crustaceans with commercial or ecological importance.

BACKGROUND INFORMATION

Life history and development of the American lobster

The natural history of the American lobster *H. americanus* is described in detail in the classic paper by Herrick (1911). Female lobsters are sexually mature when they reach a total length of about 10.5 inches; it takes from five to six or more years under natural temperature conditions to reach this length. Males become sexually

functional at a slightly smaller size. Mating (between a newly molted female and a hard shelled male) and oviposition are independent events separated by as little as one month to as much as two years (Aiken and Waddy, 1980). Fertilization occurs at oviposition. Eggs are extruded during July and August and are borne externally, attached to setae on the pleopods of the adult female, for about 10.5 months. Larvae are released primarily during May and June but hatching can extend into July.

Newly extruded eggs contain ovoverdin (Stern and Salomon, 1938), a carotenoprotein in which the carotenoid astaxanthin is non-covalently bound to the protein. Ovoverdin persists during development, at first giving the eggs a dark green and later a black appearance. Functions ascribed to this complex include use as a heat or light shield, protection of the protein from specific enzymatic action, or protection of food reserves for the embryos.

About a month prior to hatching, the carotenoprotein complex is disrupted, astaxanthin is released, and egg color changes from black to golden. The yolk is more actively metabolized during this period than earlier in development with lipid, primarily triacylglycerol, contributing the majority of the catabolized nutrients (Sasaki, 1984). Approximately one to two days before hatching the eggs swell and turn pale blue. At this point, yolk reserves are reduced but there is still some yolk present in and around the midgut gland as well as in the intestine.

During the hatching process the outer egg membrane ruptures, apparently due to osmotic swelling of the embryo, but the prelarva is

retained within an inner egg membrane and can remain attached to the female for hours. The prelarva is incapable of swimming, even if removed from the inner membrane, until ecdysis occurs and the free-swimming stage I larva emerges. Normally the prelarva is dependent on strong flicking movements of the pleopods made by the female to sweep it out of the inner membrane (Herrick, 1911; Davis, 1964). Molting often occurs at this point but it can be delayed for several hours after the prelarva is released by the female. The hatching process thus is not a discrete event but should be considered rather a developmental continuum which begins with rupturing of the outer egg membrane and ends with molting of the prelarva.

Smith (1873) and Herrick (1896; 1911) described the four planktonic larval stages of the American lobster. The first three stages are mysis stages. The stage IV larva resembles the adult aside from minor morphological differences and has been referred to as the post-metamorphic stage. Herrick (1911) considered this stage analagous to the megalopa of crabs.

The larval stages are easily distinguished on the basis of size and the presence of certain abdominal appendages. The first stage is about 8 mm in length and lacks swimmerets. The second stage is about 9 mm but possesses four pairs of swimmerets on the second through fifth abdominal segments. Stage III larvae are about 11-13 mm long; they have uropods on the last abdominal segment and the swimmerets are more well developed. Stage IV larvae are about 14-17 mm in length and, as mentioned above, have the basic adult body form.

The stage IV larva makes the transition from the plankton to the benthos. By the end of this stage the lobster develops more benthic behavior and molts to stage V which is the first postlarval or juvenile stage. It looks much like the stage IV larva but it is benthic.

Herrick (1911) reported the duration of stage I in waters around Woods Hole, MA to range from one to five days, stage II from two to five days, stage III about five days, and stage IV from ten to 19 days. The total length of the larval period is temperature dependent (Templeman, 1936). At 14°C, the fourth stage is reached in 26.5 days, at 19°C in 13.5 days, and at 24°C in 10.5 days.

Larvae reared communally in the laboratory under low density conditions at 20°C and 30 ppt salinity (S) spent from two to four days in stage I, two to four days as stage II, three to five days in stage III, and five to 11 days in stage IV; under high density conditions, stage I lasted one to two days, stage II one to six days, stage III three to six days, and stage IV six to eight days (Sastry and Zeitlan-Hale, 1977). The average duration of stage V was 10.7 ± 1.8 days for lobsters reared in the laboratory at 20°C and 28 ppt S (Logan and Epifanio, 1978).

Natural food of lobsters

The American lobster is considered an omnivore and a predator even as a larva. Analysis of the stomach contents of hatchery reared larvae stage I through IV by Herrick (1896) and Williams (1907) have shown the natural diet to consist of small planktonic organisms including diatoms, bacteria, copepods, filamentous algae, and larval

decapods, including other lobster larvae. During larval development the morphology of the mouthparts changes, an observation which Factor (1978) suggested correlated with the change in habitat and diet. Adult lobsters feed mainly on benthic invertebrates: crabs, isopods, sea urchins, sea stars, snails, clams, and polychaetes; fish, eelgrass, hydroids, ascidians, and ectoprocts form a minor part of the diet (Herrick, 1911; Weiss, 1970; and Ennis, 1973).

There are, however, variations in the composition of the diet of adults over the molt cycle (Weiss, 1970). After molting, a high proportion of crustacean and mollusk shells, presumably a source of calcium, are ingested; just before molting the lobsters tend not to feed. Dietary intake of wild adult lobsters was analysed by Leavitt et al. (1979) to provide a basis for diet formulation and the determination of nutritional requirements. Nutrient analysis of the stomach contents at three general phases of molt (hard shell, soft shell, and intermediate) showed significant ($p<0.05$) differences in crude protein and gross energy. Combined contents of the anterior and posterior chamber of the stomachs of hard shell lobsters contained 34.1% crude protein, intermediate lobsters 23.2%, and soft shell lobsters 12.2%. Gross energy (kJ/g sample) was 8.96, 6.45, and 4.35, respectively. Thus, the natural dietary intake of adult lobsters has a high protein to energy ratio.

Nutritional requirements

Most of the work on nutritional requirements of *H. americanus* has focused on postlarvae or juveniles. In view of their natural protein-rich diet, lobsters were considered to have a high protein

requirement. Castell and Budson (1974) determined that 60% protein was required for best growth of adult animals but their results were probably confounded by amino acid deficiencies in all diets. The brine shrimp Artemia sp. promotes good growth in larval and juvenile lobsters and is about 40% protein on a dry weight basis (Gallagher and Brown, 1975). Gallagher (1976) was not able to demonstrate optimum protein levels between 20-60% for postlarval lobsters but did indicate that 11% was inadequate.

The required protein level depends on the other constituents of the diet and the total energy content. Capuzzo and Lancaster (1979c) demonstrated a protein sparing effect of carbohydrate in postlarval lobsters. They varied the protein content of a formulated diet from 16.7-23.3%, the carbohydrate content from 22.9-31.3%, and the protein:carbohydrate ratio from 0.5-1.0. Protein utilization efficiency varied inversely with protein content due to the sparing effect of the carbohydrate.

Castell and Covey (1976) suggest that adult lobsters have a relatively low requirement for lipid with 5% cod liver oil in a formulated feed giving near-optimal results. Higher levels (10% and 15%) appeared to exert a slight protein sparing effect. Crustaceans have limited capacity for bioconversion or de novo synthesis of sterols. Specific lipid requirements of the lobster include cholesterol (Zandee, 1967), polyunsaturated fatty acids (PUFA) of the linolenic (ω3) and linoleic families (ω6) (Kanazawa et al.,1977; Castell and Boghen, 1979), and phosphatidylcholine (lecithin) (D'Abramo et al., 1981). Linolenic acid (18:3ω3) is more effective

at promoting growth than linoleic (18:3ω6) when fed alone. PUFA 20:5ω3 and 22:6ω3 were more effective than either 18-carbon fatty acid (Kanazawa et al., 1979; Castell, 1983). Soy lecithin, a mixture of soybean oil, phospholipids, and essential fatty acids (EFA), has been shown to improve survival rates when used in formulated lobster rations (Conklin et al., 1980).

Digestion

The process of digestion in invertebrates has been reviewed by a number of authors including Vonk (1960), Barrington (1962), van Weel (1970), Barnard (1973), Pandian (1975), and Dall and Moriarty (1983). Digestion is enzymatic cleavage of the macromolecules carbohydrate, protein, lipid, and nucleic acid to the simple metabolizable forms monosaccharides, amino acids, fatty acids and glycerol, and nucleosides, respectively. Extracellular digestion, the secretion of enzymes into a cavity, is present in all higher animals. Digestion in crustacea is considered to be entirely extracellular (Vonk, 1960; van Weel, 1970). Barker and Gibson (1977) claimed to have demonstrated that there is also an intracellular alkaline phase of digestion in *H. gammarus*. Dall and Moriarty (1983) considered this claim "unconvincing" apparently because the enzymes detected in the former study are lysosomal enzymes.

All digestive enzymes are hydrolases and can be classified as: glucosidases (carbohydrases) which act on the glycosidic bonds of carbohydrates; peptide hydrolases (proteases, proteinases, and peptidases) acting on the peptide bonds of proteins and peptides; and

carboxylic ester hydrolases (lipases and esterases) which act on ester bonds of lipids.

Carbohydrates must be broken down to monosaccharides since only these are absorbed. Digestion of starch (amylose) is accomplished in two steps: α-amylase (α-1,4-glucan-4-glucanohydrolase) hydrolyzes the intact molecule by random, multiple attacks on two to six adjacent bonds to yield maltose, some oligosaccharides, and a small quantity of glucose; then maltase (α-D-glucoside glucohydrase) breaks down the fragments to free glucose. Glycogen is similarly digested by α-amylase and maltase but yields maltose and a variety of branched oligosaccharides with α-1,6 linkages which are acted on by specific di- and oligosaccharidases. Absorption of monosaccharides across the epithelial cell membrane occurs when the di- and oligosaccharides are cleaved by membrane bound glycosidases.

Proteins are absorbed primarily as amino acids. There are three main phases of protein digestion during which the different proteolytic enzymes act sequentially. First, endopeptidases hydrolyse peptide bonds at specific sites in the protein molecule to yield shorter polypeptide chains. Examples of these enzymes are trypsin, chymotrypsin, and pepsin. Trypsin hydrolyses peptide bonds in which the carboxyl group belongs to a basic amino acid, especially lysine or arginine. Chymotrypsin and pepsin hydrolyze bonds in which the carboxyl group belongs to an aromatic amino acid, phenylalanine, tryptophan, or tyrosine. Chymotrypsin is optimally active at alkaline pH while pepsin is an acidic protease. Next, exopeptidases hydrolyze the terminal bonds in polypeptide chains removing one to three amino

acid residues at a time from either the amino-terminal end or the carboxyl-terminal end. The final stage of digestion is accomplished by specific di- and tripeptidases which are probably membrane bound rather than extracellular.

MacDonald (1985) defined proteases as general proteolytic enzymes. Proteinases refer to endopeptidase-type proteases which have specific sites that do not accept substrates with charged amino or carboxyl ends. Exopeptidases, or more simply peptidases, require one or both of these functional groups. Aminopeptidases remove amino acids one at a time from the amino terminal end of a protein or peptide. Dipeptidyl peptidases and tripeptidyl peptidases remove groups of two and three amino acids, respectively, from the amino end. Carboxypeptidases and peptidyl dipeptidases remove one or two amino acids, respectively, from the carboxyl end of a peptide. Dipeptidases cleave unsubstituted dipeptides and tripeptidases remove one amino acid from either end of unsubstituted tripeptides. Omega peptidases are a new class of exopeptidases which do not conform to the traditional definitions for aminopeptidases and carboxypeptidases, such as those which cleave N-terminal residues lacking a free α-amino group or C-terminal residues lacking a free α-carboxyl group.

Hartley (1960) proposed a classification of proteases based on the active site. The four classes of proteases are: serine, including trypsin, chymotrypsin, elastase, thrombin, plasmin, and subtilisin; thiol, including papain, ficin, bromelain, and cathepsins; acid, including pepsin and rennin; and metal, including aminopeptidases,

carboxypeptidases, and dipeptidases. Barrett (1980) has proposed a new system with the following changes in nomenclature: serine, which is the same as Hartley's category; cysteine, which replaces thiol; aspartic, which replaces acid and carboxyl; metallo, which replaces metal, and an generic catagory which includes proteinases of unknown catalytic mechanism.

Mammalian proteases are often produced as zymogens; these inactive precursors of enzymes are activated to the catalytic form when required. Trypsinogen is converted to trypsin by the selective removal of a hexapeptide from the amino-terminal end by the action of enterokinase. Enterokinase recognizes a sequence of at least three acidic amino acids just before the lysine bond which is cleaved but it does not recognize any other lysine nor does it recognize arginine. Trypsin then activates more trypsinogen and is also responsible for activating the cascade of other protease zymogens including chymotrypsinogen and pepsinogen.

Carboxylic ester hydrolases (carboxylesterases) can be subdivided into lipases (glycerol-ester hydrolases), which hydrolyze esters of glycerol and fatty acids at the ester-water interface and which preferentially hydrolyze the outer ester links, and esterases which hydrolyze simple esters. The distinction between the two is not great since neither shows marked specificity of substrate and both have fairly broad pH optima. Lipases, however, are generally considered to hydrolyze long chain (greater than twelve carbons) aliphatic acids from glycerol (Brockerhoff and Jensen, 1974). Lipase digestion

produces free fatty acids, glycerol, and mono- and diacylglycerols, all of which are absorbed.

Emulsifiers, while not enzymes, play an important role in fat digestion and resorption by reducing the particle size and increasing the total surface area exposed to hydrolysis. In vertebrates, the liver converts cholesterol into bile acids which react with nitrogenous bases to yield bile salts; after passing into the small intestine bile salts emulsify fatty acids and acylglycerols. Crustaceans, however, are incapable of cholesterol synthesis (Zandee, 1967), tend to conserve the dietary cholesterol, and thus do not produce bile acids. Instead they produce other emulsifiers with comparable functions (Holwerda and Vonk, 1973).

Digestive enzymes are produced by specialized enzyme secreting cells in the digestive tract and are released as droplets, spheres, or granules. Apocrine secretion involves almost no loss of the gland cell itself. In merocrine secretion, portions of the secretory cell are lost; however, the cell is not destroyed and it regenerates its contents. The entire gland cell is extruded during holocrine secretion.

The presence of food is the general stimulus for secretion of digestive enzymes. After the initial secretion, digestive enzyme levels fluctuate which may represent a cyclic reconstitution of active enzymes. Histological analysis performed at different times after feeding in invertebrates have revealed waves of activity of gastric juice enzymes. Two waves of secretion are reported in many invertebrates, usually one hour and again four to eight hours after

feeding. Barker and Gibson (1977) have shown three bursts of secretion in adult H. gammarus. Initial discharge of enzymes takes place within one hour of feeding by a holocrine mechanism. There are subsequent secretory maxima, most likely merocrine, one to two hours and again 3.5 to six hours after feeding. After the final wave of secretion, the secretory cells are replenished in preparation for digesting the next meal.

Resorption of the extracellularly digested food into the gastrodermal cells of crustacea occurs by absorption and to some extent pinocytosis. Absorption is surface-limited. Consequently, the surface area of the absorptive epithelial cells is increased by finger-like projections, the microvilli, which form a brush border. Some nutrients are absorbed by simple diffusion but active transport also plays a role. Certain digestive enzymes are firmly bound to the brush border and accomplish the final stages of digestion. It is thought that there is a coupled active transport of enzymes and carriers in the brush border and that absorption of monosaccharides and amino acids occurs at the time of final cleavage. Lipid is absorbed in the form of micelles which enter the cell by diffusion but whether a carrier is also involved is not known.

Digesitive enzymes of crustaceans

Crustaceans usually secrete a strongly acting α-amylase which has a pH optimum ranging from pH 5.0 to pH 7.8 and which is activated by chloride ions (van Weel, 1970). The presence of strong α-amylase seemed puzzling to Mayzaud and Conover (1976) since ß-1,3-polysaccharides, rather than starch, predominate in the sea

(Handa and Tominga, 1969). It should be remembered, however, that α-amylase also hydrolyzes glycogen which is a storage polysaccharide of marine organisms. Maltase with a pH optimum ranging from pH 4.4 to 6.2 has also been found in all crustaceans examined (van Weel, 1970). In addition, α-1,6-glucosidases are necessary for complete digestion of carbohydrates and although they have not been isolated from crustaceans, they probably are present (Dall and Moriarty, 1983).

Other carbohydrases such as saccharase (ß-fructofuranase, sucrase, or invertase), chitinase, and cellulase have been reported in crustaceans (van Weel, 1970; Gibson and Barker, 1979) usually, however, in trace amounts and only after prolonged incubation. No controls were done to preclude the possibility of contaminating micro-organisms.

A fairly detailed survey for digestive enzymes has been conducted with Astacus fluviatilis. Kooiman (1964) found α-galactosidase, cellobiase (ß-glucosidase), sacchrase, lactase, α-xylosidase, maltase, isomaltase, ß-1,3-glucanase, cellulase (ß-1,4-glucanase), mannase, and chitinase. Van Weel (1970) commented that perhaps if other species were studied as extensively, similar numbers of carbohydrases might be found. Brockerhoff et al. (1970) and Hoyle (1973) detected activities of amylase, α-glucosidase, ß-glucosidase, chitobiase (N-acetyl-ß-glucoasaminidase), ß-galactosidase, and chitinase in the adult American lobster.

Proteases, amino- and carboxypeptidases, and dipeptidases were reported by van Weel (1970) to be found in all crustacea investigated by that time. Crustacean trypsin-like enzymes have been reported to

occur in a number of species (DeVillez and Buschlen, 1967; Brockerhoff et al. 1970; Brun and Wojtowicz, 1976) and have been isolated and characterized from several others (Zwilling et al., 1969; Zwilling and Tomasek, 1970; Gates and Travis, 1969 and 1973).

Pepsin and chymotrypsin are absent from crustaceans (DeVillez, 1975); the author felt that the low activities reported by some workers for these two enzymes may have been due to a non-specific esterase. Non-specific esterases are capable of hydrolyzing amides of amino acids and can thus play a part in protein metabolism (Momin and Rangneker, 1975).

Pfleiderer et al. (1967) isolated a low molecular weight (LMW) proteinase from Astacus and, similarly, DeVillez and Lau (1970) isolated a LMW alkaline proteinase with broad substrate specificity from Orconectes virilis. The latter authors believed that the LMW proteinase acted in a compensatory manner for the absence of pepsin from the gastric fluid. Armstrong and DeVillez (1978) have presented evidence that the LMW proteinases isolated from different crustaceans are probably the same and that the slight differences in characteristics were probably due to differences in extraction procedures.

Brockerhoff et al. (1970) found "trypsin", "chymotrypsin", "carboxypeptidase A", and two proteases which were active over pH ranges of pH 4 to pH 7 and pH 2 or 3 to pH 4 in the adult American lobster. No activity was detected for carboxypeptidase B, elastase, dipeptidase, or aminopeptidase. Barker and Gibson (1977) were unable to demonstrate endopeptidase with any certainty in the midgut gland of

H. gammarus and showed only weak exopeptidase activity. They hypothesized, however, that lobster proteases could be reacting to the esterase test instead since the histochemical distinction between carboxylesterases and certain proteolytic groups is not always possible (Pearse, 1972). Barker and Gibson (1977) suggested that an exopeptidase is secreted during the latter part of extracellular digestion in the lobster but that proteolysis is completed intracellularly.

It has been reported that crustacean trypsin may not be produced in zymogen form (Gates and Travis, 1969; Brockerhoff et al., 1970). Dall and Moriarty (1983) discussed the current evidence suggesting that crustaceans in general do not produce zymogens. Recently, however, Al-Mohanna et al. (1985) reported the presence of zymogen granules in F-cells of the shrimp Penaeus semisulcatus.

Lipases and esterases have been demonstrated in many crustaceans (cf. van Weel, 1970). Simple esterases tend to dominate lipid digestion in invertebrates except in Homarus and a few others which appear to be able to digest lipids better than fatty esters (Pandian, 1975). Brockerhoff et al. (1967, 1970) found lipase in H. americanus. Barker and Gibson (1977) reported lipase and esterase activity in H. gammarus, occurring both extracellularly and intracellularly.

As mentioned above, crustaceans are incapable of cholesterol synthesis (Zandee, 1967), tend to conserve the dietary cholesterol, and thus do not produce bile salts. Instead they produce other surface-active compounds with comparable functions. Holwerda and Vonk

(1973) identified the surface-active substance from H. vulgaris as a fatty acyl-dipeptide.

The digestive tract of the lobster

The following section on the morphology of the lobster gut is based on the papers and reviews of Williams (1907), van Weel (1970), Gibson and Barker (1979), Hinton and Corey (1979), Conklin (1980), Factor (1981), Dall and Moriarty (1983), and McLaughlin (1983).

All crustaceans possess a simple intestinal tract: a straight gut with three embryologically distinct divisions, the foregut (stomodaeum), midgut (mesenteron), and hindgut (proctodaeum). The stomodaeum and proctodaeum are both ectodermal in origin and are lined with uncalcified chitin. The mesenteron develops from the endoderm, thus lacking a chitin lining.

The stomodaeum consists of the mouth, esophagus, and a two-chambered stomach. There are many tegmental glands around the labrum and in the anterior part of the esophagus which excrete a lubricating mucopolysaccharide. A tri-lobed valve separates the esophagus from the stomach. The anterior chamber of the foregut has also been referred to as the cardiac stomach. It is a bulbous widening of the foregut and includes the gastric mill, or proventriculus, at its posterior end.

Powell (1974) formulated a plausible model of foregut function in Callianassa which appears to be applicable for most decapods. After food enters the anterior chamber of the foregut, it is triturated by the gastric mill and mixed with gastric fluid from the midgut gland. The stomach is thus the principal site for extracellular digestion.

The main grinding structure of the calcified gastric mill is formed from three large, opposing teeth: two lateral and a single medial one. There is also a pair of accessory lateral teeth ventral to the lateral teeth. Two calcareous plates, the gastroliths, occur in the anterior chamber and function to store calcium during molting. The anterior chamber is separated from the smaller posterior chamber (also known as the pyloric stomach) by the cardio-pyloric valve (CPV). Food particles too large to pass over the valve are directed dorsally and are reprocessed by the gastric mill. Small particles pass laterally around the CPV and into the posterior chamber. The smallest food particles are able to pass through the gland filter of the posterior chamber into the tubules of the midgut gland. Intermediate sized particles, too large for the gland filter, pass posteriorly into the intestine. Waste debris from the midgut gland is moved by peristalsis into the intestine.

The mesenteron includes the intestine and several diverticula or caeca, the largest and most obvious of which is the midgut gland, also called the digestive gland or hepatopancreas. The term midgut gland is used in the present study rather than hepatopancreas or digestive gland, following the terminology of van Weel (1974) and McLaughlin (1983). The midgut gland synthesizes and secretes digestive enzymes and resorbs digested food as well as performing other important functions. (The midgut gland will be discussed in detail in the following section.) The anterior midgut caeca are smaller and arise along the anterior dorsal midline of the midgut; one caecum projects forward on each side of the posterior chamber. The posterior midgut

caecum arises from the left side of the intestine, just before the hindgut, and bends across to the right side.

The hindgut joins the midgut in the sixth abdominal segment. Waste food particles are compacted into feces in the hindgut. It forms a short rectum which joins to the slit-like anus opening on the ventral side of the telson.

The basic structure of the larval digestive system is the same as that of the adult (Williams, 1907; Hinton and Corey, 1979; Factor, 1981). It is more simplified but undergoes progressive development at each larval stage. There are a number of changes which occur in the gastric mill. First and second stage lobsters lack the medial and lateral teeth, possessing instead a series of pads and ridges. Heavily cuticularized teeth first appear in third stagers and the accessory lateral teeth appear in the fourth stage. Williams (1907) stated that while the gastric mill of stage IV larvae and of adults was a grinding and crushing organ, that of first and second stagers was a tearing and shredding organ. The developmental changes in the gastric mill and also those which occur in the mouthparts correlated well with the changes in diet and habitat which accompanied the metamorphosis from planktonic larvae to benthic juveniles (Factor, 1978). Gastroliths are absent until the fourth stage.

The complexity of the midgut gland also increases with development (Factor, 1981; Biesiot, 1983), as both the number and length of tubules increases. The diameter of the lumen, relative to the size of the gland, decreases and the thickness of the walls increases.

According to Factor (1981), the stage I larva does not have a posterior midgut caecum, the stage II larva bears a rudimentary outpocketing of the intestinal wall, whereas the stage III larva has a well-formed caecum with a lateral bend as in the adult. Hinton and Corey (1979), however, reported that in stages I, II, and III the caecum consists of a small fold in the dorsoposterior wall of the midgut and that stages IV and V have a caecum extending about one quarter the length of the hindgut.

The midgut gland

The midgut gland, also referred to as the hepatopancreas, gastric gland, or the digestive gland (cf. van Weel, 1974; McLaughlin, 1983) for discussion of the appropriate name), is the most obvious structure in the cephalothorax of the lobster. In the introduction to their extensive review paper on the decapod midgut gland Gibson and Barker (1979) state:

> That the hepatopancreas is a vital and major organ in the Crustacea is beyond a doubt; involved in diverse metabolic activities, it is primarily responsible for the synthesis and secretion of digestive enzymes and subsequent uptake of nutrient materials, but is also implicated in excretion, the moulting cycle, the storage of inorganic reserves, and lipid and carbohydrate metabolism, and at least a proportion of its functions are under neuroendocrine control.

In view of the principal role played by the midgut gland in the process of digestion, the following is a detailed discussion of its structure and function in adult lobsters, based primarily on the review by Gibson and Barker (1979) and their study (Barker and Gibson, 1977) on digestion in the European lobster Homarus gammarus.

The midgut gland is a paired structure flanking the stomach and anterior part of the intestine. Each half of the midgut gland is

composed of three lobes, simply named the anterior, dorsal, and posterior. The two halves open independently from the gut at the junction of the pyloric stomach and midgut through a pair of primary ducts which branch into secondary ductules in each of the lobes. The ductules subdivide extensively in each lobe to form a complex of blind-ended tubules.

The midgut gland is closely associated with the hemolymph system. Paired hepatic arteries leave the ventral surface of the heart and run to the midgut gland (Phillips et al., 1980). The intertubular areas are filled with blood sinuses, basophilic connective tissue, polysaccharide deposits, and "wandering cells" (Barker and Gibson, 1977).

A myoepithelial layer sheathes the tubules at the hemolymph sinus interface and forms a uniform reticulated array of broad circular muscle fibers connected by smaller longitudinal muscle fibers (Leavitt and Bayer, 1982). These muscles contract sequentially to move materials in and out of the lumen of the tubules (Loizzi, 1971).

The tubules are lined by an epithelium which is one cell layer thick except at the distal, closed end. Four cell types make up the epithelium. They were described and named by Jacobs (1928): Embryonalenzellen ("embryonic cells"), Fibrillenzellen ("fibrillar cells"), Blasenzellen ("blister cells"), and Restzellen ("remaining cells"). Hirsch and Jacobs (1928) renamed the Restzellen to Resorptionzellen ("resorptive cells") after further study of their function. This nomenclature is the basis of the modern labelling of hepatopancreatic cell types as E-cells, F-cells, B-cells, and R-cells.

E-cells are the smallest cells and are confined to the distal ends of the tubules. They are undifferentiated cells, produced continuously by mitosis, and contain organelles typical of unspecialized cells: short lengths of developing endoplasmic reticulum (ER), both rough (RER) and smooth (SER), small spherical mitochondria with few cristae, single membrane bound vesicles, and numerous Golgi bodies. The large smooth-surfaced nucleus contains a single acidophilic nucleolus.

F-cells synthesize digestive enzymes and store them in supranuclear vacuoles. F-cells appear striated because of their extensively developed RER. In young F-cells the cisternae are long and narrow but when the cells mature the ER develops further and the cisternae dilate as they fill with material. F-cells are strongly basophilic because of the large numbers of free and membrane-bound ribosomes. Mitochondria are numerous and uniformly distributed in the cytoplasm. The many Golgi bodies are always enlarged; the complex is surrounded by small, dense vesicles. All of these characteristics point to the participation of F-cells in protein or enzyme synthesis. There is a brush border of microvilli on the lumenal surface. Older F-cells characteristically develop one or more supranuclear vacuoles distal to the nucleus. Each first appears as a small vacuole adjacent to one of the Golgi complexes and contains numerous inclusion bodies which eventually coalesce into a single mass. Meanwhile, the cytoplasm and RER become condensed and displaced distally, coated vesicles appear near the lumenal border, and pinocytotic vesicles and channels appear. In young F-cells, the nucleus is located medioproximally but as the cell ages it becomes more central and takes on a wrinkled appearance.

B-cells develop from F-cells; they are the largest cell type and contain a huge (80-90% of total cell volume) blisterlike vacuole enclosed by a thin layer of dense cytoplasm with RER. The vacuole contains digestive enzymes and is formed by further enlargement of the vacuole in the distal cytoplasm of an F-cell. An apical complex is located at the distal edge of B-cells. It is composed of a brush border with a sparse enteric coat, small accumulations of vacuoles, pinocytotic vesicles, small mitochondria, and bundles of microtubules. The nucleus of mature B-cells is compressed near the proximal cell border.

Secretion of the B-cell contents occurs by two different methods depending on the nutritional state of the animal. Loizzi (1966) working with the crawfish Orconectes virilis first suggested the sequence of events which was later also noted for the adult lobster H. gammarus by Barker and Gibson (1977). In previously starved animals, B-cells mature rapidly after a meal. Violent peristalsis in the midgut gland squeezes intact B-cells into the tubule lumen within 0.5-1.5 h of feeding. Thus, initial secretion in this case is holocrine; however the later waves of enzyme secretion are merocrine. Under normal feeding conditions, both the initial and secondary waves of enzyme secretion are merocrine. Merocrine discharge results in a B-cell that resembles an F-cell and is followed by cyclical restitution of the digestive enzymes which replenishes the B-cells.

Presenting an opposing viewpoint to the model outlined above, Hopkin and Nott (1980) suggested that B-cells are not secretory. Working with Carcinus maenus, they found numerous B-cells and B-cell vacuoles in the feces of the crab within 12 to 48 h after feeding. These authors believe

that F-cells synthesize and secrete digestive enzymes and that B-cells develop from F-cells but function to store and then excrete metabolic waste products produced by the F-cells. Al-Mohanna et al. (1985) proposed that F-cells of Penaeus semisulcatus produce digestive enzymes in zymogen granules, secrete the enzymes, and then transform into B-cells by endocytosis of nutrients from the lumen of the midgut gland.

R-cells are the most abundant cell type in the midgut gland and characteristically contain large numbers of irregularly shaped vacuoles. They function in the absorption of digested food molecules. The brush border of R-cells is slightly longer and bears a surface enteric coat reportedly consistently thicker that that of F-cells. Immediately below the brush border is an area nearly free of intracellular organelles, called the terminal web. Below the terminal web are concentrations of mitochondria. Proteinaceous granules have been found in the distal cytoplasm in H. gammarus. There is scanty RER in young R-cells, with short lengths of cisternae which bud off to form chains of vesicles; there are fewer ribosomes than on F-cell RER. The Golgi bodies are stacks of flattened lamellae. R-cells contain large amounts of glycogen and lipid. Older R-cells, more proximally situated in the tubules, contain more and larger lipid droplets than young R-cells and less glycogen. R-cells were also implicated as the site of intracellular digestion of lipids and proteins in H. gammarus by Barker and Gibson (1977).

The epithelial cells show a differential distribution along the length of midgut gland tubules. E-cells are found only in the distal end. As they differentiate into the other types, the cells migrate

proximally down the tubule. F-cells and R-cells evolve first, as they comprise all the cells of the mediodistal region; all the evidence indicates that F- and R-cells develop independently of each other. The cells go through a maturation process as they move down the tubules. Young F- and R-cells occur more distally than obviously older ones. The medial and medioproximal portion is comprised of F-, R-, and B-cells. The position and histology of B-cells thus indicate that they arise from F-cells, not independently. The most proximal end of the tubule is primarily F- and R-cells with some degenerating B-cells present. Cell death occurs at the base of the tubules; old cells are cast out into the lumen to make room for the younger cells migrating down and are eliminated with other waste materials in the intestine. It is hypothesized that lipid emulsifying compounds are synthesized in R-cells and are liberated when the senescent cells rupture. The secondary ductules of *H. gammarus* are composed entirely of R-cells.

REVIEW OF THE LITERATURE: DIGESTIVE ENZYMES OF MARINE INVERTEBRATES

Trophodynamic field studies

The digestive enzyme activities of marine zooplankton populations have been measured in an attempt to provide information on feeding and nutritional status of the organisms under field conditions. Activities of specific digestive enzymes have been related to the trophic level of natural populations and a decrease in activity has been correlated with food deprivation (Boucher and Samain, 1975; Boucher et al., 1976). Since trypsin degrades protein and amylase degrades carbohydrate, changes in the amylase to trypsin (A/T) ratio have been used as a measure of changes in the food composition. Species with low A/T ratios were assumed to

metabolize protein more efficiently than carbohydrate while those with high A/T ratios were presumed to use starch and protein with equal efficiency (Gaudy and Boucher, 1983).

Early work showed positive correlations between components of the diet and certain enzyme activities which suggested that digestive enzyme synthesis was substrate induced. Boucher and Samain (1974) observed that amylase activity may be higher in herbivorous copepods and that there may be a diurnal rhythm of activity. Mayzaud and Conover (1976) surveyed the biochemical composition (protein, carbohydrate, and lipid) of the natural particulate matter available as food and the activity of digestive enzymes of neritic zooplankton during summer and fall. Activities of carbohydrases including amylase, ß-galactosidase, and α- and ß-glucosidases and the proteases cathepsin and trypsin varied in association with seasonal variations in the food supply. Michaelis constants (K_m) showed an inverse relationship between the carbohydrases and proteases, suggesting the possibility that large amounts of protein in the diet reduced the affinity of the carbohydrases and vice versa. No phospholipase activity was detected.

The results of Mayzaud and Conover (1984) also generally support the hypothesis that the amount and biochemical composition of the food supply can affect zooplankton digestive enzymes but with an important corollary. That study was undertaken during the spring bloom and sampled zooplankton populations over depth. Enormous changes in the population structure of the near-surface waters took place while conditions in deeper waters were more stable. There was no correlation between digestive enzymes and food supply in samples taken from surface waters,

indicating factors other than dietary induction were controlling activity. Thus, the authors warn, problems in the interpretation of such studies can occur if the community structure is not well-defined and if the spatial and temporal distributions of the organisms are not carefully studied.

Harris et al. (1986) have recently shown that under food-saturated conditions, there is a compensatory mechanism between digestive enzyme levels and the type of substrate ingested. Amylase activity in field caught Calanus helgolandicus gradually declined when the copepods were fed algae with low levels of starch but it increased, after a short lag period, when they were fed starch-rich algae. Trypsin activity showed the opposite pattern, with a rapid initial decline in activity followed by a constant level among those fed algae with a higher protein content and a slight increase among those fed algae with a lower protein content. In contrast, Bamstedt (1984) reported no correlation between amylase activity and carbohydrate concentration or between trypsin activity and protein concentration in Calanus glacialis.

Diel rhythms in digestive enzyme activity have been reported in a number of field studies (Boucher and Samain, 1974; Boucher et al., 1976; van Wormhoudt, 1977; van Wormhoudt and Ceccaldi, 1976; Mayzaud et al., 1984). Bamstedt (1984) and Head et al. (1984), however, found no such cyclic rhythms in their studies.

Digestive enzymes in relation to composition of the diet: laboratory studies

Yonge (1937) reported a definite correlation between the food of an animal and the nature and activity of its digestive enzymes. There is recent evidence in support of this statement. The activity of

laminarinase in the omnivorous euphausiid Euphasia pacifica could be reduced to zero by feeding the euphausiid only animal food and could be increased if phytoplankton was fed (Cox, 1981). Cox and Willason (1981) determined that laminarinase activity in the copepod Calanus pacificus was induced by the presence of phytoplankton or particulate laminarin in the diet. Induction appeared to have a lag time of about one day. Starvation reduced enzyme activity but not below a constitutive level.

There are also reports in the literature on marine fishes that the specific proteolytic activity in the digestive tract is proportional to the protein content of the diet and is thus higher in carnivorous than in herbivorous species. Some workers, however, have found poor correlation between the type of diet and the enzymes present. Hofer (1982) pointed out the following paradox: since herbivorous fish ingest such low amounts of protein, special mechanisms of nutrient extraction are required, one of which would be secretion of large amounts of protease. In experiments with adult and juvenile roach Rutilus rutilus the author found higher daily proteolytic production in those feeding on grass than those feeding on meal worms.

There is other evidence contrary to Yonge's (1937) statement. Van Weel (1959) showed that a continued high protein diet caused a suppression of protease activity in snails. During a study on the amylase and proteinases of decapods, Sather (1969) discovered higher protease activity in omnivores than in carnivores. Hoyle (1973) examined enzyme secretion in fasted adult lobsters and in those fed diets containing 0, 5, and 20% starch. The author found little pattern to the variation of total enzymes in digestive juice with respect to diet except

that fed animals seemed to have higher specific activities of proteinase and lipase than fasted ones. The exclusion of starch from the diet for periods of 20-30 days did not seem to lower the total amylase production. The author suggested that a low level of production of all major enzymes would allow for immediate digestion of almost any available food which could be supplemented by a subsequent increase in enzymes necessary to deal with a substantial quantity of an unusual food material as it became available.

Head and Conover (1983) studied levels of digestive enzymes in diapausing stage V Calanus hyperboreus. Three factors contributed to the changes observed in digestive enzyme levels: (1) the termination of diapause; (2) the molting of stage V copepodites to feeding females and non-feeding males; and (3) the effect of food on each of the different stages. They determined that feeding induced synthesis of a suite of digestive enzymes. There was a lag of one week at 8-9°C and of two weeks at 1-2°C. Animals molting to females had higher levels of digestive protease and laminarinase than animals molting to males. The males had digestive enzyme levels similar to starved controls. The type of substrate in the diet did not affect levels of digestive enzymes among females nor did ten days of starvation significantly depress activity.

A recent hypothesis states that zooplankton acclimate to ambient food concentrations such that higher digestive enzyme activities and higher maximum ingestion rates occur at higher food levels (Poulet, 1974; Mayzaud and Conover, 1976; Mayzaud and Poulet, 1978; Hirche, 1981). Hassett and Landry (1983) have reported a positive correlation between ingestion rate and digestive enzyme activities in Calanus pacificus.

However, the copepods had higher ingestion rates and higher enzyme activities at lower food concentrations.

Samain et al. (1981) proposed a general model which may reconcile these different observations. They studied Artemia which were fed the same species of phytoplankton at the same concentration. The phytoplankton, however, had different biochemical compositions, either low carbohydrate-high protein or high carbohydrate-low protein ratios. Two processes were suggested to regulate feeding ecology and behavior in zooplankton. Ingestion rate was regulated by the quality of the food rather than the quantity. Protein tended to be ingested at a relatively constant rate; large differences in the amount of carbohydrate ingested therefore occurred depending upon the food source. The assimilation of these two nutrients was then controlled by the levels of digestive enzymes. Experimental results indicated that digestive enzymes have greater efficiency at lower substrate concentrations.

The general consensus suggests that regulation of protease and amylase synthesis are independent of each other but that amylase synthesis is correlated with the amount of carbohydrate in the diet (van Praet, 1982). This may be due to the fact that carbohydrate is a relatively minor component of the natural diet of marine organisms. Induction of a specific carbohydrase as needed could be an adaptation for efficient use of a rare or seasonally available food source. Protein, however, is a major proportion of the natural diet. Marine organisms are probably adapted to this substrate and always produce proteases.

It is ironic, therefore, that both Brockerhoff et al. (1970) and Barker and Gibson (1977) commented that they were unable to demonstrate

the presence of much protease activity in the lobster. Brun and Wojtowicz (1976) acknowledged that they were unable to detect either protease or lipase in fresh extract of lobster midgut gland although the enzymes were present in lyophilized gastric juice. However, the assay conditions in these studies may not have been optimal for detection of the particular enzymatic activity.

Changes in lipase activity in relation to the diet have not been examined, partly because the analysis is both difficult and tedious. The fact that lipid is always less abundant than protein or carbohydrate in marine particulate matter, the natural food for planktonic grazers (Mayzaud and Martin, 1975), has led most investigators to omit lipases from their analyses. However, lipids are important dietary components for herbivores as well as for carnivores and omnivores. Many marine invertebrates incorporate the dietary lipid unchanged into their depot lipid (Kattner et al., 1981).

Digestive enzymes in relation to the molt cycle

Trellu and Ceccaldi (1976) determined the activity of digestive enzymes during the molt cycle of _Palaemon serratus_ "by a semi-quantitative method." Although they were not able to detect a number of digestive enzymes known to occur in this species, they did report that a number of other enzymes showed minima and maxima at specific stages in the molt cycle. Van Wormhoudt (1983) measured amylase activity of the prawn _Palaemon serratus_ during the molt cycle at different times of the year. During the summer, amylase activity increased twofold between intermolt and premolt stages reaching a maximum at D_1.... During the winter, no significant variation were measured.

Trypsin activity was at a maximum during D_2D_3 in Penaeus japonicus and at a minimum during A (Galgani et al., 1985). No work has been conducted which correlates other digestive enzyme activities over the course of the molt cycle of larval crustaceans but it is possible that molt stage specific changes in digestive enzyme activities may occur.

CHAPTER 2: GENERAL MATERIALS AND METHODS

Experimental animals: gravid females

Ovigerous lobsters ("eggers") were obtained offshore from New Bedford, MA, from the Massachusetts State Lobster Hatchery, Vineyard Haven, MA, and from the Division of Marine Fisheries, Sandwich, MA. They ranged in size from 0.6 to 1.5 kg; carapace lengths ranged from 90 to 120 mm. The lobsters were held at the Environmental Systems Laboratory (ESL) of the Woods Hole Oceanographic Institution (WHOI), Woods Hole, MA until the eggs hatched. The holding tanks had flowing natural seawater of 30 to 31 ppt S and were equipped with shelters, either cinder blocks or pieces of PVC pipe. The lobsters were fed frozen squid *Loligo pealei* two or three times a week and occasionally menhaden *Brevoortia tyrannus* or mussels *Mytilus edulis*.

Experimental animals: embryos and prelarvae

Embryos at different stages of development (black, gold, and blue eggs) were sampled by cutting small masses of eggs from the pleopods of the female. The eggs were transferred to finger bowls containing filtered seawater and examined under a binocular dissecting scope to confirm viability. Individual eggs were cut from the clumps with small dissecting scissors and as much of the extraneous attachment membrane as possible was trimmed away.

Prelarvae were obtained by one of several ways. Occasionally they were ejected from the egg mass by the pleopod flicking motions of the female which normally occurred during the course of hatching. Prelarvae were then netted from the bottom of the aquarium. They were

also removed directly from the egg mass of the female with small, flexible forceps or else dissected from the swollen inner chorion egg membrane under the binocular scope.

Experimental animals: lobster larvae

Females bearing eggs in advanced stages of development were put into separate 95 l glass aquaria. Discrete hatches of sibling lobsters from a single female could thereby be netted from each aquarium. The overflow tubes were covered with fiberglass screen to prevent the siphoning loss of larvae.

Unfed first stage larvae were needed for some of the experiments. To ensure that they did not engage in the cannibalism of their siblings, larvae were netted within two to four hours of hatching and segregated into individual containers until they were well into molt stage C (intermolt), about 12-24 h.

Large numbers of individual compartments were made by cutting off the bottoms of plastic ice cube trays and attaching a rectangle of fiberglass screen by heat sealing it in place. The screen permitted water exchange and, in the case of the fed controls, allowed feces and particles of uneaten food to drop out of the compartments. To improve flotation, three or four narrow strips of styrofoam were inserted in the gaps between the compartments and the screening. The modified ice cube trays were floated in water tables 2.4 m x 0.7 m x 10 cm deep through which flowed 10 um filtered seawater. Two or three large airstones in each water table aided water circulation.

Larvae used for the morphological studies and for the studies on enzyme activity during development were raised communally from the

first to the fourth stage in 40 l fiberglass plankton kreisels (Hughes et al., 1974). The kreisels were supplied with natural seawater at sufficient water flow to keep the animals and their food suspended. The center standpipe and kreisel bottoms were cleaned daily. Every third day the larvae were transferred to a bucket for a brief time while the tanks were thoroughly scrubbed. Temperature was ambient in the summer (20-23°C) or heated to 19-22°C during the winter and spring. The light regime was 14 h light and 10 h reduced illumination.

The lobster larvae were fed frozen adult brine shrimp *Artemia* sp. two or three times a day at approximately 8 h intervals. The brine shrimp were thawed in a container of seawater and then rinsed several times with fresh seawater to remove debris. A feed supplement composed of 20% cod liver oil, 10% homogenized brine shrimp, and 70% gelatin (Sasaki, 1984) was given once a day either by itself or in combination with the brine shrimp. Sasaki and Capuzzo (1984) have shown that frozen *Artemia* sp. is deficient in the essential fatty acids 20:5ω3 and 22:6ω3. Cod liver oil has a high percentage of these fatty acids (Ackman and Burgher, 1964) and is known to promote growth in lobsters (Castell and Covey, 1976). Without this feed supplement, there was poor survival of larvae molting from stage III to stage IV (Sasaki, 1984).

By the time the fourth stage larvae were seven days old or older, they appeared physically and behaviorally capable of settling to the benthos. They were strong swimmers and exhibited directed swimming activity against the water current in the kreisels. Occasionally they clung to the center standpipe or nestled on the bottom below the water

jets in the standpipe. At this point in their development, the larvae were moved to individual fiberglass screen cages, cylinders about 4 cm in diameter and 11 cm tall. The cages were placed in compartmented trays set in the water tables described above; the trays had a screen bottom for sea water exchange. The survival rate of these older stage IV larvae after transfer to the cages was very good (>85%). Feeding with Artemia sp. was decreased to once a day and the feed supplement was discontinued. All subsequent postlarval and juvenile stages (stage V and older) were reared in these cages.

The larval and postlarval lobsters were molt staged by the method of Sasaki (1984) which was based on the method described by Aiken (1973, 1980) for molt staging adult lobsters.

Summer-hatch and winter-hatch larvae

Female lobsters with well-developed gold embryos were collected in the spring and early summer and held in the laboratory at ambient natural seawater temperatures (19-22°C). Larvae from these lobsters hatched during the summer (May through July or early August). Eggers with newly-extruded green or black eggs were obtained in late summer and fall. They were held in the laboratory over the course of the winter at ambient natural seawater temperature; seawater temperature dropped to 2°C or less in midwinter and rose to about 15°C by late spring. Larvae from these lobsters hatched in May and early June. Larvae which hatched in the laboratory during the normal hatching period in nature constituted the so-called "summer-hatch larvae", regardless of whether the females over-wintered in the wild or in the laboratory.

Because the duration of embryonic development is temperature dependent (Templeman, 1940), it is possible to obtain lobster larvae outside the natural hatching season by exposing the eggers to seawater temperatures warmer than ambient (Branford, 1978; Waddy and Aiken, 1984). Fall-caught lobsters with newly extruded eggs were held in heated seawater (19-22°C); their larvae hatched in December and January. Lobsters held at about 17°C produced larvae in January and February. Such larvae are hereafter referred to as "winter-hatch larvae".

Storage of samples for digestive enzyme analysis

Replicate samples of lobsters at the appropriate stage of development for a particular experiment were taken. The lobsters were rinsed quickly in distilled water to remove salts and blotted dry on paper towels. They were transferred to 1.0 or 1.5 ml plastic disposable microcentrifuge tubes and frozen in liquid nitrogen. The samples were stored in an ultra-cold freezer (-85°C) until the enzyme assays were performed. There was no significant influence on the enzyme activities due to freezing and thawing the samples (cf. Appendix B).

Preparation of samples for digestive enzyme analysis

On the day of analysis the samples were removed from the ultra-cold freezer and kept on ice during transfer to the lab. The foregut and midgut gland tissues were trimmed from the carcass under a binocular dissecting scope. The intestine (hindgut) was not included because it is not involved in digestive enzyme synthesis or secretion. The abdomen was cut off, the periopods were removed, and

the mouthparts and eyes snipped off. This "trimmed" portion of the lobster thorax contained the foregut and midgut gland tissues, the carapace, a small bit of musculature, some nervous tissue, and the heart. A homogenate of the digestive tract tissues was made using ice-cold 0.1 M citrate phosphate buffer, pH 5.5. Enzyme activity calculated on the basis of the amount of protein in the trimmed thorax was not significantly different from that determined on the basis of protein in the foregut and midgut gland (cf. footnote c, Table 4.1 in Chapter 4); the carcass contained no detectable digestive enzyme activity.

Statistical analysis

Statistical analyses (Zar, 1984) including analysis of variance (ANOVA), the Student-Newman-Keuls test, nonparametric Kruskal-Wallis ANOVA, the Mann-Whitney test, and the median test which is based on a χ^2 contingency table were performed in the appropriate instances.

CHAPTER 3:
CHANGES IN MIDGUT GLAND MORPHOLOGY ASSOCIATED WITH DEVELOPMENT IN EARLY STAGES OF THE AMERICAN LOBSTER HOMARUS AMERICANUS

INTRODUCTION

The functional morphology and development of the digestive tract of marine crustacean embryos and larvae have not received much attention compared to the large number of studies performed on adult animals. Reddy (1935) studied the developing gastric mill in the stomatopod *Squilla nepa*. Le Roux (1971a and 1971b) described the foregut in larval and juvenile shrimp *Palaemonetes varians*. Walley (1969) and Rainbow and Walker (1977) worked on the development of the digestive tract of larval barnacles *Balanus* spp. More extensive work has been conducted with the American lobster *Homarus americanus*. Smith (1873) and Bumpus (1891) briefly mentioned changes occurring in the embryonic digestive tract. Herrick (1911) gave a rudimentary description of the developing digestive tract of embyronic and larval lobsters. Williams (1907) described the larval lobster stomach, including the foregut, the musculature, the gastric mill, and the formation of gastroliths. Hinton and Corey (1979) performed a more detailed analysis of the mouthparts and developing digestive tract of American lobster larvae as did Factor (1979, 1981) in a similar study. These latter studies focused on aspects of development in the midgut and hindgut as well as re-examining the foregut. However, neither group made more than cursory mention of changes in the midgut gland. In adult decapod crustaceans the midgut gland, also called the hepatopancreas or digestive gland, is responsible for synthesis and secretion of digestive enzymes, for resorption of digested nutrients,

and for storage of energy reserves and thus plays a crucial role in digestion (cf. Gibson and Barker, 1979 for review and Chapter 1 of the present work for a more detailed discussion).

In adult crustaceans the midgut gland is composed of a series of blind-ended tubules, each of which is lined by an epithelium one cell layer thick except at the distal, closed end. Four cell types make up this epithelium, the E-, R-, F-, and B-cells (Jacobs, 1928; Hirsch and Jacobs, 1928). E-cells are undifferentiated and produced continuously by mitosis. As they migrate down the tubules, E-cells differentiate into R- or F-cells. The R-cells are the most abundant cell type and characteristically contain large numbers of irregularly shaped vacuoles. Immediately below the brush border is an area called the terminal web which is nearly free of intracellular organelles. R-cells function in the absorption of digested nutrient molecules and in the storage of both glycogen and lipid. F-cells appear striated because of the extensively developed RER; they are strongly basophilic because of the large numbers of ribosomes. F-cells synthesize digestive enzymes and store them in supranuclear vacuoles. Further down the tubule is a zone composed of R-, F-, and B-cells. B-cells develop from F-cells and are responsible for secretion of the digestive enzymes. They are the largest cell type and contain a huge vacuole which is formed by further enlargement of the F-cell vacuole. An apical complex is located at the distal edge of B-cells. It is composed of a brush border, small vacuoles, pinocytotic vesicles, small mitochondria, and bundles of microtubules. The nucleus of B-cells is compressed near the proximal cell border.

The present study was undertaken to examine and describe certain aspects of development of the midgut gland in early developmental stages of the American lobster H. americanus. Knowledge of the extent and timing of developmental changes in the different cell types of the midgut gland should provide a basis for assessing changes in the digestive capabilities of this species.

MATERIALS AND METHODS

Morphology of the developing midgut gland: light microscopy

Eggs, prelarvae, larvae, and postlarvae of the American lobster were examined to describe morphological changes which occur in the mdigut gland during development. Embryos and prelarvae were removed from the egg mass of female lobsters at the appropriate stage of development as described in Chapter 2. Larvae were raised communally in kreisels and the postlarvae reared in separate cages, also as described earlier. The larvae and postlarvae were molt staged and only intermolt (stage C) animals were used.

Tissues to be used for light microscopy were fixed in 3% phosphate-buffered gluteraldehyde, pH 7.2 for 1.5-2 h at 7°C. The vials were swirled occasionally during this period. The tissues were washed three times in cold 0.1 M phosphate buffer for 10 min each and then dehydrated at room temperature. Dehydration commenced with 50% ethanol for 1 min, then two changes of 50% ethanol for 5 min each, followed by 15 min each in 70, 80, 90, and 95% ethanol.

The vials were put on a shaker table at low speed during washing, dehydrating, and infiltration. Plastic embedding media, either Sorvall or JB-4, was used. Tissues were infiltrated for about 1.5 h

each in two changes of media and then embedded in embedding media mixed according to the manufacturer specifications.

Plastic embedding medium does not polymerize in the presence of oxygen. Therefore, either melted Paraplast® was dripped over the exposed surface of the plastic to exclude oxygen or the filled embedding trays were slipped into plastic bags filled with nitrogen.

The tissues were sectioned at 2 or 3 um on a Sorvall JB-4 microtome with 3/8 inch-wide glass knives and mounted on clean glass microscope slides. The sections were routinely stained with Lee's methylene blue-basic fuchsin (Bennett et al., 1976) which gives results similar to hematoxylin and eosin. Micrographs were taken using Technical Pan film 2415, ASA 100.

Unfed stage I lobster larvae were utilized to demonstrate the presence of yolk material stored in the midgut gland. Newly hatched larvae were held separately for 12 h to reach molt stage C before being sacrificed. The larvae were fixed in 0.1 M phosphate buffered 3% gluteraldehyde, pH 7.4 for one to two h on ice. The fixative contained 0.75 g/ml sucrose. After fixation, the midgut gland was dissected and washed five times for 10 min each in buffer containing sucrose. The tissues were stored in the final buffer wash at 7°C for a maximum of two days. The tissues were post-fixed for 2 h on ice in 1% osmium tetroxide buffered with 0.1 M phosphate buffer plus sucrose and then washed two times in cold buffer for 10 min each. The tissues were dehydrated, infiltrated, and embedded in plastic as described above.

Morphology of the developing midgut gland: scanning electron microscopy

The midgut gland from intermolt stage IV larval lobsters was dissected out and fixed for 1 to 3 h at 7°C in 3% gluteraldehyde buffered with 0.1 M phosphate buffer, pH 7.2 or buffered with seawater which had been filtered to 0.45 um. The tissues were washed in buffer three times for 10 min each, dehydrated in an ethanol series as above, and critical point dried, either in Freon or in CO_2.

Each midgut gland was attached to a stud with double stick Scotch tape, coated with gold in a vacuum sputterer, and observed with a JSM-U3 scanning electron microscope at an accelerating voltage of either 15 or 25 kV. Micrographs were taken using Polaroid Type 55 P/N film.

RESULTS

The gross structure of the midgut gland of larval lobster H. americanus is much like that of the adult lobster except that it has far fewer and shorter tubules. Since very minor variations occurred among the four larval stages, only the results obtained with the stage IV larva are presented here.

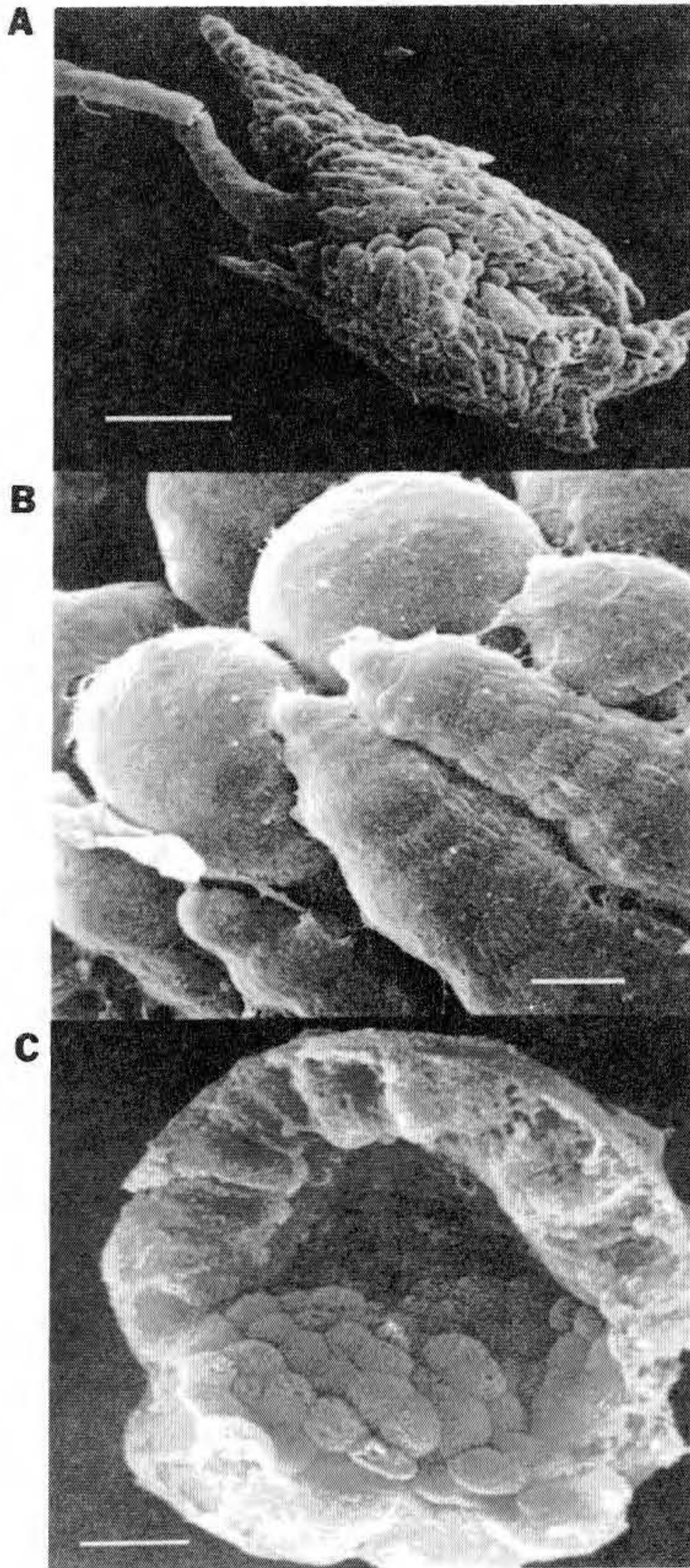
The tubules making up the midgut gland are obvious in Fig. 3.1.A; the midgut (intestine) projects up and to the left in the micrograph. Relaxed tubules as well as those with various degrees of constriction can be seen. The diameter of the tubules was very similar between stage IV larvae and adults but the relative lengths were quite different. Measurements taken of fixed tissues indicated that the length to diameter ratio was about 5 in the stage IV larva but it was

Figure 3.1. Scanning electron micrographs of the midgut gland of the stage IV lobster larva *H. americanus*.

A. Ventral view of the midgut gland. Scale bar = 0.5 mm.

B. Relaxed and contracted tubules of the midgut gland. Scale bar = 50 um.

C. Embryonic cells comprising the distal portion of a tubule in the midgut gland. Scale bar = 25 um.


A
B
C

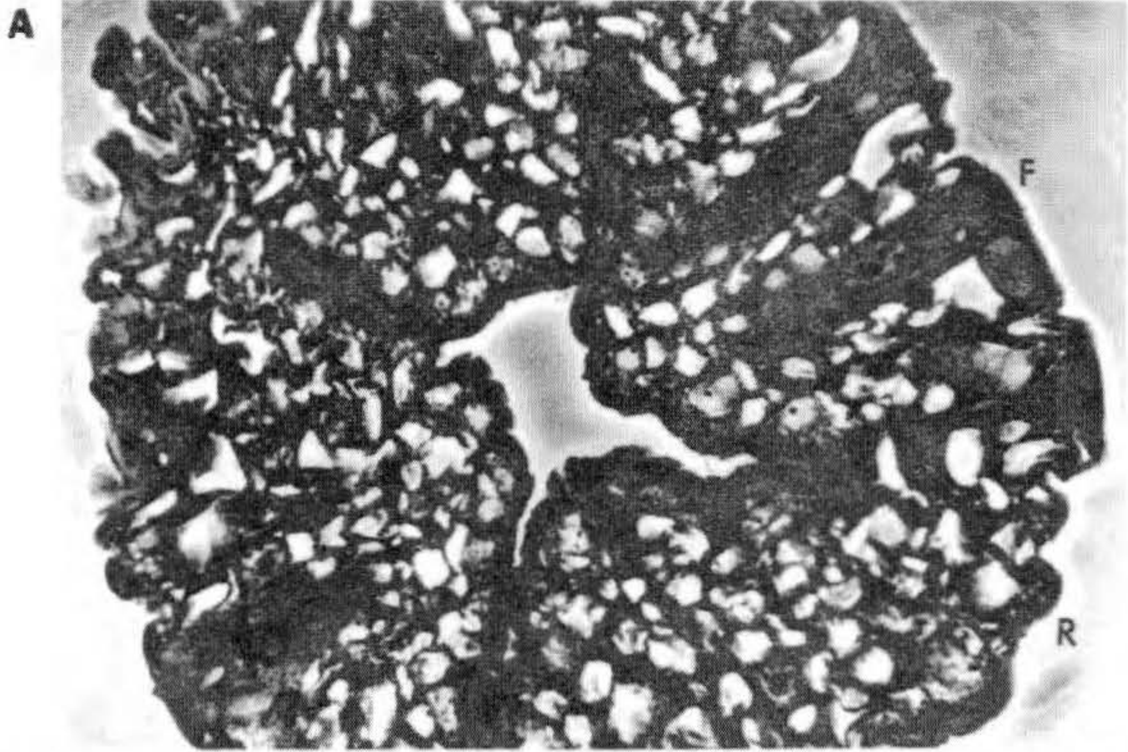
about 50 in the adult. In this respect the morphometrics of the tubules are quite different between larvae and adults.

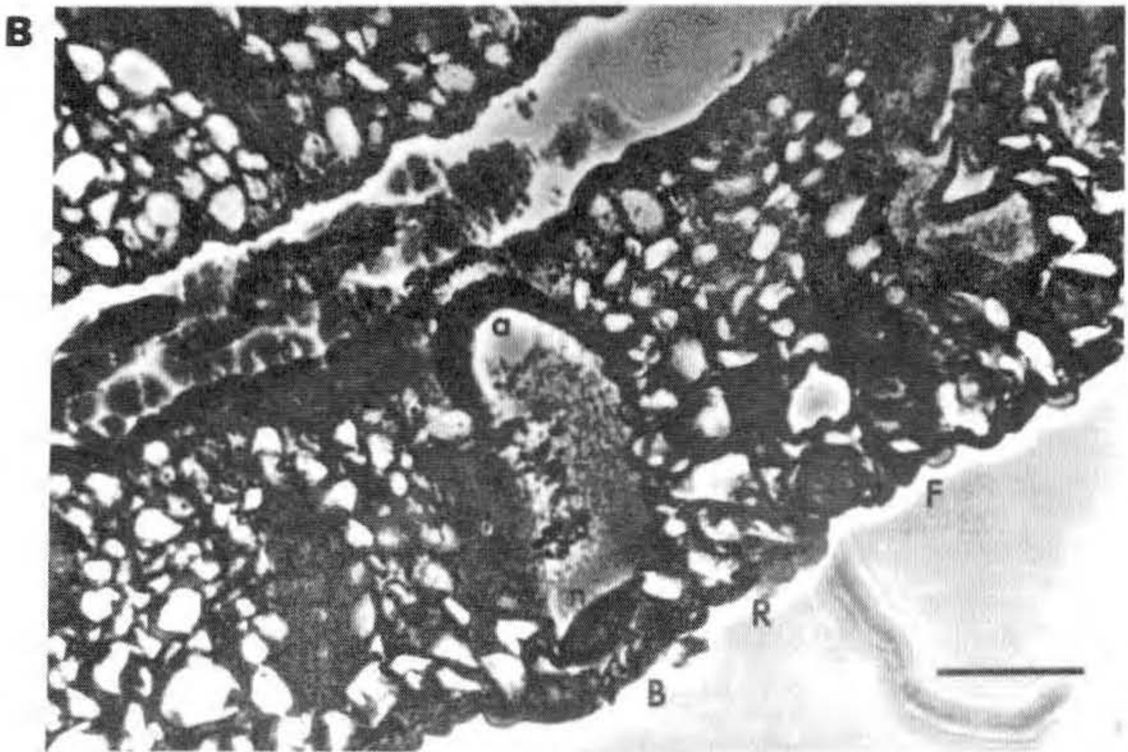
Both relaxed and contracted midgut gland tubules are shown in Fig. 3.1.B. Although the myoepithelium surrounding the tubules obscures them somewhat, there are circular muscle bands approximately 5 um wide and spaced at 17 to 20 um intervals evident in the contracted tubules. Near the tips of these tubules, the circular bands appear to be somewhat closer together. Smaller longitudinal muscle fibers approximately 1 um wide and spaced at 7 um intervals occur between the circular bands. The uppermost right contracted tubule shows a pronounced constriction compared to the others.

The distal end of a tubule from the midgut gland is illustrated in Fig. 3.1.C. E-cells are located at the tip; whether the more proximal columnar cells have begun to differentiate cannot be determined from this preparation. The microvilli covering the epithelial cells are apparent. At this point the cells range from about 7 to 11 um in diameter and are approximately 25 um deep.

A cross section (A) and a sagittal section (B) of midgut gland tubules from a well-fed adult female lobster are presented in order to demonstrate the classic appearance of the R-, F-, and B-cell types (Fig. 3.2). Especially prominent are the numerous small lipid vacuoles in the R-cells. Dark staining F-cells are apparent in both Fig. 3.2.A and 3.2.B whereas B-cells can be seen in Fig. 3.2.B. The apical complex of the B-cell is easily distinguished. Darkly stained granular material is present in the vacuole.

Figure 3.2. Cell types comprising the midgut gland of the adult American lobster H. americanus. R, R-cell; F, F-cell; B, B-cell; a, apical complex of B-cell; n, nucleus. Scale bar = 5 um.
A. Cross section.
B. Sagittal section.


A
F
R


B
a
F
n
R
B

Sections of the midgut gland of a well-advanced gold embryo (A), of a blue egg (B), and a prelarva (C) are shown in Fig. 3.3. The gold egg was sampled approximately three days prior to the initiation of hatching among its siblings, the blue egg was sampled early in the hatching period with the outer chorion still attached, and the prelarvae was sampled within hours of molting to the stage I larva. Primarily R-cells are evident in the midgut gland epithelium of the gold embryo (Fig. 3.3.A) although a few F-cells are also present. Most of the R-cells appear to contain only one, if any, lipid vacuole situated above the nucleus although occasionally cells are seen with a vacuole below the nucleus as well. This description of R-cells lacking numerous lipid vacuoles has not been reported previously. Aside from lacking numerous lipid vacuoles, the R-cells of gold embryos fulfilled the other characteristic criteria of this cell type including the fact that they are most numerous and that they have a terminal web immediately below the brush border. By the blue egg stage (Fig. 3.3.B) B-cells made their appearance in the midgut gland but there was no change in the appearance of the R-cells. Fully mature B-cells were also present in the prelarva (Fig. 3.3.C) along with R- and F-cells. Thus, if form is an indication of function in this case, it appears that the young lobster probably produces digestive enzymes before hatching.

The vacuoles present in R-cells of the early developmental stages of the lobster contain lipid (Fig. 3.4). Embryos themselves could not be used to demonstrate this point because they were too brittle to section properly after osmium tetroxide treatment; the yolk remnants

Figure 3.3. Cell types comprising the midgut gland in embryonic stages of the American lobster H. americanus. R, R-cell; F, F-cell; B, B-cell; a, apical complex of B-cell.

A. Gold egg, well-advanced embryo (approximately three days prior to hatching). Scale bar = 5 um.

B. Blue egg, early in hatching process. Scale bar = 5 um. Inset: B-cell of blue egg. Scale bar = 2 um.

C. Prelarva, late in hatching process. Scale bar = 5 um.

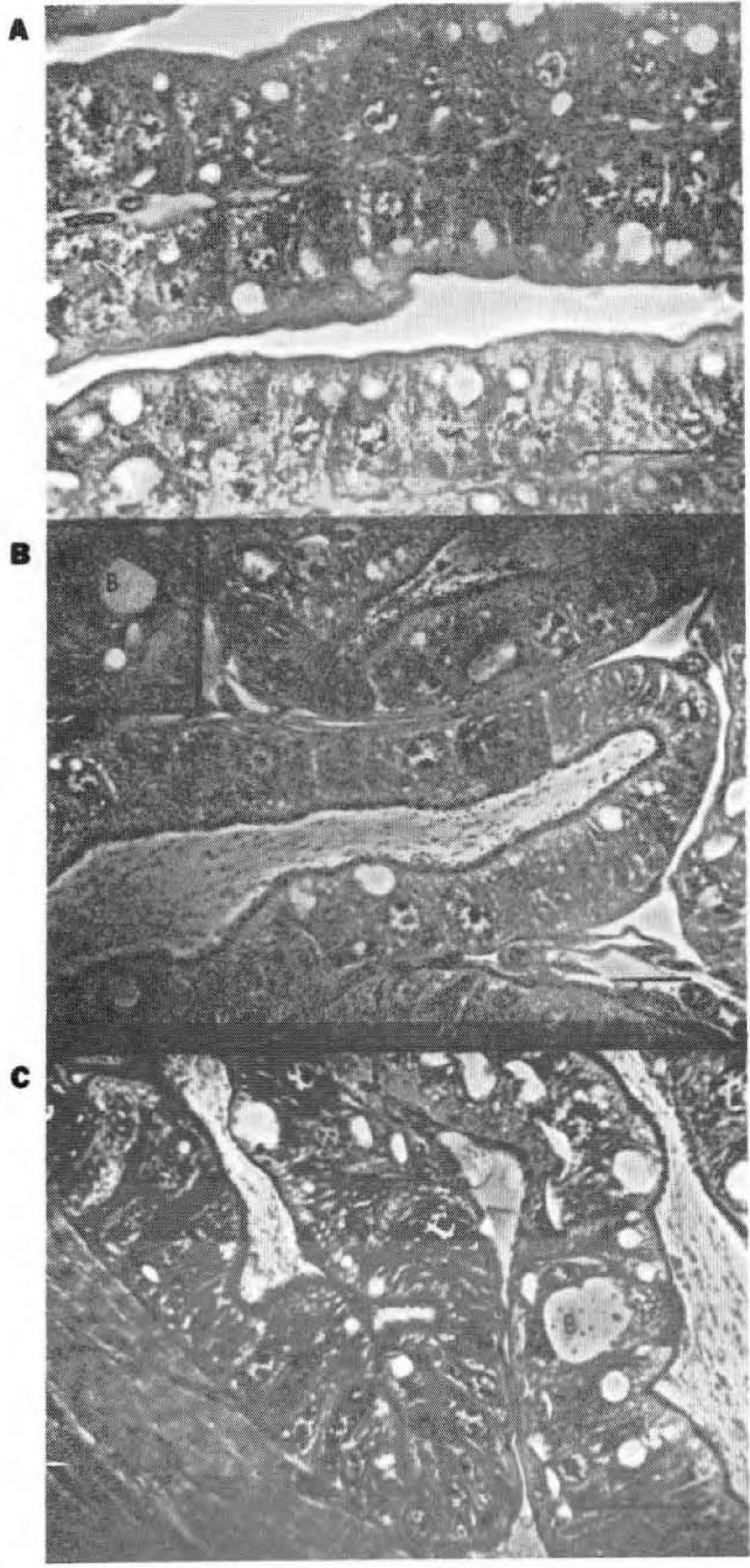
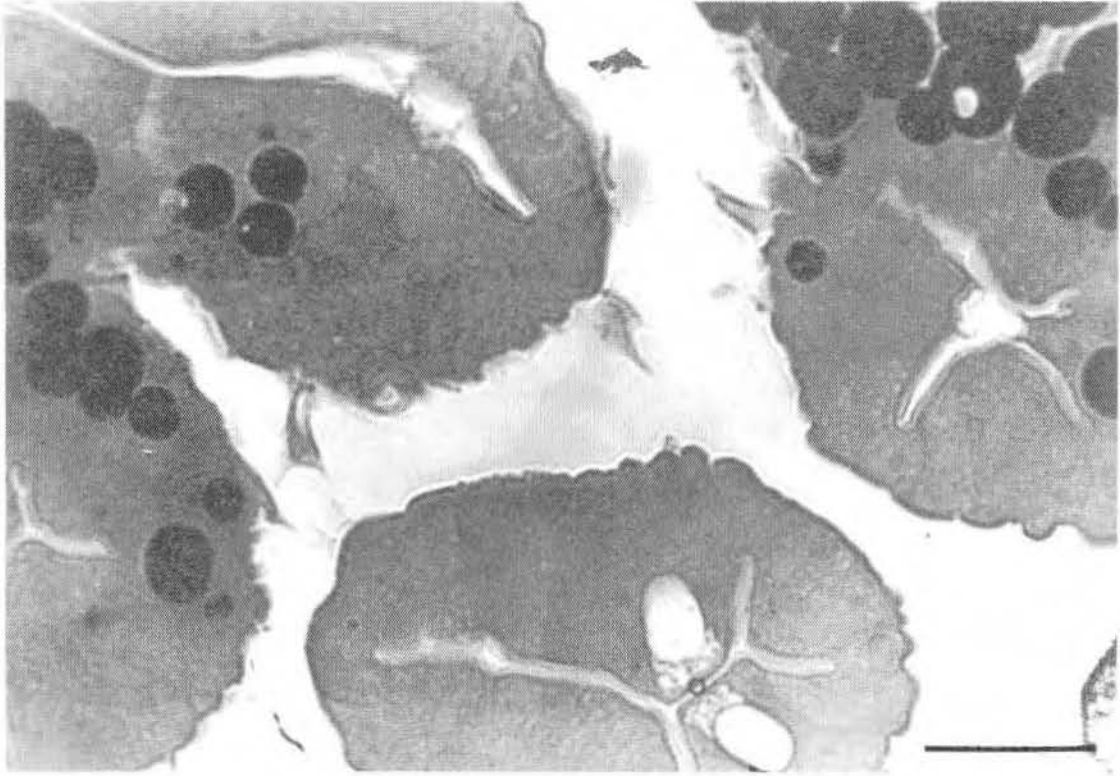

A
B
C

Figure. 3.4. Lipid vacuoles in R-cells of unfed intermolt stage I lobster larva post-fixed with osmium tetroxide. R, R-cell; F, F-cell; B, B-cell; L, lipid droplets; a, apical complex of B-cell. Scale bar = 5 um.



in and around the midgut gland were much more extensive than in the stage I larva. Osmium complexes with lipid, turning it hard and black. A number of black vacuoles can been seen in the R-cells. F-cells are also faintly discernible after this treatment. The cells containing unstained vacuoles can be classified as B-cells by the presence of the apical complex. Since this histological section was obtained from an unfed stage I lobster larva, it is presumed that the lipid present in the R-cells of the very early stages must be obtained by resorption of yolk material.

No obvious changes occur in the morphology of F- or B-cells in lobster larvae or postlarvae (Figs. 3.5 and 3.6, respectively). The number of B-cells in the stage I larva, however, is greater compared to those occurring in the prelarva. This increase occurs regardless of whether or not the larva has fed.

Morphology of the R-cells changes over the course of lobster development. R-cells of the stage I larvae are similar to those of the well-advanced gold embryo in that only a few large lipid vacuoles occur (Fig. 3.5.A). There are a number of very small lipid vacuoles in the R-cells of the stage II larva (Fig. 3.5.B). But there are very few, if any, lipid vacuoles in the midgut gland of the stage III larva (Fig. 3.5.C). Most of the R-cells of the second and third larval stages completely lack lipid vacuoles or have very small ones. By stage IV, however, the lobster apparently begins to store excess lipid obtained from the diet; a few lipid vacuoles can be seen in the R-cells (Fig. 3.5.D). The lipid reserves stored in R-cells increased during stage V (Fig. 3.6.A.) but it was not until stage VI (Fig.

Figure 3.5. Cell types comprising the midgut gland of intermolt larval stages of the American lobster H. americanus. R, R-cell; F, F-cell; B, B-cell. Scale bar = 5 um.
A. Stage I. B. Stage II. C. Stage III. D. Stage IV.

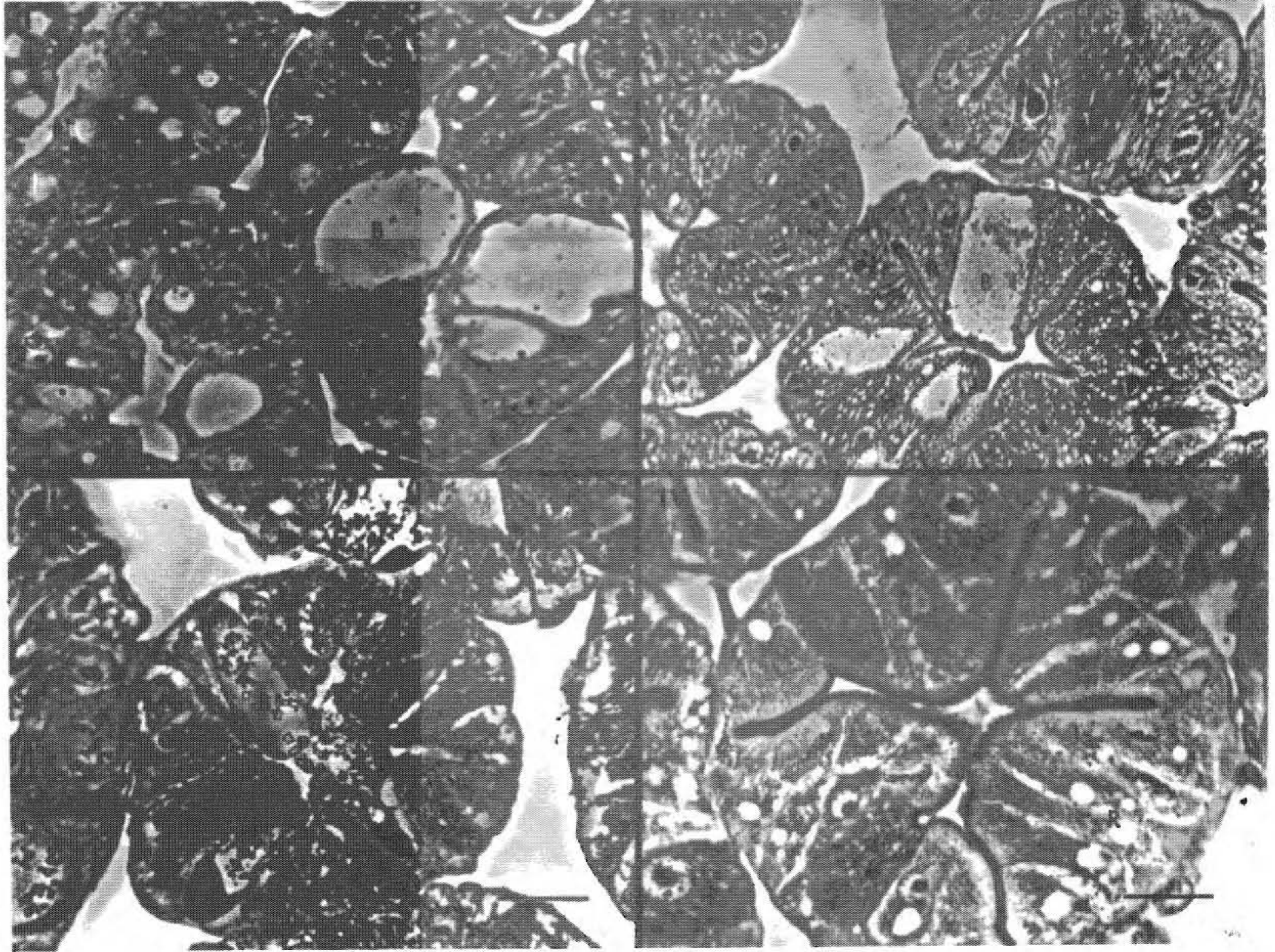
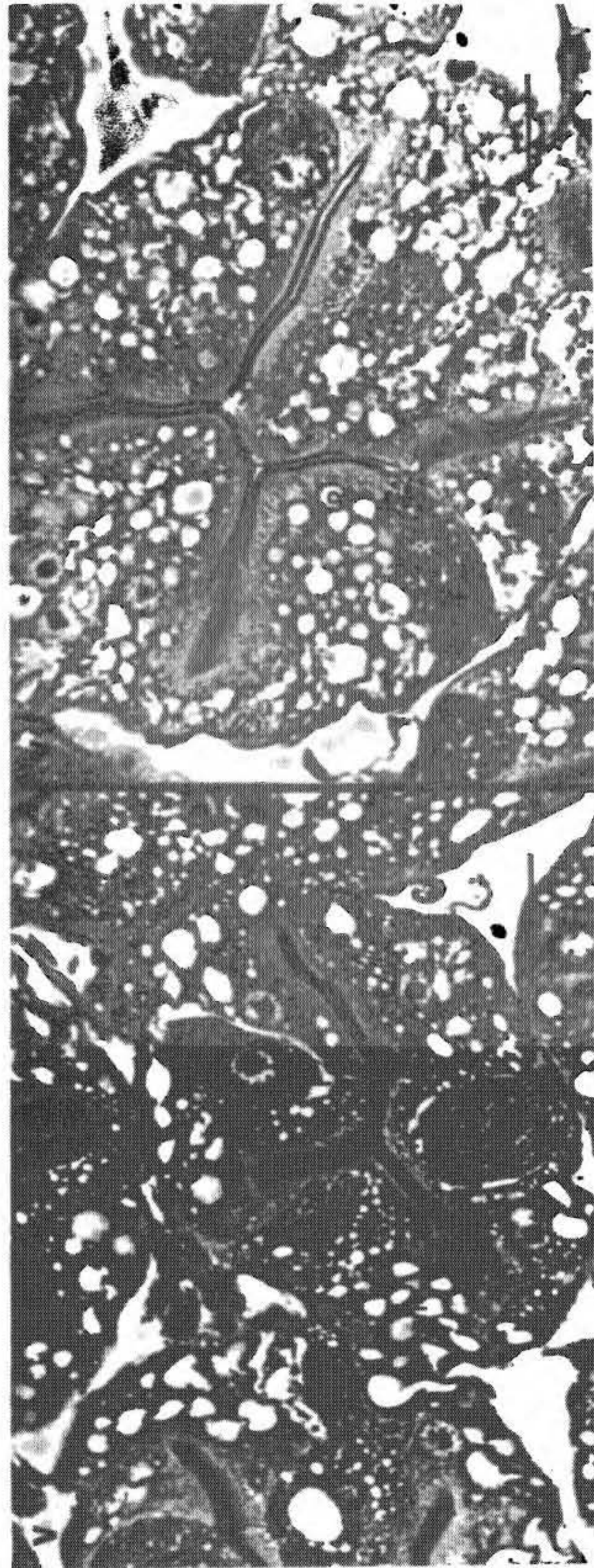

A
B
C
D

Figure 3.6. Cell types comprising the midgut gland of intermolt postlarval stages of the American lobster H. americanus. R, R-cell; F, F-cell; B, B-cell. Scale bar = 5 um.
A. Stage V. B. Stage VI.


B
A

3.6.B.) that the R-cells truly resembled those of the adult in that numerous vacuoles were present.

A series of sections through a discharging B-cell is presented in Fig. 3.7. The apical complex at the distal end of the cell, visible in Fig. 3.7.A, has been torn aside and the entire vacuole is entering the lumen of the tubule (Fig. 3.7.B and C). Thus, B-cell secretion is merocrine in larval lobsters.

DISCUSSION

The presence of muscle fibers in the myoepithelial layer (or myoendothelial layer, <u>sensu</u> Leavitt and Bayer, 1982) surrounding the tubules of the midgut gland of crustaceans was noted long ago. Pump (1914) summarized the previous research on this muscle network and concluded that two types of fibers were present. The first type is characterized by thick striated mononucleate circular fibers evenly spaced along the tubule length except at the distal tip where they were more closely arranged. The second type is characterized by slender, usually non-striated, longitudinal fibers which appear to connect two adjacent circular muscles and which appear at times to divide into two or three branches. The circular fibers probably contract to move material in or out of the tubules whereas the longitudinal connectives maintain the integrity of the tubule during contraction and relaxation of the circular fibers. They may also serve to conduct the contraction impulses through consecutive circular bands and to establish a contractile peristaltic wave (Leavitt and Bayer, 1982).

Figure 3.7. Sequential series demonstrating merocrine secretion of B-cell contents into lumen of midgut gland tubule. Intermolt stage II lobster larva. R, R-cell; F, F-cell; B, B-cell; a, apical complex of B-cell. Scale bar = 5 um.

A

B

C

The muscle network observed in the stage IV larva is similar in many respects to that of the adult. The circular muscle fibers in the tubules of the lobster larvae are approximately 17 um apart but are more closely spaced at the tips. In adult Orconectes virilis and Procambarus clarkii the circular fibers were arranged at 10 um intervals (Loizzi, 1971) whereas in adult H. americanus they were arranged at 15 um intervals (Leavitt and Bayer, 1982). Even though the tubules of larval lobsters are short relative to those of adults, the spacing of the circular muscle fibers is almost the same as is the width of the bands, 5 um in both stage IV larvae and adults. The width and spacing of the longitudinal fibers are also the same in the larvae and adults.

Also retained by the adult lobster is the method of secretion of the B-cell contents. Barker and Gibson (1977) noted that enzyme secretion in the adult European lobster was merocrine. In merocrine secretion, portions of the secretory cell are lost but the cell is not destroyed and can regenerate its contents. Merocrine secretion was observed in the stage IV larvae in the present study.

The sequence of development reported herein for the R-cells of embryonic through postlarval lobsters has not been previously noted. The lipid-storing function of R-cells is one which is present to a small degree among the embryos but which increases during ontogeny. In embryonic stages of the lobster, the lipid reserves are in the form of lipoprotein yolk which eventually becomes contained within the developing midgut. The primary role of the yolk lipid is to provide energy for development. Near the end of embryonic development, there

is yolk material both inside and outside the midgut gland. It is not illogical to hypothesize that some of the degraded yolk lipid can be reabsorbed and stored in the R-cells of the embryonic midgut gland before it is metabolized. In this manner, the R-cells of the embryo perform a function directly analogous to the lipid resorption and storage function of older stages except that the lipid is derived from the yolk rather than from exogenous dietary sources. The amount of yolk lipid available for this type of storage is limited because of intense metabolic demands for energy particularly during the latter part of embryogenesis. Thus, the presence of a small number of lipid storage vacuoles in the R-cells of the embryos is explained. This hypothesis also explains the presence of lipid vacuoles in the R-cells of stage I larvae which had not yet fed. Since the presence of lipid vacuoles in R-cells is an indication of lipid storage, the histological evidence presented here suggests that the first three lobster larval stages do not store dietary lipid but instead metabolize it immediately. Increased storage of lipid derived from the diet began among the post-metamorphic stage IV larva and increased further in successive postlarval stages.

The increased amount of lipid storage in R-cells of the stage IV larvae and older postlarval stages compared to earlier stages is reflected in the changing biochemical composition and metabolic patterns exhibited by these stages. Sasaki (1984) measured the biochemical changes which occurred during embryonic and larval development of the American lobster. The embryo contained mostly protein; total lipid was about one-third the total protein while total

carbohydrate was about one-tenth the total protein. The author showed that lipid, primarily triacyglycerol and to a lesser extent phosphatidyl choline, supplied most of the energy for embryogenesis. Among the larval stages, protein was also the principal biochemical component. It tended to increase steadily through the first two larval stages with an even greater rate of increase starting in stage III and continuing through stage IV. There was a lag in the increase of carbohydrate among the stage I and II larvae followed by an exponential increase in stage III through the middle of stage IV when it leveled off. Total lipid in larval American lobsters did not begin to rise above levels observed immediately after hatching until the middle of stage II. There was a slight increase among stage III larvae followed by a dramatic three-fold increase in total lipid by the middle of the fourth stage. This was due almost entirely to an increase in triacyglycerol which is the primary storage form for lipid.

Capuzzo and Lancaster (1979a,b) have shown that lipid was important in energy production during development of lobster larvae but that it tended to be replaced in importance by protein just prior to metamorphosis. The weight-specific respiration rates of larval American lobsters increased with each larval stage, I through IV, but decreased slightly just before the molt to the first postlarval stage, V. Ammonia excretion rates followed a similar pattern. The authors used the O:N ratio as an index of substrate utilization for energy production in H. americanus stages I through V. This ratio reflects the catabolic balance of carbohydrates, lipids, and protein in an organism. The theoretical minimum of 8.0 indicates total dependence

on protein catabolism; higher ratios indicate greater dependence on carbohydrates and/or lipids up to infinity if lipids and/or carbohydrates are used exclusively (Conover and Corner, 1968). (The relative contribution from each source--lipid or carbohydrate--cannot be determined by this method.) Capuzzo and Lancaster (1979b) determined that there were no significant differences in the O:N ratios of the first three larval stages (O:N ≃ 26.5) but there was a significant reduction ($p<0.01$) in stages IV (O:N = 22.1) and V (O:N = 23.6). Thus, whereas protein was the principal source of energy there was some energy yielded from lipids and/or carbohydrates. Protein utilization increased at metamorphosis to the benthic stage. Pandian (1970) suggested that lipid oxidation was the principal source of energy during embryonic development of the lobster, a situation which was confirmed by Sasaki (1984). Capuzzo and Lancaster (1979a) speculated that the slightly greater utilization of lipid during early larval development was a carry-over from embryonic development when lipid utilization was most important.

Sasaki et al. (in press) have suggested that accumulation of energy reserves may be related to the mode of existence whereby small, rapidly growing organisms with fast molt recoveries store less reserves than larger, slower growing animals which have prolonged molt recovery periods. Thus, the changes in energy reserves observed during the transition from the early larval stages to the postmetamorphic stage may reflect different energetic strategies for the planktonic and benthic habitats. Premetamorphic larvae, stages I through III, showed little evidence of lipid storage and appeared to

catabolize lipid very rapidly for metabolic needs. Postmetamorphic lobsters accumulated significantly greater proportions of lipid than the earlier larval stages, particularly during stages C to D_1 of the molt cycle. The reason for this is unknown but may be related to preparation for a new habitat with its attendant new food supply. The stage IV larva makes the transition from the plankton to the benthos and therefore must search for suitable substrate and construct a burrow. The authors suggested that accumulation of lipids in the stage IV larva was advantageous since it conferred a degree of starvation resistence which may be important in adjusting to the new habitat.

Among pelagic marine invertebrate larvae, planktotrophy and lecithotrophy represent two different nutritional strategies. Planktonic larvae obtain energy by feeding in the plankton during their development while lecithotrophic larvae metabolize the stored energy reserves contained in the yolk (Thorson, 1950). Crisp (1976) preferred to use the terms feeding and non-feeding larvae, respectively. Planktotrophic larvae generally have a small amount of stored yolk reserves at hatching which they can metabolize in addition to the dietary sources but in general they are obligate feeders totally dependent upon finding food in the plankton or starving to death. In contrast, lecithotrophic larvae are unable to feed. Often the mouth and anus of non-feeding larvae are non-functional; material seen in the intestine of these species is yolk. These larvae are generally present in the plankton for short periods of time, from hours up to about three days. They are not normally subject to

starvation per se while in the plankton but must find suitable substrate in order to metamorphose and start feeding.

Based on body size alone, the two larval types represent different energetic strategies. Under conditions of low but steady food supply, small body size may be advantageous in reducing the total energy requirements of the individual. Conversely, large body size reduces the weight specific rate of energy expenditure per unit time as well as permitting larger amounts of energy products to be stored (Taniguchi, 1973). The basic differences between planktotrophic and lecithotrophic larvae lie in the immediate source of their energy reserves (exogenous dietary source as opposed to maternally derived stored yolk reserves), in the length of larval life, and in the relative body size. There are, however, similarities both in the storage and in the utilizaton of the energy reserves of marine larvae, regardless of its source. Lipid tends to be the primary energy source, at least in the earlier larval stages, followed by protein (Holland, 1978). As development proceeds, protein can become as important as lipid or more so.

Larvae of the American lobster hatch at an advanced stage, having undergone naupliar development in the egg. Previous morphological studies (Williams, 1907; Hinton and Corey, 1979; Factor, 1981) have demonstrated that the digestive tract of the stage I lobster larva is well-developed. The results of the present study on changes occuring in the midgut gland indicate that the digestive tract is probably functional when the lobster larva hatches. The presence of F-cells in the well-advanced gold egg stage was indicative of the potential for

digestive enzyme synthesis. That mature B-cells were observed in the hatching stages is evidence that in fact such enzyme synthesis may have occurred. Presumably active digestive enzymes were produced during the hatching process in the absence of an exogenous nutrient supply and as a consequence of genetic programming. Since lobster larvae are planktotropic, the possession of functional digestive capability immediately upon hatching, but before feeding, is an advantage. However, the activities of digestive enzymes must be measured among these early developmental stages before it can be concluded that digestive capabilities are fully functional.

CHAPTER 4: OPTIMUM ASSAY CONDITIONS FOR DETERMINATION OF THE DIGESTIVE ENZYME ACTIVITIES OF EARLY DEVELOPMENTAL STAGES OF THE AMERICAN LOBSTER HOMARUS AMERICANUS

INTRODUCTION

Over the years a succession of literature reviews has appeared in which studies dealing with the identification and properties of various crustacean digestive enzymes have been summarized (Vonk, 1960; van Weel, 1970; Pandian, 1975; Dall and Moriarty, 1983). Very few of these digestive enzymes have been characterized in any detail, a situation far different from the mammalian system in which the digestive enzymes have been well-characterized. Levels of activity, pH range and optima, and kinetics are all important to enzyme function.

There have been a few studies dealing with properties of the digestive enzymes of the adult American lobster H. americanus. Brockerhoff et al. (1970) surveyed the digestive enzymes present in lyophilized gastric fluid and determined the molecular weights and pH optima for a number of these enzymes. Hoyle (1973) also determined the pH optima for some of the same digestive enzymes using freshly obtained gastric fluid. Wojtowicz and Brockerhoff (1972) isolated amylase from lobster gastric juice and determined its specific activity, molecular weight, pH optimum, and the effect of some activators. Aside from these studies there is meager information about the properties of adult lobster digestive enzymes and nothing about the enzymes of earlier developmental stages.

The activities of lobster protease, lipase, and amylase reported in this study are chemical estimations of the amount of product formed

during a specific period of time. The reaction conditions for such assays which measure the rate of enzymatic reaction "should, where practicable, be optimal" (Report of the Commission on Enzymes of the International Union of Biochemistry, 1961). With this caveat in mind, standard methods to measure digestive enzyme activities were selected and a series of control experiments performed in order to determine the optimum reaction conditions for each hydrolase. Although it was beyond the scope of the present study to perform detailed characterizations of lobster digestive enzymes, the influence of a number of chemical and physical factors on the reaction rate was investigated. Factors considered during the course of these tests included the concentrations of both enzyme and substrate, linearity of reaction rate over time, and the influence of pH, temperature, and ionic strength on the rate of reaction. The effect of some activators was also determined. The final protocols used to assay protease, lipase, and amylase activities (as described in Chapter 5) are based on the findings of these control tests.

MATERIALS AND METHODS

Experimental animals

Winter-hatch lobster larvae were reared communally in kreisels. Only well-fed intermolt stage I larvae were used in these tests. Depending upon the availability, either live larvae or those previously frozen in liquid nitrogen and stored at -85°C were sampled. Details for rearing larvae, for molt-staging, and for storing samples are given in Chapter 2.

The digestive tract tissues of the larvae were trimmed as described in Chapter 2 and a homogenate made in ice-cold 0.1 M citrate phosphate buffer, pH 5.5. Generally, either three or six larvae, as described below, were pooled per ml of homogenate. The crude homogenate was centrifuged for 2 min and the supernate decanted off. The supernates were kept on ice until the analyses were performed.

Protein measurement

Protein was determined using the Hartree (1972) modification of the Lowry et al. (1951) assay. For this analysis, 100 ul of lobster digestive tract supernate were diluted with 0.9 ml of distilled water. Bovine serum albumin (BSA) was used as the standard. All samples were run in duplicate and the mean value used for calculations.

Protease activity: method of analysis

Protease activity was assayed using Azocoll (Calbiochem-Behring Doc. No. 3805-483) as a substrate. Azocoll is insoluble, powdered cowhide to which a red azo dye is bound. When a protease cleaves any of the peptide linkages in the collagen, the dye is released into the medium. The rate at which dye is released can be used to measure the relative activity of the enzyme by measuring the increase in absorbance at 520 nm.

The standard protocol for measuring the digestive action of a protease on Azocoll suggests suspending 50 mg of Azocoll in 5 ml of 0.1 M phosphate buffer, pH 7.0, containing proteolytic enzyme. The reaction is allowed to proceed for 15 min at 37°C and is stopped by filtering off the substrate. Absorbance of the soluble reaction products is read at 520 nm.

Chavira et al. (1984) have recommended prewashing the Azocoll in buffer and vigorously agitating the reaction tubes to obtain reproducible, linear assays as a function of time. Accordingly, the Azocoll was washed for 2 h in buffer at a concentration of 10 mg/ml, filtered through Whatman No. 1 filter paper, and resuspended in fresh buffer to the final desired substrate concentration just before use.

In practice, it proved difficult to quickly filter off the insoluble substrate to stop the reaction, especially when many samples were being run. Instead, the capped reaction tubes were plunged into an icewater bath for 10 to 20 min. Then the cold tubes were centrifuged for 2 min to compact the substrate into a pellet and the supernate pipetted off into separate test tubes. There was no increase in absorbance even if the reaction tubes were left on ice for over an hour before removing the substrate.

Lobster protease activity is herein reported as increase in absorbance units (A_{520}) per mg protein per h. Since it is not possible to directly determine the micro-equivalents of Azocoll peptide bonds cleaved, the protease activity must be reported in the above manner. The digestive enzyme responsible for the activity reported here has not been identified. Thus, rather than arbitrarily choosing a protease as a reference standard to which the absorbance values are converted, the absorbance values themselves are used.

Protease activity: control experiments

In order to show that the protease activity detected by this method is due to digestive enzymes rather than to intracellular enzymes, control tests were done using homogenates of (1) whole

larvae; (2) larvae dissected into two components--the foregut plus midgut gland and the remaining carcass; and (3) larvae trimmed into two components--the thorax and the residual carcass. Three larvae (or the parts of three larvae) were homogenized in 1.0 ml of cold 0.1 M phosphate buffer, pH 7.5. The homogenates were centrifuged and 100 ul of the supernates were used as the enzyme source. The reaction was carried out at 37°C for 60 min at an Azocoll concentration of 10 mg/ml.

For all of the remaining control tests, homogenates of trimmed lobster digestive tracts (comprising the foregut and midgut gland) were made as described in Chapter 2. The homogenates were centrifuged and the resulting supernates kept on ice until they were used in the assays.

The effect of different concentrations of enzyme solution was tested. Digestive tract tissues from six larvae were homogenized in 1.0 ml of 0.1 M phosphate buffer, pH 4.0. Both 100 ul and 200 ul of the supernate were assayed separately at 38°C at a substrate concentration of 5 mg/ml. Replicate time course samples (n=2) were taken at 15, 30, and 60 min.

In order to determine whether the substrate concentration recommended in the standard protocol (10 mg/ml) was saturating, assays were run at substrate concentrations from 2.5 to 30.0 mg/ml in 0.1 M phosphate buffer, pH 7.5. Digestive tract tissues from six larvae were homogenized in 1.0 ml of buffer; 200 ul of the supernate were used as the enzyme source. Temperature of the reaction was 38°C; duration was 60 min.

To determine the optimum pH, time course assays were carried out from pH 4 through pH 8 at approximately 0.5 pH unit intervals. Citrate phosphate buffer, 0.1 M was used in the range of pH 4.2 to 6.3 whereas 0.1 M phosphate buffer was used for the range pH 5.9 to pH 7.9. Digestive tract tissues from six larvae per ml were homogenized in the respective buffers; 200 ul of the supernates were used as the source of protease. Temperature was 38°C and the substrate concentration 9 mg/ml. Replicate time course samples (n=4) were taken after 15, 30, 60, and 90 min at each pH.

The effect of temperature on protease activity was tested by conducting assays at temperatures ranging from 25° to 50°C at 5°C intervals. The assays were run for 60 min at pH 5.5 with 0.1 M citrate phosphate buffer at 10 mg/ml Azocoll. Digestive tract tissues from three larvae per ml of homogenate were pooled for the tests; 200 ul of supernate were used for the assays. Replicate samples (n=3) were taken for each temperature point.

Assays were also conducted to determine the effect of ionic strength on protease activity. Digestive tract tissues from three larvae per ml of homogenate were pooled for each sample (n=3); 200 ul of supernate were used as the enzyme source. The reaction was run at 37°C for 60 min at a substrate concentration of 10 mg/ml and pH 5.5, 0.1 M citrate phosphate buffer. NaCl was added to increase ionic strength.

Protease activity is reported as absorbance units at 520 nm (A_{520}) per mg protein per h. For comparative purposes, however, the assay was run using purified bovine pancreatic trypsin (Sigma) as a

reference standard. Reaction conditions were 0.1 M phosphate buffer, pH 7.5, 37°C, and an Azocoll concentration of 10 mg/ml. BSA was added at 0.35 mg/ml.

Lipase activity: method of analysis

Lipase activity was determined by a novel method which was developed for these studies. This method is based on the review papers by Wills (1965), Desnuelle (1972), Brockerhoff and Jensen (1974), and Jensen (1983) and included precautions suggested by these authors. A one-paragraph summary of the protocol will be presented first, followed by a detailed explanation of the various steps.

Free oleic acid, 2-monolein, and 1,2-diolein are the products of pancreatic lipase action on triolein. Brockerhoff et al. (1967) showed that the action of lobster lipase on triolein was similar to that of pancreatic lipase. The present method for determination of lipase activity entailed incubation of lobster digestive tract supernate in a stabilized emulsion of purified triolein, the universal lipase substrate. Both H^+ and Na^+ ions and a free fatty acid (FFA) acceptor were added to the reaction mixture. BSA was added to stabilize the lipase during the reaction. The reaction was stopped by the addition of organic solvent. The lipids were extracted and the lipid classes separated by thin layer chromatography (TLC) on SII chromarods (hereafter referred to as rods) and quantified by a flame ionization detector (FID) using an Iatroscan TH-10 Mark 3. Lipase activity is reported as umoles free oleic acid (OA) liberated from the triolein per mg protein per h. All materials used in this assay were

made of glass or Teflon and were solvent-rinsed, first in methanol and then in hexane.

Gum arabic (acacia) was used to prepare the stabilized emulsion but it was difficult to dissolve completely. Best results were obtained when the acacia was well macerated with a stirring rod in a few drops of buffer. More buffer was added dropwise at first and then a few ml at a time, stirring continuously. The solution was gently warmed and stirred for 3 h with a stir bar. The mixture was allowed to settle overnight and the translucent solution transferred to another container and stored in the refrigerator at 7°C. The rate of lipolysis was slightly better when 1% acacia was used than with 2, 3, or 4% acacia.

One of the first control tests to be done was selection of a suitable free fatty acid acceptor. A FFA acceptor is needed since accumulation of FFA usually inhibits lipases. Brockerhoff and Jensen (1974) and Jensen (1983) indicated that Ca^{++} or Mg^{++} are suitable for lipase assays at concentrations from 0.02 to 0.10 M. The effect of Ca^{++} concentration on lipase activity was studied by adding $CaCl_2$ at concentrations of 0.0 to 0.10 M at 0.02 M intervals with NaCl at 0.0, 0.14, or 0.28 M. The lipase reaction mixture contained 1 umole of triolein emulsified in pH 7.5 phosphate buffer (0.02 M final concentration) with 0.35 mg/ml BSA. Gastric fluid from juvenile lobsters was used as the enzyme source. The reaction was carried out at 37°C for 30 min. The reaction rate was fastest with 0.02 M $CaCl_2$ and 0.28 M NaCl.

Since $CaCl_2$ produced a precipitate in the reaction mixture, $MgCl_2$ and $MgSO_4$ were also tried as FFA acceptors under the conditions described above. There was no difference in reaction rate when $CaCl_2$ or $MgCl_2$ was used but the rate was decreased by a factor of 2 with $MgSO_4$. Therefore, $MgCl_2$ was used routinely as the FFA acceptor.

At low concentrations, BSA is a FFA acceptor and also serves to protect lipase from denaturation, yet at higher concentrations it can be inhibitory (Brockerhoff, 1971). Using the reaction mixture developed for this study, BSA completely inhibited lipid hydrolysis by lobster lipase at 3.5 mg/ml, was slightly inhibitory at 1.0 mg/ml, but salutary at 0.35 mg/ml; BSA concentrations of 0.01 to 0.20 mg/ml had no effect on rate of lipolysis.

The lipid extraction procedure followed the Sasaki and Capuzzo (1984) adaptation of the combined methods of Folch et al. (1957) and Bligh and Dyer (1959). The first extraction, which also served to stop lipolysis, was done by adding 3.75 ml chloroform:methanol (C:M) at the ratio 1:2 to the reaction mixture. About 40 ug of oleic acid methyl ester (OAME) was also added at this time as an internal standard. The tube was flushed with nitrogen, capped, and inverted occasionally during the next 15 to 30 min to mix the contents.

The tube was centrifuged for 2 min and the supernate pipetted into a second 10 ml graduated screw-top centrifuge tube. The pellet remaining in the first tube was extracted a second time for 15 to 30 min with 3.75 ml of C:M 2:1. This tube was centrifuged for 2 min and the supernate added to that already present in the second tube. Then

2.375 ml of 0.7% NaCl were added to the combined extracts in the second tube. The contents of the second tube were mixed on a vortex mixer and then put on ice for 30-60 min to improve separation of the solvent layers. The cold tube was centrifuged for 2 min and the volume of the bottom, chloroform layer (which contained the lipids) was recorded.

The upper, methanol layer was removed by aspiration as was any interfacial material. A measured aliquot (normally about 3.7 ml) of each chloroform extract was removed to a separate 4.0 ml vial. Each vial was flushed with nitrogen and stored in the freezer at -20°C until analysis. On the day of Iatroscan TLC analysis the aliquot was evaporated to dryness under nitrogen at 40°C and resuspended in 50 ul of C:M 1:1. This material is hereafter referred to as concentrated extract.

A set of ten SII chromarods was matched for solvent flow characteristics and for similar lipid peak area determination following the guidelines of Sasaki (1984). The matched rods were kept in a metal rack and stored in a humidity chamber when not in use. This humidity chamber contained distilled water to a depth of about 1 cm; the fritted portion of the rods was in contact with the water. To activate the rods, they were dried at 110°C for 5 min and then run through the Iatroscan twice.

Eight ul of the concentrated extract were spotted onto each of duplicate rods. Four samples plus a standard can be run in duplicate at one time, as the rack holds ten chromarods. The rack of rods was suspended above a saturated NaCl solution in a humidity chamber for 10

min. The rods were then transferred to a developing tank where they were suspended above the developing solvents for 10 min. The developing system was hexane:diethyl ether:formic acid (42:8:0.06), one of the solvent systems tested by E. M. Becker Co. (the distributor of Iatroscan in the United States) for separation of triacylglycerol and its degradation products on the Iatroscan. A single development in this solvent system gave complete separation of triacylglycerol (TAG), free fatty acid (FFA), 1,3-diacyglycerol (1,3-DAG), 1,2-diacylglycerol (1,2-DAG), and monoaclyglycerol (MAG). Oleic acid methyl ester (OAME) also produced a separate peak and was used as an internal standard.

The rods were lowered into the developing solvent system and developed until the solvent front had progressed to the 10 cm mark. The rods were removed from the developing tank and dried at 110°C for 4 min. The rods were cooled for 2 min at room temperature and put into the Iatroscan. Operating parameters were the following: scan speed 4 (0.42 cm/sec), hydrogen pressure 8 kg/cm^3, and airflow 2000 ml/min.

After the first scan, during which peak areas were determined, the rods were scanned a second time to remove the final traces of triolein. The other lipid classes were completely burned off during the first scan. After the rods had been used for four or five separations, they were put into a glass rod holder and cleaned for 1 h in Chromerge, rinsed ten times with tap water, and soaked for 30 min (or overnight) in distilled water. The rods were put back into the

metal rack and then dried and scanned as above to activate them for the next series of samples.

The peak area for each lipid class was integrated on a Hewlett Packard 3390A integrator and converted to ug lipid using standard curves. Separate curves were generated for each lipid class, using pure standards: triolein, 1,2-diolein, 1,3-diolein, 1-monolein, oleic acid, and oleic acid methyl ester. These standards were prepared according to Sasaki (1984). They were dissolved in C:M 1:1 and stored in the dark under nitrogen at -20°C.

2-monolein is not available commercially so 1-monolein was used as the standard. This was justified because the standard curve for 1,2-diolein is essentially the same as that for 1,3-diolein; by analogy, the curve for 1-monolein should be the same as for 2-monolein. Since 1- and 2-monolein are known to co-migrate in some solvent systems (Tatara et al., 1983), the choice of a standard is moot.

The triolein and OAME standard curves ranged from 0.0 to 10.0 ug while the free oleic acid, monolein, and diolein standard curves covered the range from 0.0 to 5.0 ug. The relationship between the amount of lipid spotted (x) and the peak area (y) was a power function ($y=ax^b$).

Using an internal standard, OAME in this case, improved the accuracy of the lipid class determinations. The actual amount of OAME added to the samples was determined gravimetrically and a correction factor, $OAME_{gravimetric}$: $OAME_{Iatroscan}$, was applied to the Iatroscan values obtained for the other lipid classes.

In order to obtain reproducible results on the Iatroscan, new standard curves were made every few weeks. The characteristics of the rods (reported peak areas) changed with use. Generally, standard curves made consecutively did not vary much from each other but over time the curves were different from the first ones generated.

Usually all the rods in a matched set changed at the same rate but sometimes one rod yielded anomalous values. Thus, it is essential that the rods be re-matched periodically (every few weeks) using standards. That is, the Iatroscan values should be compared to values obtained gravimetrically. Care must be taken to pair the rods so that the mean values obtained for each lipid class using the Iatroscan are within 10% or less of the gravimetric values.

Lipase activity: control experiments

Assays were conducted to determine the effect of substrate concentration on the lipase reaction rate. Triolein concentrations of 0.01 to 1.0 umoles/ml were tested at pH 5.5, 0.1 M citrate phosphate buffer; the reaction mixture contained 0.28 M NaCl, 0.35 mg/ml BSA, and 0.02 M $CaCl_2$ or $MgCl_2$. The reaction was run at 37°C for 30 min. Digestive tract tissues from twelve larvae were homogenized per ml of buffer and 100 ul of supernate used as the enzyme source.

The effect of pH on the rate of lipolysis was assayed from pH 4.0 to pH 10.0 at approximately 0.5 pH unit intervals. Citrate phosphate buffer was used for the range pH 4.0 to pH 5.5, phosphate buffer for the range pH 5.5 to pH 8.0, and carbonate bicarbonate buffer for the range pH 9.2 to pH 10.0. The final concentration of all buffers was 0.04 M. The reaction mixture included 0.5 umoles/ml of emulsified

triolein combined with 0.28 M NaCl, 0.35 mg/ml BSA, and 0.02 M $MgCl_2$. Digestive tract tissues from twelve larvae were homogenized per ml of buffer and 100 ul of supernate used as the enzyme source. The reaction was run at 37°C for 30 min.

To determine the influence of temperature on the rate of reaction, assays were run from 25° to 50°C at 5°C intervals. The concentration of reactants was 0.5 umoles/ml triolein, 0.28 M NaCl, 0.35 mg/ml BSA, and 0.02 M $MgCl_2$ in 0.1 M citrate phosphate buffer, pH 5.5. Digestive tract tissues from twelve larvae were homogenized per ml of buffer and 100 ul of supernate used as the enzyme source. The reaction was stopped after 30 min.

The influence of ionic strength on the reaction rate was determined over the range 0.2 to 0.9. The assay was run at 37°C for 30 min. The concentration of reactants was 0.5 umoles/ml triolein, 0.35 mg/ml BSA, and 0.02 M $MgCl_2$ in 0.1 M citrate phosphate buffer, pH 5.5. Digestive tract tissues from twelve larvae were homogenized per ml of buffer and 100 ul of supernate used as the enzyme source. NaCl was added to increase ionic strength.

As a final check, a time course study was conducted at 37°C to demonstrate whether the reaction was linear. Twelve larvae were homogenized per ml of buffer and 100 ul used as the enzyme source. The reaction mixture contained 0.5 umole/ml triolein emulsified with 1% acacia, 0.28 M NaCl, 0.35 mg/ml BSA, and 0.02 M $MgCl_2$ in 0.1 M citrate phosphate buffer, pH 5.5. Samples were taken after 15, 30, 45, and 60 min.

Amylase activity: method of analysis

Amylase activity was assayed according to a modification of the method of Bernfeld (1955). In the original procedure, 1 ml of "properly diluted" enzyme was incubated for 3 min at 20°C with 1 ml of substrate solution (10 mg/ml soluble starch) mixed with 0.02 M phosphate buffer, pH 6.9, containing 0.0067 M NaCl. The reaction was stopped by adding 2 ml of dinitrosalicylic acid reagent (which is alkaline). The tubes containing the mixture were heated for 5 min in boiling water and then cooled in running tap water. After adding 20 ml of distilled water, absorbance was read "using a green filter". The blank was prepared the same way but without enzyme. A standard curve was made with maltose from 0.2 to 2.0 mg (which corresponds to 0.3 to 2.9 micro-equivalents of maltose) in 2 ml of distilled water and used to convert the absorbance values to mg maltose. The rate of liberation of reducing sugars was reported as maltose units, mg maltose liberated per mg protein per 3 min.

The following changes were made in the standard method. The reaction tubes were heated in boiling water for 10 min and cooled in an icewater bath. The reaction mixture was not diluted before the absorbance values were determined. Absorbance was read at 540 nm. The standard maltose curve was prepared with 1 ml of starch substrate and 1 ml of maltose solution ranging from 0.2 to 1.2 mg. Finally, amylase activity was reported as micro-equivalents of maltose liberated per mg protein per h.

Amylase activity: control experiments

Preliminary tests determined that the 3 min assay time was too short to produce a measurable signal under the conditions used. Therefore, a time course experiment was conducted to ascertain whether the reaction was linear for longer incubation times. Assays were conducted at 37°C with 0.1 M citrate phosphate buffer, pH 5.5 with 0.05 M NaCl at a substrate concentration of 5 mg/ml starch. Digestive tract tissues from three lobster larvae were homogenized in 1.0 ml of buffer; 200 ul of the supernate were used as the enzyme source. Replicate samples (n=3) were taken at 6 min intervals from 0 to 36 min.

To determine the relation between amylase activity and substrate concentration, assays were performed at starch concentrations of 2.5, 5.0, 10.0, and 20.0 mg/ml. Starch was mixed with buffer at a concentration of 20 mg/ml with 0.2 M citrate phosphate buffer, pH 5.5. The lower substrate concentrations were made by diluting the stock substrate solution with buffer. NaCl was added to produce a final concentration of 0.05 M. Digestive tract tissues from three larvae were homogenized per ml of buffer and 200 ul of supernate used as the enzyme source. The assays were conducted for 30 min at 37°C.

To determine the effect of pH on amylase activity, assays were done at approximately 0.5 pH unit intervals from pH 4.5 to pH 8.0. Citrate phosphate buffer was used for pH 4.4 through pH 6.8 and phosphate buffer used for pH 5.9 through pH 8.0. Digestive tract tissues from three larvae were homogenized per ml of distilled water; 200 ul of the supernate were used for the reaction. Starch was mixed at 30 mg/ml with distilled water and diluted with 0.2 M buffer to

produce a substrate concentration of 15 mg/ml and a buffer concentration of 0.1 M for each buffer tested. NaCl was added to give a final concentration of 0.05 M. The reaction was run for 30 min at 37°C.

Amylase assays were performed at temperatures from 25° to 50°C at 5°C intervals. Digestive tract tissues from six larvae were homogenized in distilled water and 100 ul of the supernate used in the reaction. The reaction was conducted for 30 min at a starch concentration of 15 mg/ml; 0.1 M phosphate buffer, pH 6.5, with 0.05 M NaCl was used.

The effect of ionic strength on the reaction rate was tested over the range of ionic strength from 0.0 to 0.7. Digestive tract tissues from six larvae were homogenized in distilled water and 100 ul of the supernate used in the reaction. The reaction was conducted for 30 min at 37°C at a starch concentration of 15 mg/ml; 0.1 M phosphate buffer, pH 6.5 was used. NaCl was added to increase ionic strength.

RESULTS AND DISCUSSION

Protease activity

The data presented in Table 4.1 demonstrate that the protease activity measured by the present method was due to digestive enzymes contained in the stomach and/or in the midgut gland. This included both secreted enzymes in the foregut and the tubules of the midgut gland as well as the unsecreted enzymes present in F- and B-cells. Only the digestive tract tissues of larval lobsters show any protease activity; there is none detectable in the carcass. The slight protease activity attributed to the carcass of the "dissected larvae"

Table 4.1.

Protease activity in stage I larval lobsters.

Treatment	Protein (mg)	Protease activity (A_{520}/mg protein/h)
Whole larvae	0.07±0.10[a]	0.70±0.09[b]
Dissected larvae		
Foregut & midgut	0.02±0.003	1.94±0.23[c]
Carcass	0.05±0.001	0.06±0.05
Total	0.07±0.004[a]	0.59±0.08[b]
Trimmed larvae		
Thorax	0.04±0.004	1.81±0.53[c]
Carcass	0.04±0.002	0.00
Total	0.08±0.006[a]	0.85±0.22[b]

[a]No significant difference in amount protein at α=0.05 (Kruskal-Wallis ANOVA).

[b]No significant difference in protease activity at α=0.05 (Kruskal-Wallis ANOVA).

[c]No significant difference in protease activity at α=0.05 (χ^2 median test).

Values expressed are mean±standard deviation (n=3).

was most likely due to contaminating gastric fluid from the stomachs which ruptured during the dissection.

Under the conditions of the present assay, activity of any intracellular enzymes was not detectable. Whole animal homogenates could thus have been used with impunity. The data, however, are reported here as specific enzyme activities (enzyme units per mg protein per h). The carcass contained 2.5 times more protein than did the "dissected" foregut plus midgut gland. The "trimmed" carcass contained as much protein as did the digestive tract tissues. Enzyme activities reported on the basis of whole animal protein rather than on the basis of the digestive tract protein will not reflect relatively small changes in digestive enzyme activity. Therefore, to obtain the most precise enzyme activity measurements, digestive tracts should be dissected and used. When this dissection was first attempted, however, it became obvious that the digestive tract tissues could not be removed intact, i.e. without rupturing the stomach and releasing gastric fluid, unless an extremely meticulous and time-consuming dissection was done. Thus, to maximize the amount of digestive enzyme containing tissue and yet retain economy of effort, the larvae were trimmed rather than dissected. The enzyme activity calculated on the basis of the amount of protein in the trimmed thorax was not significantly different from that determined on the basis of the dissected foregut and midgut gland (cf. footnote c, Table 4.1). For this reason, all the enzyme assays in this study were performed using the trimmed digestive tract tissues.

As shown in Fig. 4.1, the rate of proteolysis is linear from 0 to 60 min, regardless of whether 100 ul or 200 ul of enzyme solution is used. The reaction rate is also linear with respect to the concentration of enzyme. The dashed line in Fig. 4.1 represents the curve obtained when the activity measured for 200 ul of supernate is halved; this line is not different from that produced when 100 ul of supernate was assayed.

As can be seen in Fig. 4.2, activity of the lobster protease continued to increase linearly with an increase in substrate concentration well above the recommended substrate concentration of 10 mg/ml. Protease activity thus shows first-order kinetics with respect to substrate concentration, at least for Azocoll concentrations up to 30 mg/ml, rather than zero-order kinetics which is normally desired for enzyme assays. At 30 mg/ml Azocoll, the reaction mixture contained so much insoluble substrate that it was almost a slurry. It may be that all the peptide bonds of Azocoll are not available to the enzyme and thus the protease reaction was not saturated with substrate.

Since activity of the lobster protease continued to increase linearly with increase in substrate concentration well above the standard recommended amount, it was decided to conduct the assays in this study at the standard concentration of 10 mg/ml Azocoll. This was done primarily to facilitate comparison of the results with other studies in the literature, most of which have been done at this substrate concentration. Because of the cost of this substrate very few studies are conducted at higher concentrations. In fact, there

Figure 4.1. Activity of lobster protease ($\bar{X} \pm SD$, n=2) in relation to enzyme concentration. Reaction conditions are 38°C, 5 mg/ml Azocoll, and pH 4.0, 0.1 M phosphate buffer. The dashed line represents the curve obtained when the activity produced using 200 ul is divided by 2.

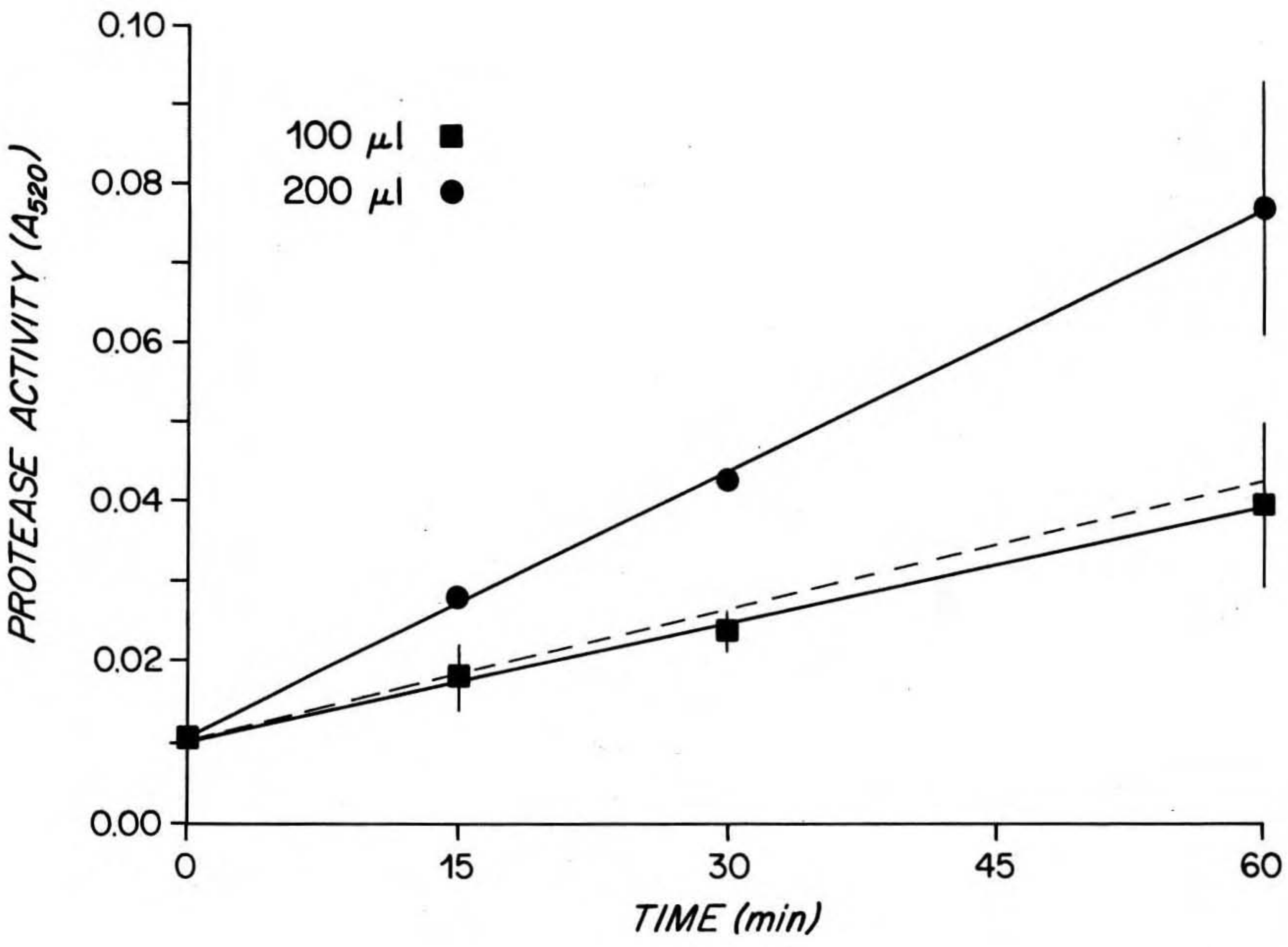
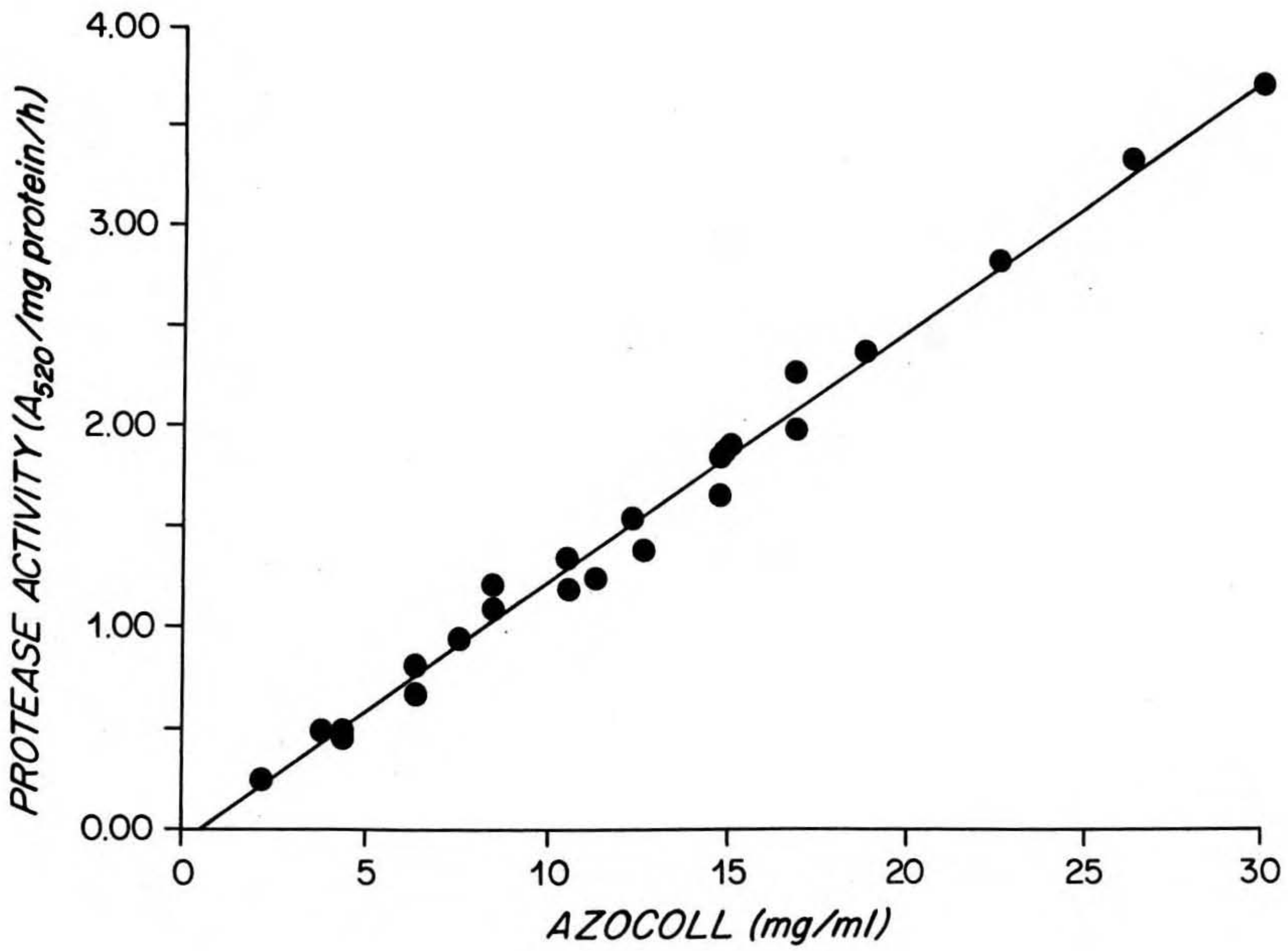

100 µl
200 µl
PROTEASE ACTIVITY (A520)
0.10
0.08
0.06
0.04
0.02
0.00
0
15
30
45
60
TIME (min)

Figure 4.2. Activity of lobster protease in relation to substrate concentration. Reaction conditions are 38°C and pH 7.5, 0.1 M phosphate buffer.



PROTEASE ACTIVITY (A_{520} /mg protein/h)
4.00
3.00
2.00
1.00
0.00
0
5
10
15
20
25
30
AZOCOLL (mg/ml)

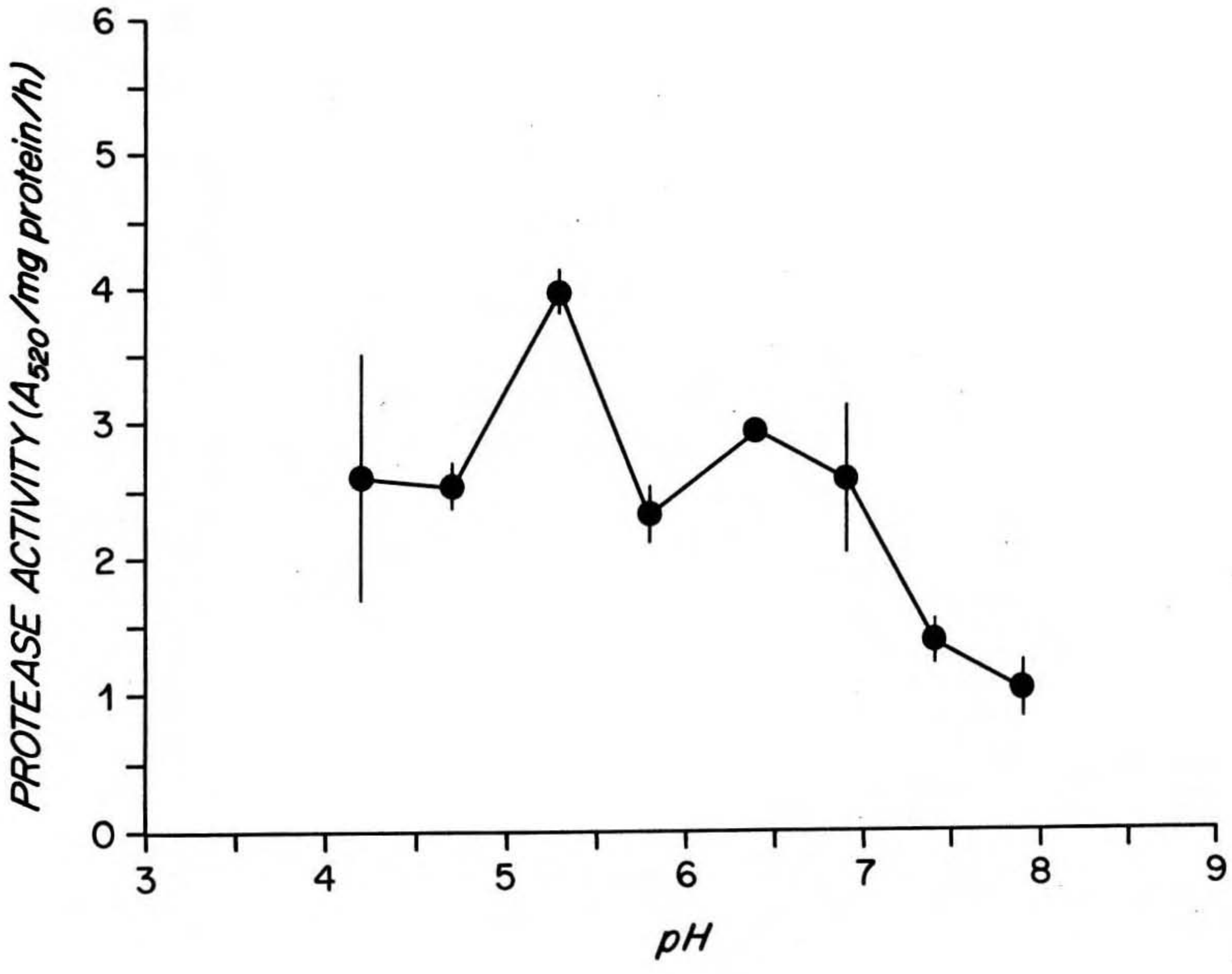
are many which used concentrations of 5 mg/ml, presumably for reasons of economy.

Two pH optima for lobster protease are evident in Fig. 4.3, one at pH 5.3 and another, smaller peak at about pH 6.4, probably indicating the presence of two different proteolytic enzymes. Selection of the most appropriate pH for the assay of lobster protease activity was thus somewhat problematic. In the past, acid proteases have been generally considered to be rare in crustacea and often to appear cathepsin-like when found (van Weel, 1970). Many crustacean digestive enzymes are reported to have alkaline pH optima. Crustacean trypsin-like enzymes which have been isolated and characterized from several different organisms have had pH optima ranging from pH 7 to 9 (cf. review of Dall and Moriarty, 1983). Likewise, the low molecular weight (LMW) proteases isolated by DeVillez and Lau (1970) and by Pfleiderer et al. (1967) had pH optima at pH 8.0.

Barnard (1973) cautioned that peaks of protease activity at acidic pH could be due to intracellular enzymes rather than to extracellular digestive enzymes, particularly if extracts of whole organisms are used. As has been discussed above, this is not the case in the present study. The evidence suggests that the proteases detected in the present control tests are indeed digestive enzymes.

In fact, there have been a number of other reports of acidic digestive proteases in decapods. DeVillez (1965) reported a peak of protease activity at pH 6.0 using gastric juice extracts from the crayfish <u>Orconectes</u> <u>virilis</u>. Sather (1969) found two peaks of digestive protease activity, at pH 5.6 and pH 7.9, in the crab

Figure 4.3. Activity of lobster protease ($\bar{X} \pm SD$, n=4) in relation to pH. Reaction conditions are 38°C, 9 mg/ml Azocoll, and 0.1 M buffers.


PROTEASE ACTIVITY (A520 /mg protein/h)
0
1
2
3
4
5
6
pH
3
4
5
6
7
8
9

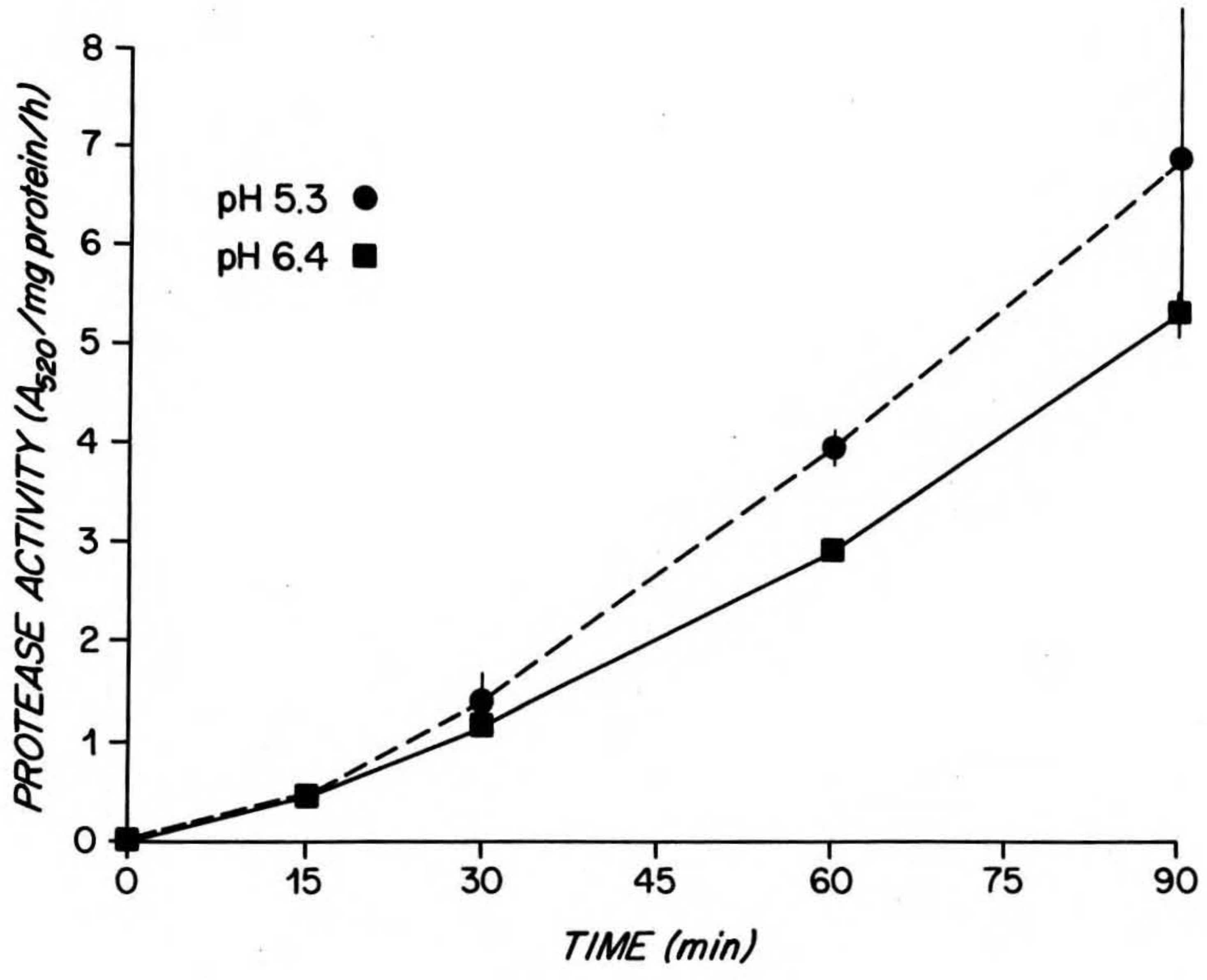
Podophthalmus vigil; cathepsin activity in this species showed a pH optimum at pH 6.0. Muramatsu and Morita (1981) have demonstrated the presence of four trypsin-like enzymes from the crab Eriocheir japonicus; two of these enzymes have acidic pH optima, one at pH 5.8 and the other at pH 6.3.

Brockerhoff et al. (1970) reported finding several proteolytic enzymes in Homarus gastric fluid, two or three of which showed pH optima at about pH 4.0. (The pH optima they obtained when Azocoll was used as the substrate were pH 4.0 and pH 8.0.) Hoyle (1973) also found two pH optima with Azocoll, at pH 5.5 and pH 7.5 when he assayed for adult lobster protease activity. He suggested that these peaks corresponded to those found by Brockerhoff et al. (1970) who used freeze-dried gastric fluid which had been adjusted to pH 8 and dialyzed; the properties of this "slightly refined preparation of lobster gastric juice" may have been altered by the treatment.

In view of the fact that other workers have found acid digestive proteases in crustaceans and because the pH of the gastric fluid of laboratory-reared juvenile lobsters is around pH 5.5 (cf. Appendix A), pH 5.5 was chosen for the present protease assays.

Time course data for protease activity at pH 5.3 and 6.4 are plotted in Fig. 4.4. There is a lag in the early portion of the reaction which continues to a slight degree up to 30 min. This lag occurred consistently at all the pH values that were assayed. It could be due to activation of a zymogen form of the protease. However, it is generally considered that crustaceans do not produce zymogens (cf. Dall and Moriarty, 1983). The same lag was seen when

Figure 4.4. Time course of lobster protease activity ($\bar{X} \pm SD$, n=4). Reaction conditions are 38°C, 9 mg/ml Azocoll, and 0.1 M citrate phosphate buffers.


pH 5.3
pH 6.4
PROTEASE ACTIVITY (A_{520}/mg protein/h)
0
1
2
3
4
5
6
7
8
TIME (min)
0
15
30
45
60
75
90

the enzyme source was pre-incubated for 15 min at 37°C before the time course samples were taken; thus the lag did not appear to be due to activation of the protease. It is possible that the reaction rate increased with time because the peptide bonds of the Azocoll become more available to the protease after digestion had proceeded for a short while.

As shown in Fig. 4.5, the activity of lobster protease increased with temperature from 25° to 50°C at pH 5.5. There is an indication that the reaction rate may have been starting to decrease at 50°C. Most of the studies in the literature which used Azocoll as an assay for non-specific proteases were done at 37°C, even when non-mammalian enzymes were studied. Although this temperature may not be physiologically relevant for animals which live at temperatures below 22°C, the lobster protease was not denatured at this temperature as evidenced by the high activity after 90 min during the time course experiments. Thus, because the enzyme was not inactivated at 37°C and because most other studies in the literature were conducted at 37°C, this temperature was chosen for the present assays.

Ionic strength had no effect on the activity of the enzyme (Fig. 4.6). Thus, 0.1 M buffer was used.

The dilution curve generated for bovine pancreatic trypsin after 60 min of incubation is given in Fig. 4.7. The reaction rate was linear from 0 to 60 min. It can be seen that 0.10 ug of mammalian trypsin produced an absorbance of 0.11 at 520 nm after a 60 min incubation. In other words, under the conditions of the Azocoll

Figure 4.5. Activity of lobster protease ($\bar{X} \pm DS$, n=3) in relation to temperature. Reaction conditions are 10 mg/ml Azocoll and pH 5.5, 0.1 M citrate phosphate buffer.

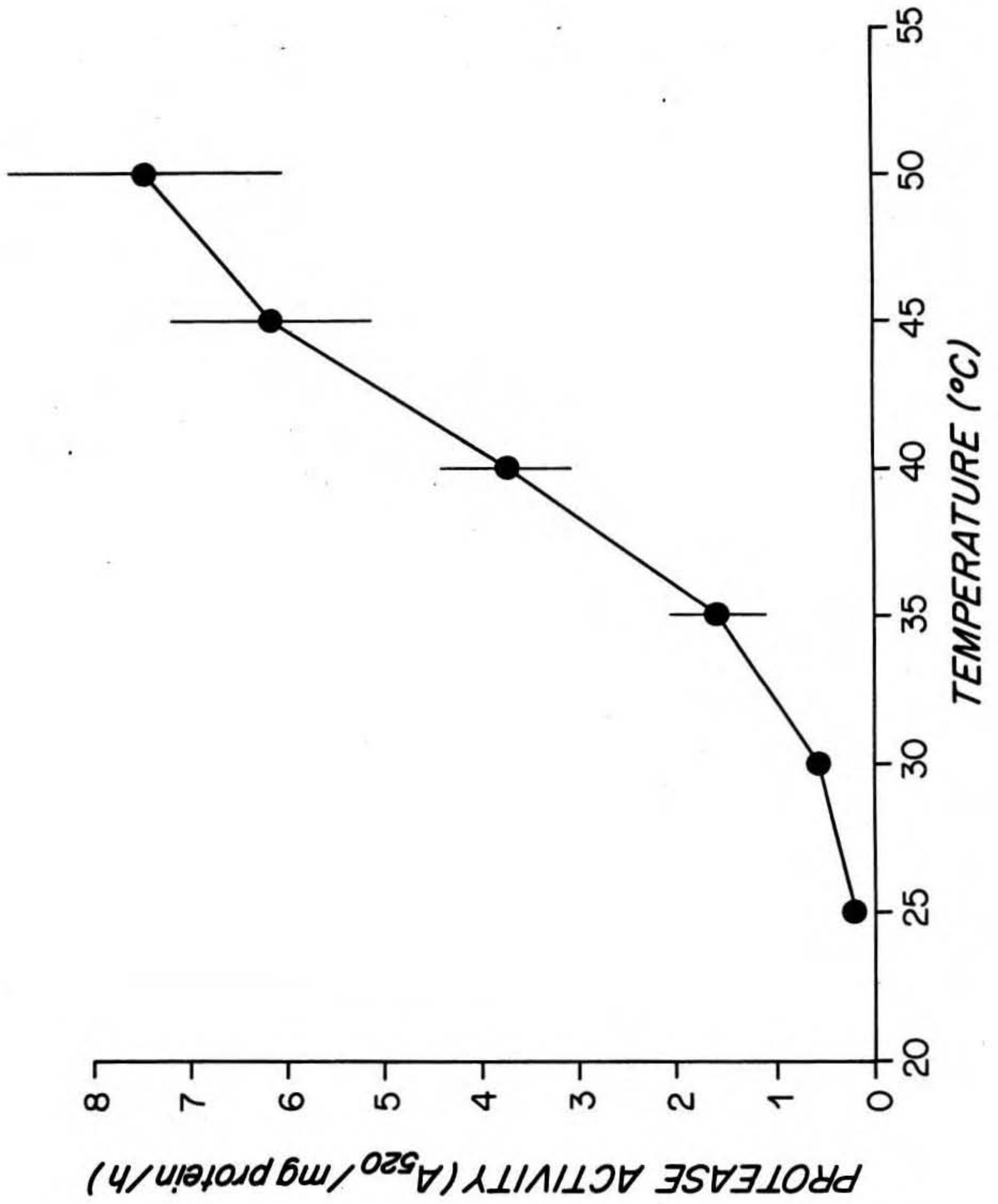

PROTEASE ACTIVITY (A520 / mg protein/h)
0
1
2
3
4
5
6
7
8
TEMPERATURE (°C)
20
25
30
35
40
45
50
55

Figure 4.6. Activity of lobster protease ($\bar{X}\pm$DS, n=3) in relation to ionic strength. Reaction conditions are 37°C, 10 mg/ml Azocoll, and pH 5.5, 0.1 M citrate phosphate buffer.

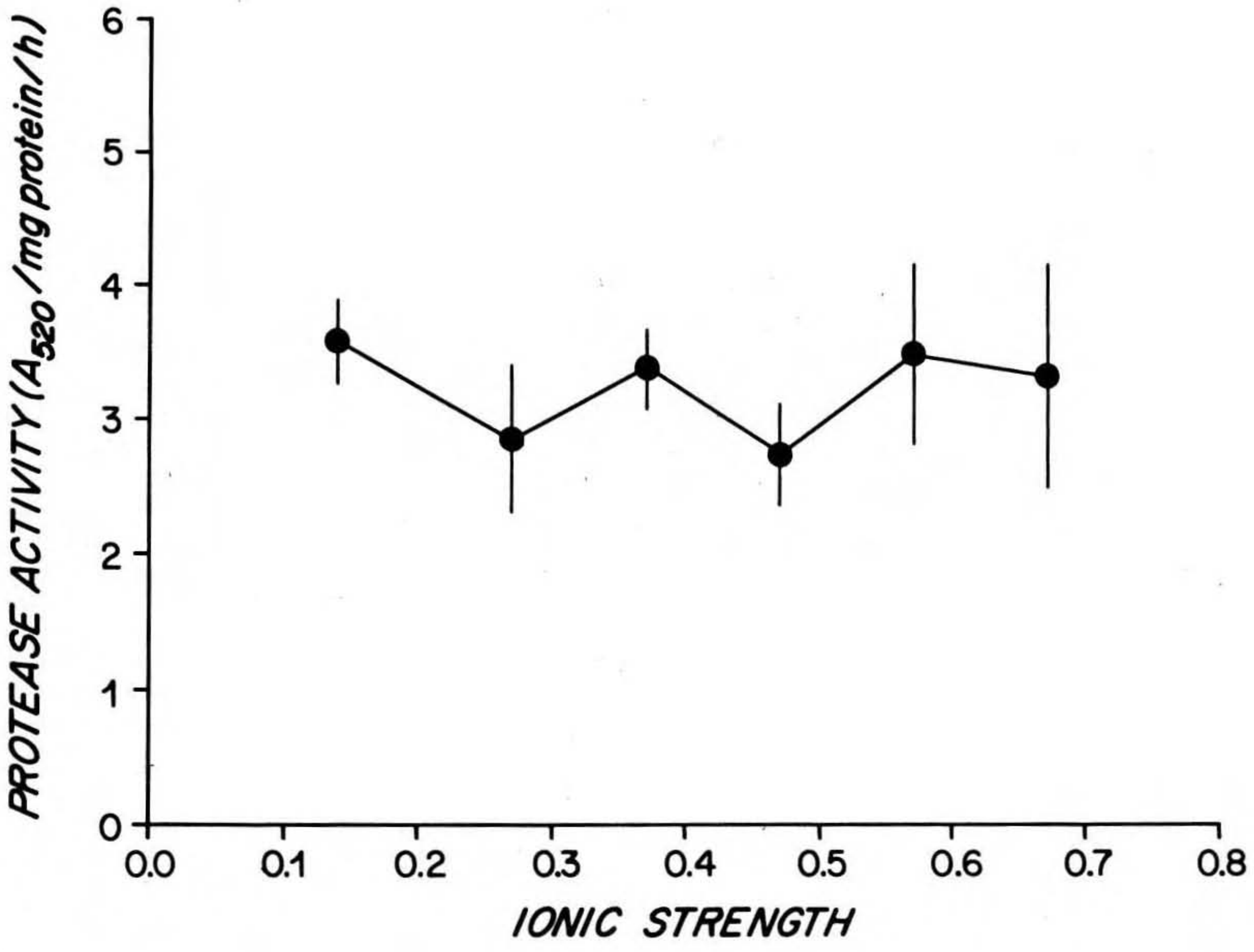
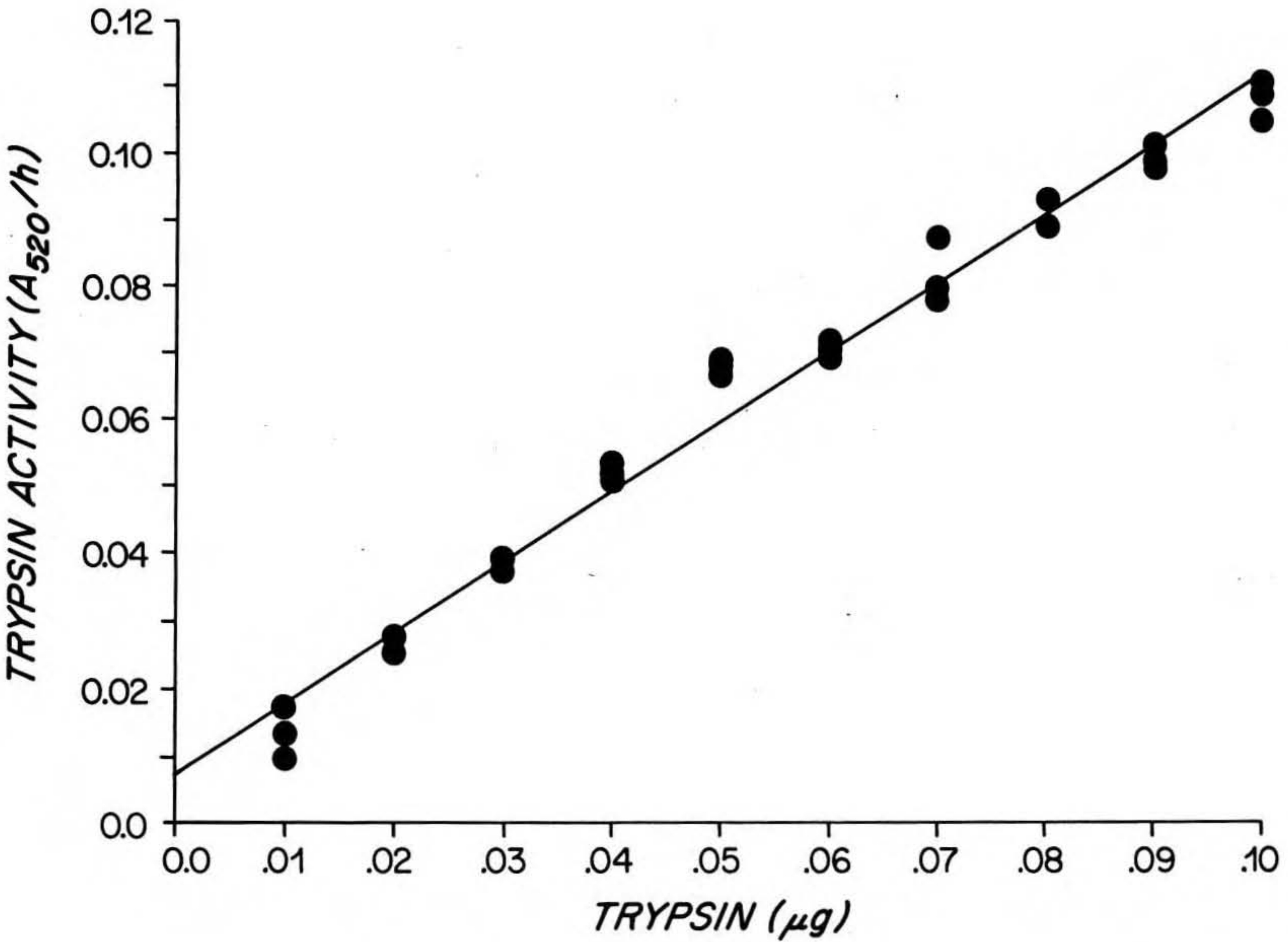

PROTEASE ACTIVITY (A_{520} /mg protein/h)
6
5
4
3
2
1
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
IONIC STRENGTH

Figure 4.7. Standard curve for Azocoll hydrolysis by bovine pancreatic trypsin. The curve is generated by incubating trypsin with 10 mg/ml Azocoll for 60 min at 37°C and pH 7.5, 0.1 M phosphate buffer. BSA is added at 0.35 mg/ml.



0.12
0.10
0.08
0.06
0.04
0.02
0.0
TRYPSIN ACTIVITY (A_{520}/h)
0.0
.01
.02
.03
.04
.05
.06
.07
.08
.09
.10
TRYPSIN (μg)

assay, an increase in absorbance at 520 nm of 0.10 after 60 min was equivalent to 0.09 ug of purified vertebrate trypsin.

Lipase activity

Lipase activity peaked at a concentration of 0.5 umoles/ml triolein (Fig. 4.8); thus, this substrate concentration was chosen for the rest of the lipase assays. Activity declined at 1.0 umoles/ml triolein, possibly as a result of substrate inhibition.

The results of the optimum pH test are presented in Fig. 4.9. The reaction showed a peak of activity at pH 5.5. Most lipases studied in detail show neutral to slightly alkaline pH optima (Wills, 1965; Jensen, 1983). Brockerhoff et al. (1970) showed that optimum activity of partially purified lipase from adult Homarus was at pH 7. There are, however, examples of digestive lipases, including those of marine invertebrates, with acidic pH optima. Data compiled by Mansour-Bek (1954) suggest pH optima for lipases from the genus Astacus ranging from pH 5.2 to pH 6.5. The lipase(s) of crawfish Cambarus virilis show pH optima at pH 4.0 and pH 9.0 (Berner and Hammond, 1970). In view of the fact that the optimum pH for lobster lipase found in the present control test was the same as the pH of juvenile gastric fluid (pH 5.5), the remainder of the lipase assays conducted as part of the present study were done at pH 5.5.

The effect of temperature on the reaction rate is shown in Fig. 4.10. The rate of lipolysis increased from 20° to 45°C. The rate of increase, however, appeared to diminish somewhat between 30° and 45°C; the reaction rate decreased between 45° and 50°C. Jensen (1983) reported the usual range of optimal lipase activity to be 30°

Figure 4.8. Activity of lobster lipase in relation to substrate concentration. Reaction conditions are 37°C and pH 5.5, 0.1 M citrate phosphate buffer. The reaction mixture contains 0.28 M NaCl, 0.35 mg/ml BSA, and 0.02 M $CaCl_2$ or $MgCl_2$.

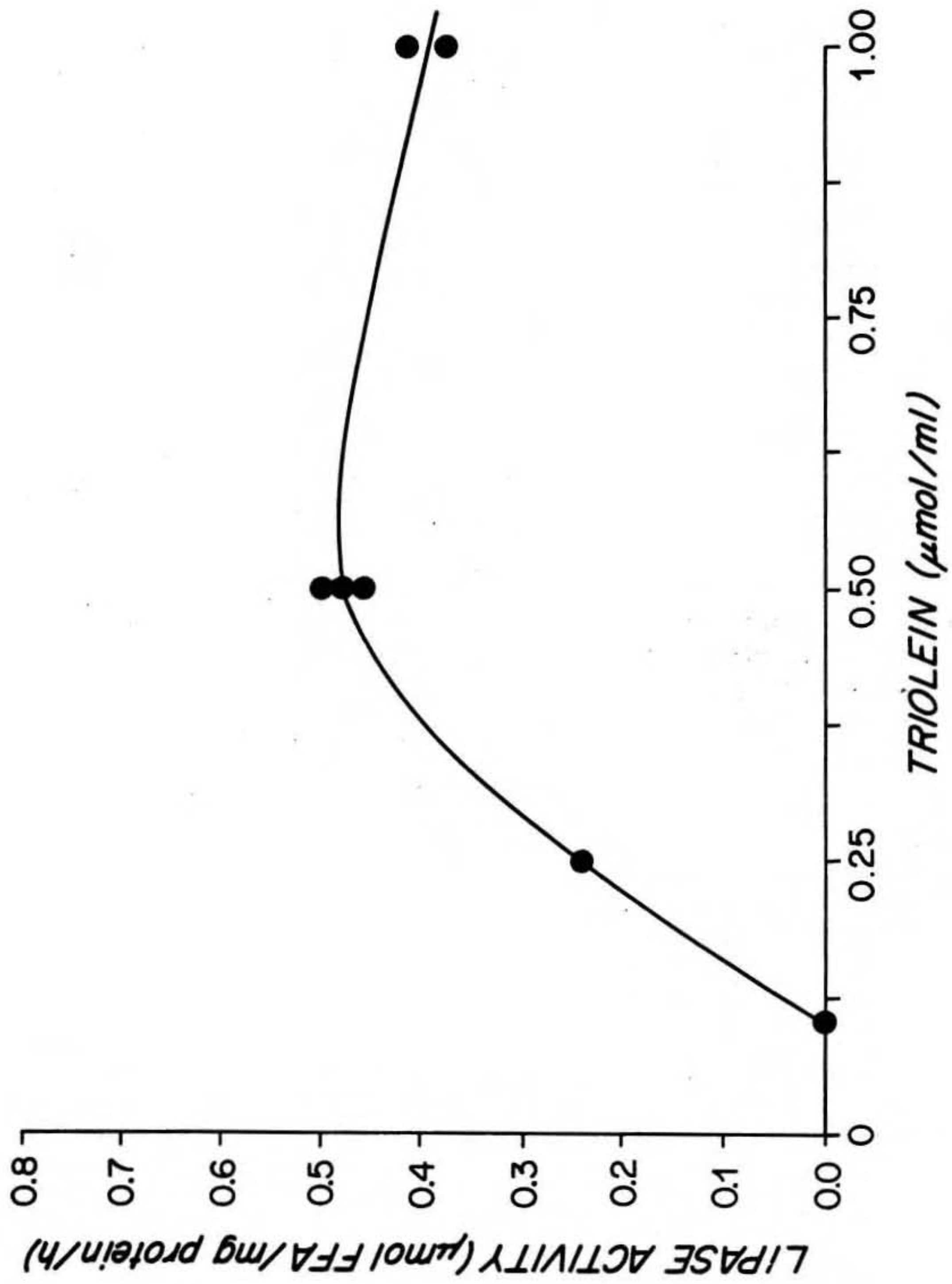

LIPASE ACTIVITY (µmol FFA/mg protein/h)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
TRIOLEIN (µmol/ml)
0
0.25
0.50
0.75
1.00

Figure 4.9. Activity of lobster lipase ($\bar{X} \pm SD$, n=3) in relation to pH. Reaction conditions are 37°C and 0.5 umole/ml triolein. 0.04 M buffers are used. The reaction mixture contains 0.28 M NaCl, 0.35 mg/ml BSA, and 0.02 M $MgCl_2$.

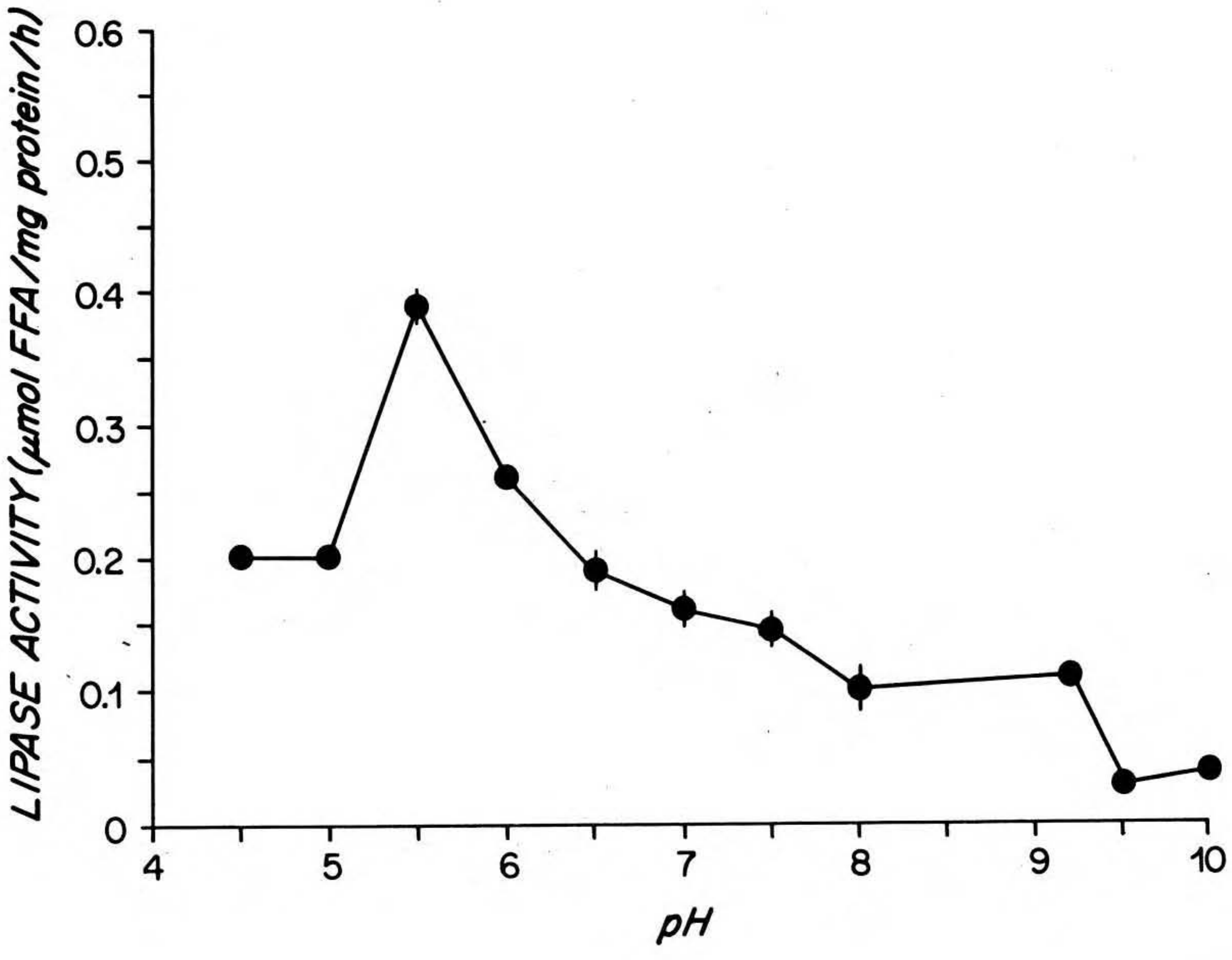
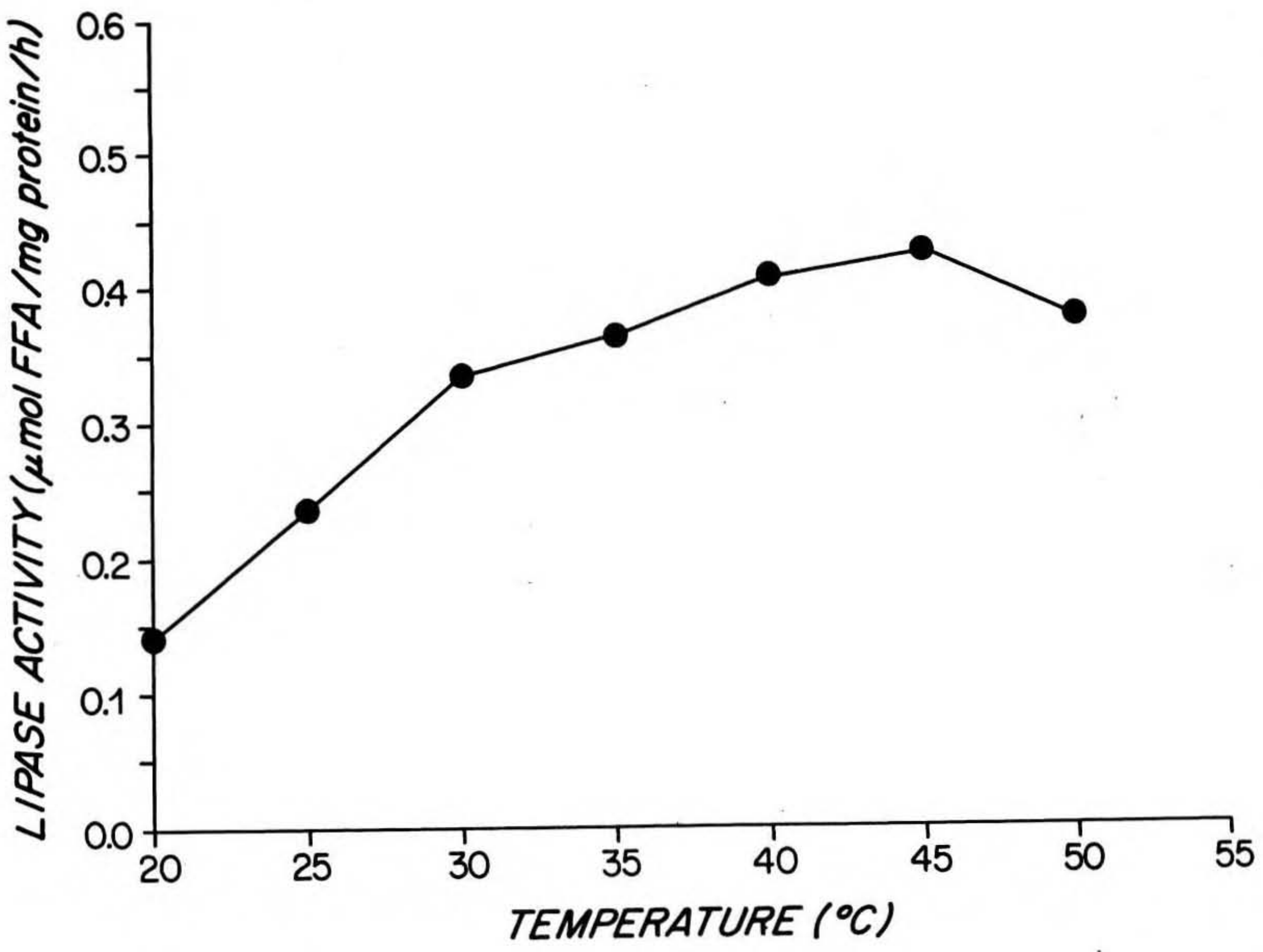

LIPASE ACTIVITY (µmol FFA/mg protein/h)
0.6
0.5
0.4
0.3
0.2
0.1
0
4
5
6
7
8
9
10
pH

Figure 4.10. Activity of lobster lipase ($\bar{X}\pm SD$, n=3) in relation to temperature. Reaction conditions are 0.5 umole/ml triolein and pH 5.5, 0.1 M citrate phosphate buffer. The reaction mixture contains 0.28 M NaCl, 0.35 mg/ml BSA, and 0.02 M $MgCl_2$.



LIPASE ACTIVITY (μmol FFA/mg protein/h)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
20
25
30
35
40
45
50
55
TEMPERATURE (°C)

to 45°C. Because a temperature of 37°C had been used for the protease assays, this temperature was also chosen for the lipase assays conducted in the present study.

Ionic strength had very little influence on the lipase reaction rate (Fig. 4.11); 0.1 M buffer was used for the assays.

Lipase activity was essentially linear with time (Fig. 4.12). Lipase assays, therefore, were routinely run for 60 min to take advantage of the increased production of oleic acid over time.

Amylase activity

It can be seen from Fig. 4.13 that amylase activity was linear from 0 to 36 min. A reaction time of 30 min was chosen for the amylase assays of the present study because a strong signal of maltose production was generated by this time.

The standard starch concentration of 5 mg/ml was not saturating for lobster amylase when the reaction proceeded for 30 min at 37°C (Fig. 4.14); substrate was not saturating under the conditions of the assay until 10 mg/ml or more. Therefore, starch at 15 mg/ml was routinely used for the rest of the amylase assays.

Crustaceans usually secrete a strongly active amylase with pH optima which range from pH 5.0 to pH 7.8 (van Weel, 1970). Lobster amylase from stage I larvae showed a relatively broad pH optimum, from about pH 6.0 to pH 7.0 with a peak between pH 6.5 and 7.0 (Fig. 4.15). This corresponds reasonably well to the pH optimum of pH 6.2 determined for amylase from laboratory-reared juvenile lobsters (Biesiot, unpublished data). Wojtowicz and Brockerhoff (1970) demonstrated a pH optimum of pH 5.2 for adult American lobsters.

Figure 4.11. Activity of lobster lipase ($\bar{X} \pm SD$, n=3) in relation to ionic strength. Reaction conditions are 37° C, 0.5 umole/ml triolein, and pH 5.5, 0.1 M citrate phosphate buffer. The reaction mixture contains 0.35 mg/ml BSA and 0.02 M $MgCl_2$.

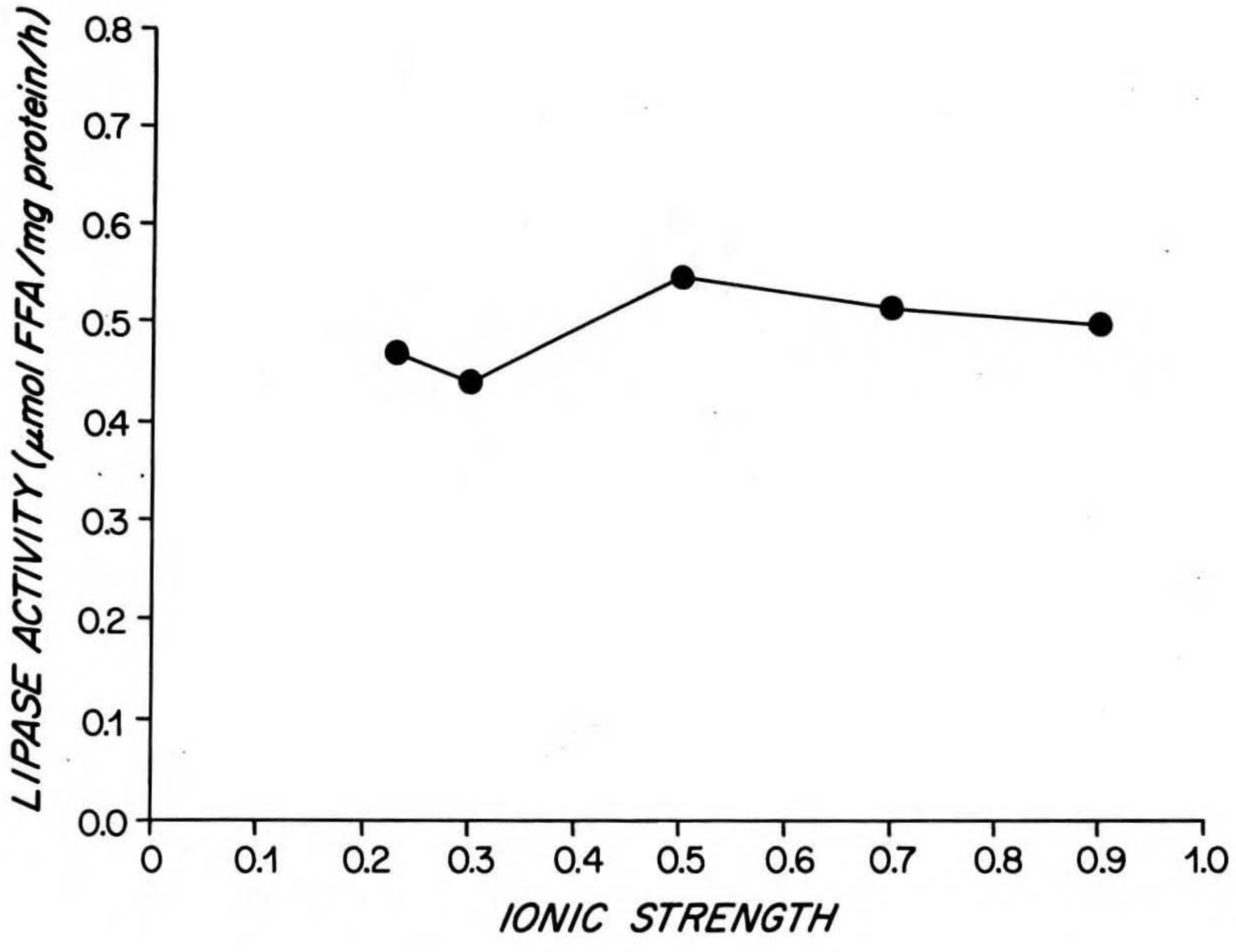

LIPASE ACTIVITY (μmol FFA/mg protein/h)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
IONIC STRENGTH

Figure 4.12. Time course of lobster lipase activity ($\bar{X}\pm SD$, n=3). Reaction conditions are 37°C, 0.5 umole/ml triolein, and pH 5.5, 0.1 M citrate phosphate buffer. The reaction mixture contains 0.28 M NaCl, 0.35 mg/ml BSA, and 0.02 M $MgCl_2$.

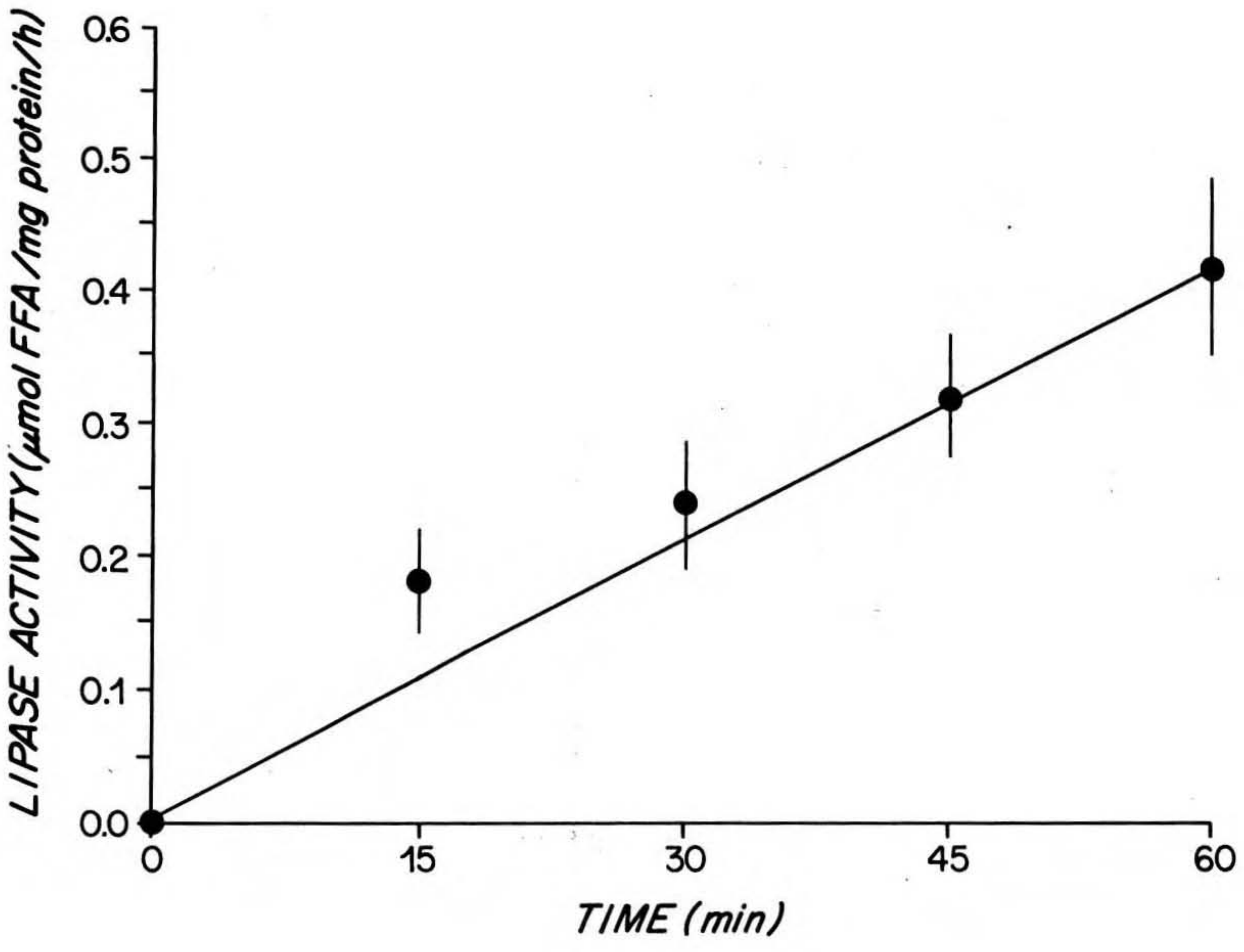

LIPASE ACTIVITY (µmol FFA/mg protein/h)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
15
30
45
60
TIME (min)

Figure 4.13. Time course of lobster amylase activity. ($\bar{X}\pm$SD, n=3). Reaction conditions are 37°C, 5 mg/ml starch, and pH 5.5, 0.1 M citrate phosphate buffer with 0.05 M NaCl.

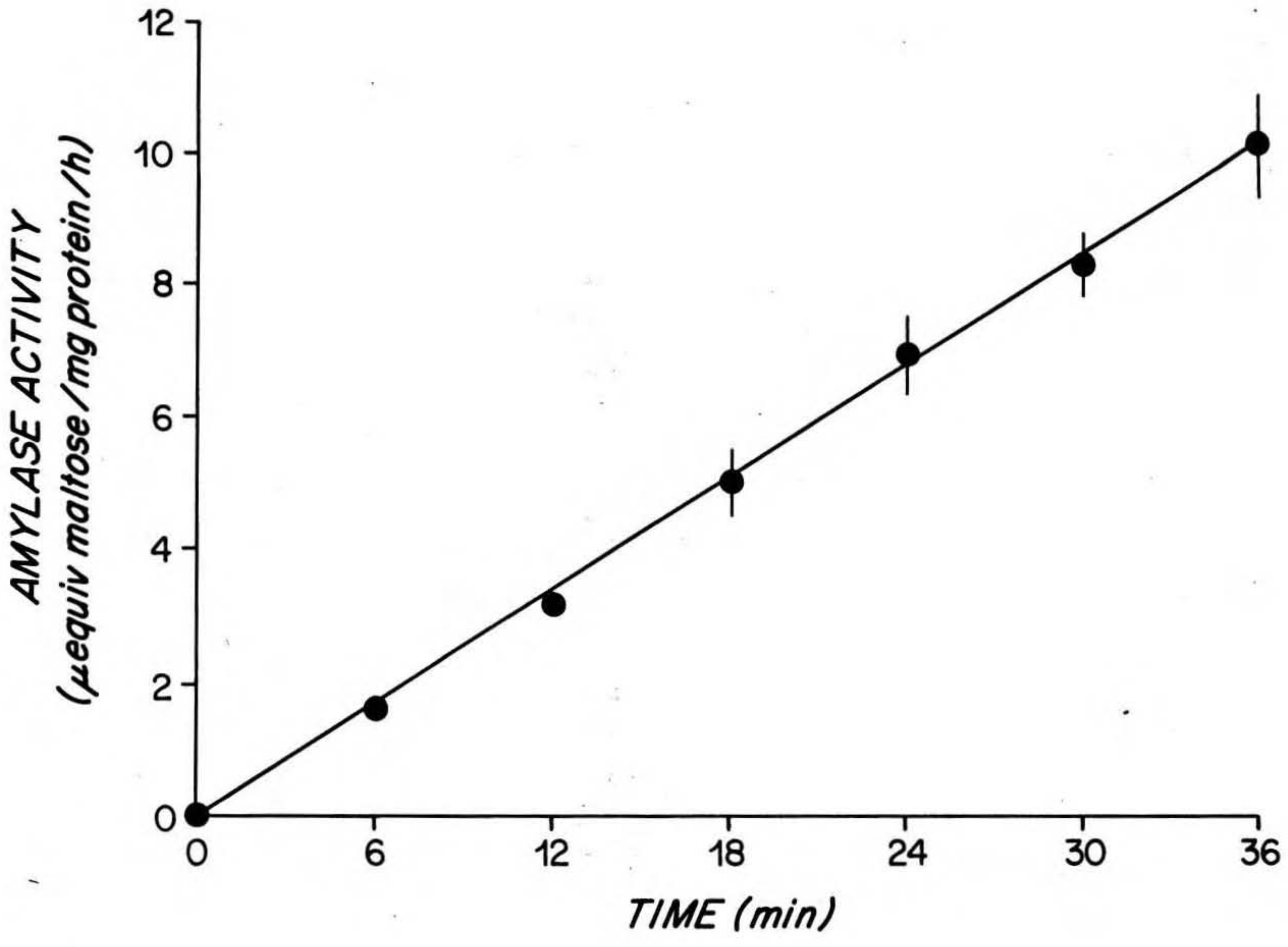

AMYLASE ACTIVITY
(μequiv maltose/mg protein/h)
12
10
8
6
4
2
0
0
6
12
18
24
30
36
TIME (min)

Figure 4.14. Activity of lobster amylase ($\bar{X} \pm SD$, n=3) in relation to substrate concentration. Reaction conditions are 37°C and pH 5.5, 0.1 M citrate phosphate buffer with 0.05 M NaCl.

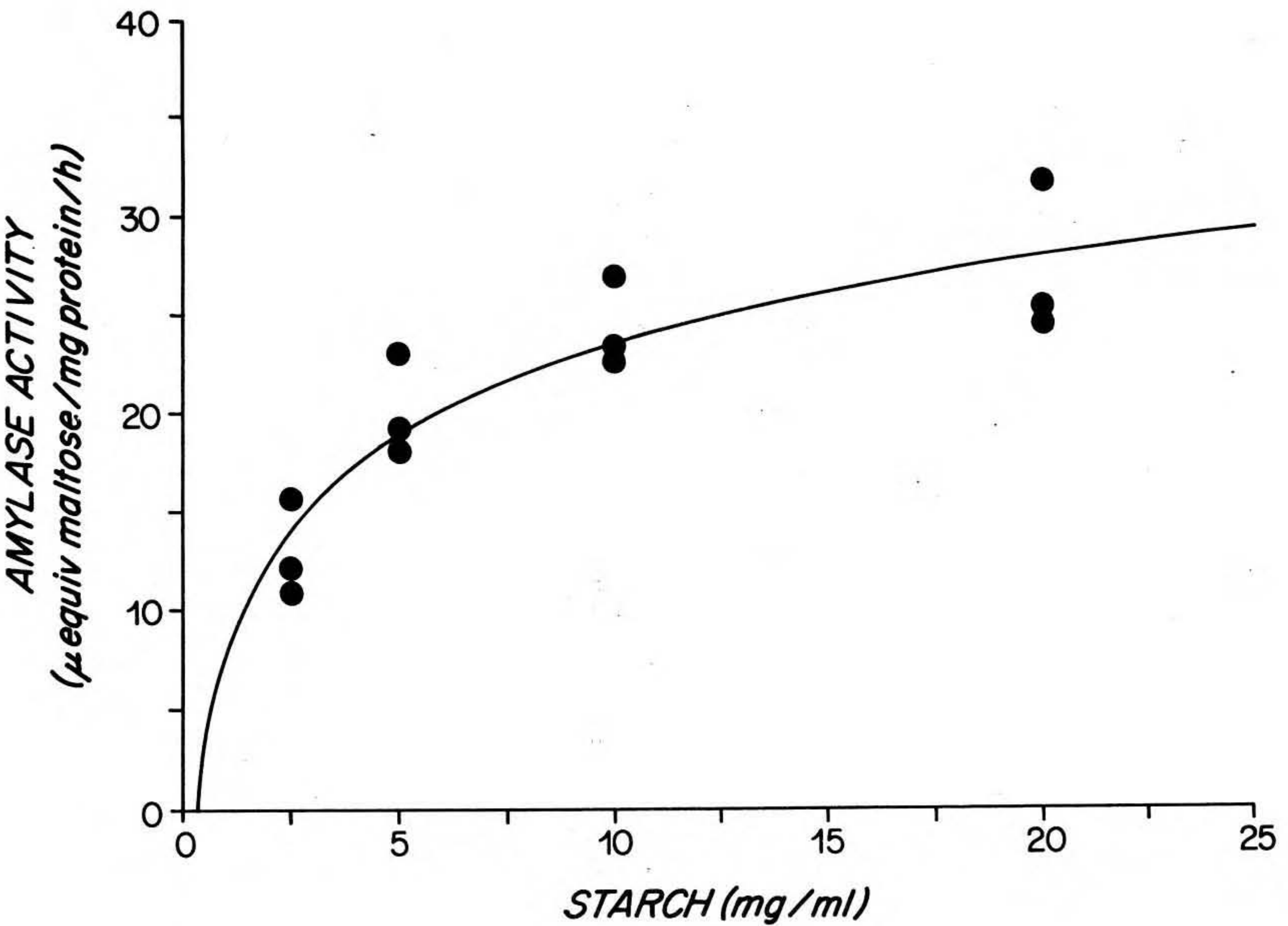
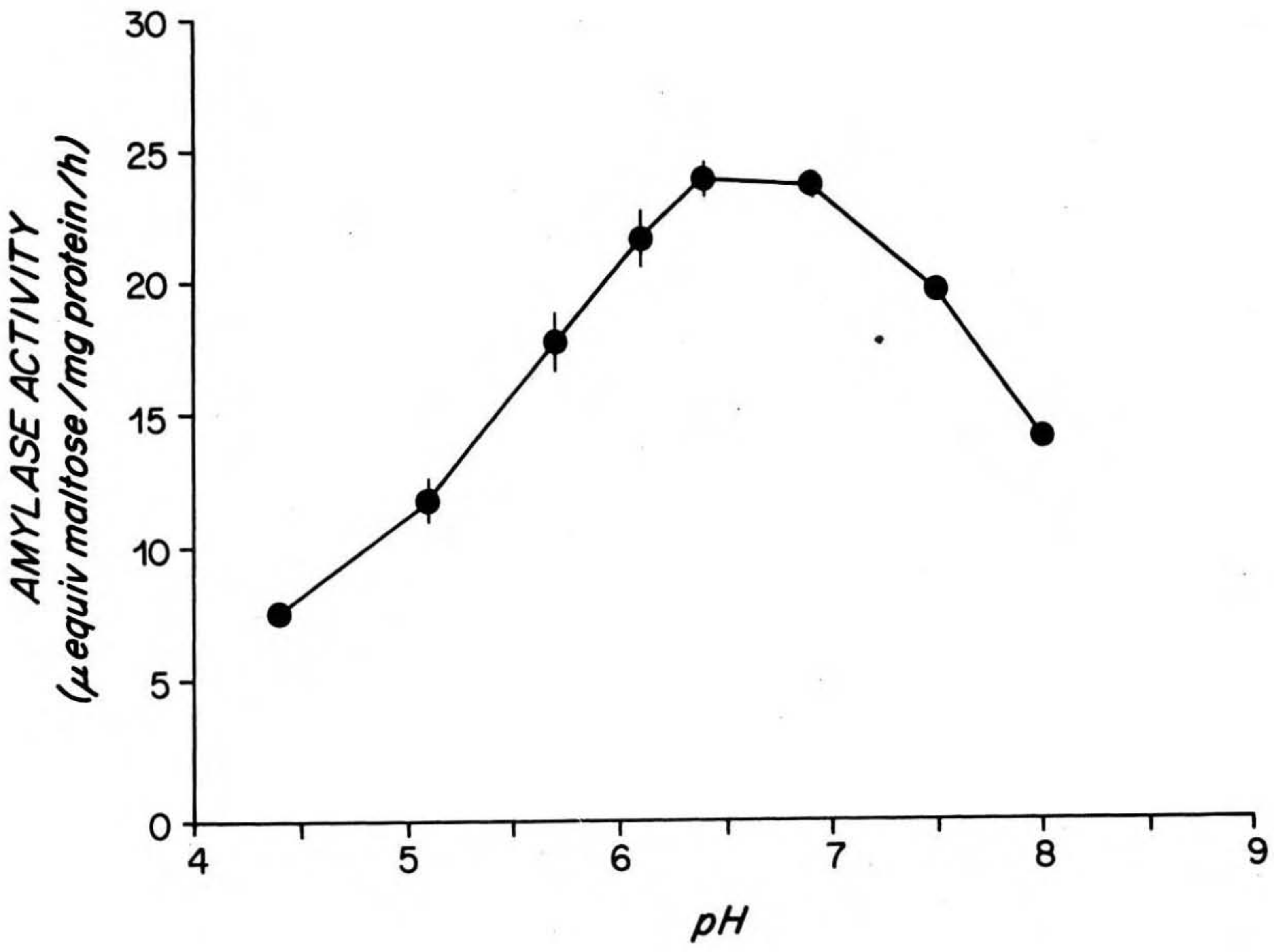

AMYLASE ACTIVITY
(μequiv maltose/mg protein/h)
0
10
20
30
40
0
5
10
15
20
25
STARCH (mg/ml)

Figure 4.15. Activity of lobster amylase ($\bar{X} \pm SD$, n=3) in relation to pH. Reaction conditions are 37°C and 15 mg/ml starch. 0.05 M NaCl is added to 0.1 M buffers.


AMYLASE ACTIVITY
(µ equiv maltose / mg protein / h)
0
5
10
15
20
25
30
pH
4
5
6
7
8
9

Hoyle (1973) showed a broad pH optimum for adult lobster amylase, from pH 5.0 to pH 6.0. In both cases the lobsters had not fed for over a week. In the present instance, however, the lobsters had been well-fed. The amylase assays conducted for the present study were therefore performed at pH 6.5.

As demonstrated in Fig. 4.16, amylase activity increased with temperature over the range 25°to 50°C. Because the other enzyme assays were conducted at 37°C, it was decided to use this temperature for amylase as well.

An ionic strength of about 0.35-0.45 appeared to be optimal for the lobster amylase (Fig. 4.17). Therefore, the amylase assays were conducted using 0.1 M phosphate buffer (final concentration) with 0.05 M NaCl added.

Figure 4.16. Activity of lobster amylase ($\bar{X} \pm SD$, n=3) in relation to temperature. Reaction conditions are 15 mg/ml starch and pH 6.5, 0.1 M phosphate buffer with 0.05 M NaCl.

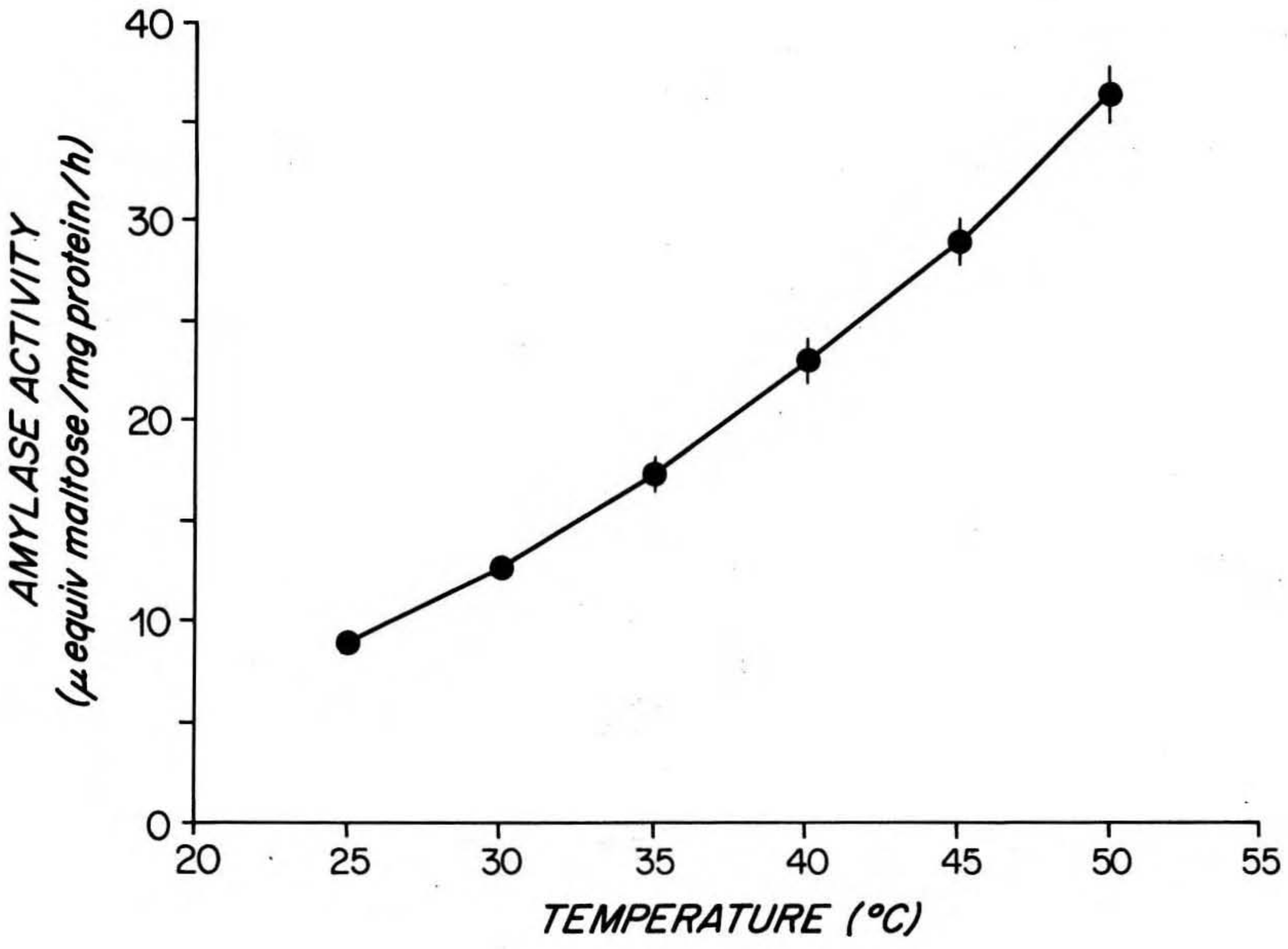
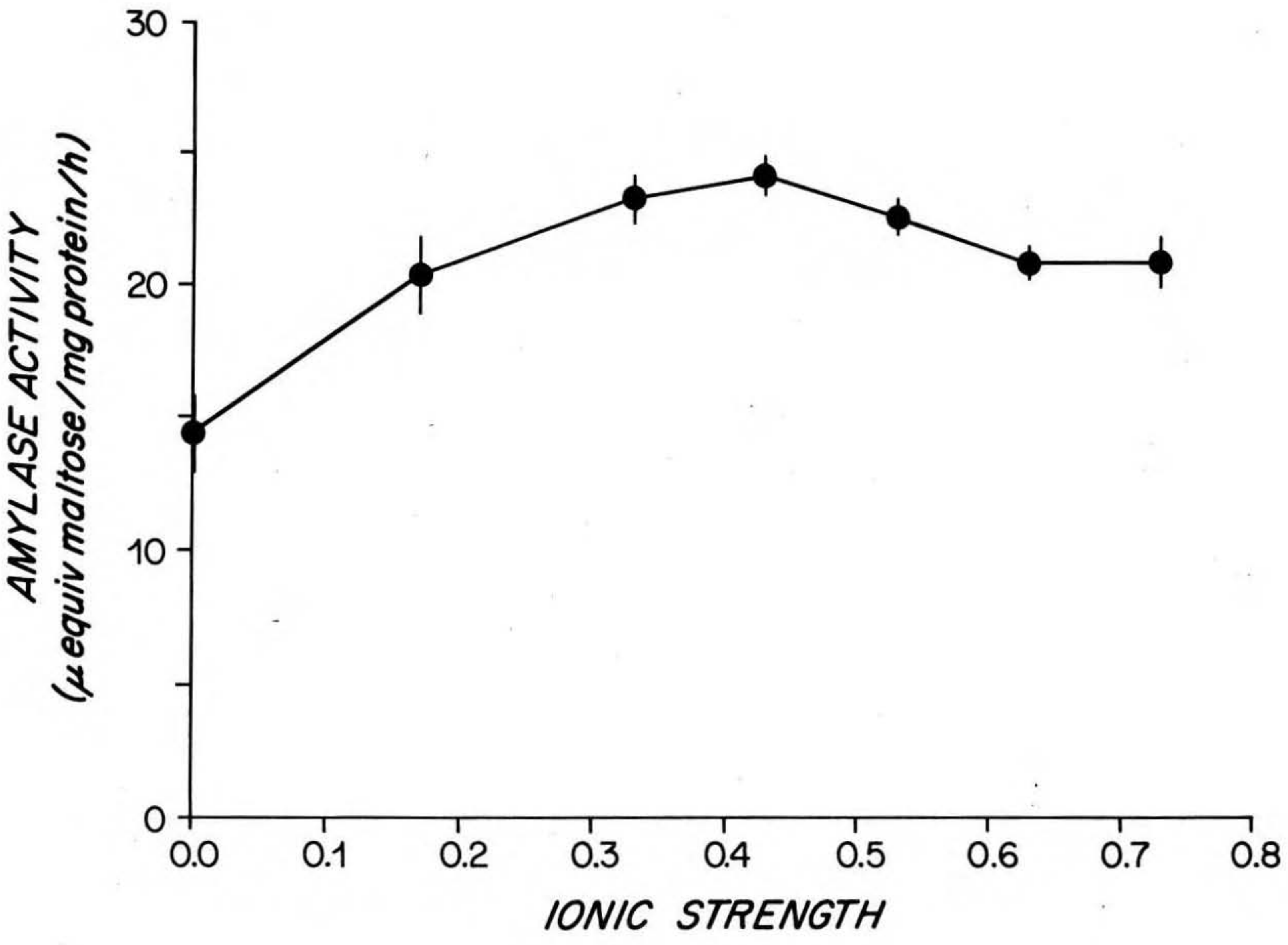

AMYLASE ACTIVITY
(μ equiv maltose/mg protein/h)
40
30
20
10
0
20
25
30
35
40
45
50
55
TEMPERATURE (°C)

Figure 4.17. Activity of lobster amylase ($\bar{X} \pm SD$, n=3) in relation to ionic strength. Reaction conditions are 37°C, 15 mg/ml starch, and pH 6.5, 0.1 M phosphate buffer.


AMYLASE ACTIVITY
(μ equiv maltose / mg protein/h)
30
20
10
0
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
IONIC STRENGTH

CHAPTER 5:
DIGESTIVE ENZYME ACTIVITIES DURING THE DEVELOPMENT OF EARLY STAGES OF THE AMERICAN LOBSTER HOMARUS AMERICANUS

INTRODUCTION

An understanding of basic digestive physiology is important for investigations of nutritional requirements and feeding ecology of marine invertebrates. To date, however, research on development of digestive function by the early life history stages of these organisms has been limited.

Most attention has been paid to characterizing the proteolytic enzymes detected during development of Artemia sp. gastrulae and nauplii. Green (1971) summarized the experimental work done to date on enzyme activity during the embryonic development of crustacea. Enzymes detected before and after hatching of brine shrimp included amylase, dipeptidase, proteinase, lipase, acid and alkaline phosphatase, and ribonuclease. There was, however, no distinction made in these studies between yolk-metabolizing enzymes and true digestive enzymes produced by the embryonic digestive tract.

Embryonic nutrient reserves of crustaceans are in the form of proteinaceous yolk granules and lipid vesicles (Holland, 1978). The proteinaceous yolk is in the form of lipoprotein aggregates, the lipid fraction of which is about 30% of the protein; the lipids are primarily triacylglycerols and phospholipids. In lobsters, components of the yolk are apparently synthesized both by the oocyte itself and in the midgut gland of the female; the latter circulates in the hemolymph and is absorbed by the oocyte by pinocytosis (Schade and Shivers, 1980). Yolk-metabolizing enzymes are presumed present in lysosomes within the

yolk of crustaceans but whether they are produced by the adult female, the oocyte, or both is unknown.

Acid hydrolases of lysosomal origin are involved in the degradation of yolk in Artemia sp. (Perona and Vallejo, 1985). Cathepsin B-like proteinase was responsible for primary degradation of the lipovitellin component of yolk granules. Other acid hydrolases detected during embryonic and naupliar development included acid ribonuclease, acid deoxyribonuclease, acid phosphatase, acid phosphodiesterase, ß-glucosidase, ß-N-acetylgalactosaminidase, and acid lipase. Maximum activities of these enzymes occured among the nauplii, when yolk degradation was maximum.

Earlier, Osuna et al. (1977) had reported that extracts from encysted gastrulae and from developing embryos of Artemia sp. contained almost undetectable levels of protease activity. However, after hatching there was an increase in activity of four enzymes, protease A, B, C, and D, which showed sequential timing of induction during early larval development. The authors determined that proteases B and C were induced shortly after hatching while D and A were induced several hours later. Subsequent experiments (Garesse et al., 1980) have suggested that these proteases are not induced but instead are present in the Artemia embryo during development in an inhibited form that is gradually activated in the larvae after hatching. Olalla et al. (1978) determined that A is a chymotrypsin-like serine protease and that B is a trypsin-like serine protease. Proteases A, B, and C are optimally active at alkaline pH. Burillo et al. (1982) partially purified protease D and determined that it is not inhibited by soybean trypsin inhibitor and that the optimum pH

is 8. Protease A is also an esterase, B also has amidase and esterase activity, C shows amidase and esterase activities but does not hydrolyze casein, and D digests casein but not synthetic esters with leucine or arginine (Osuna et al., 1977). All four larval proteases lysed yolk granules but whether these proteases were products of the developing digestive system or intracellular enzymes was not determined (Sillero et al, 1980). Using different proteins as substrates, Ezquieta and Vallejo (1985) recently found two proteases rather than four in Artemia embryos. The protease responsible for the major enzymatic activity was a trypsin-like serine protease and was localized in the yolk granules associated with lipovitellin. The same protease was also found in the larvae. Therefore, at least one of the four alkaline proteases detected in Artemia nauplii is involved in yolk digestion and is not a digestive enzyme but the status of the other three is still unknown.

Variations in protease and amylase activity during larval development have been shown for the shrimp Palaemon serratus (van Wormhoudt, 1973) and Penaeus japonicus (Laubier-Bonichon et al., 1977). In Palaemon serratus, amylase activity reached a high level during the second larval stage whereas an increase in protease activity occurred only at the fifth larval stage; these changes were correlated to modifications of the natural diet (van Wormhoudt, 1973). Galgani and Benyamin (1985) measured the activity of trypsin throughout larval development of the shrimp Penaeus japonicus. Activity was very low among the non-feeding nauplii, increased almost 300% among the zoea 2, then declined to half this activity by the postlarval stage. The authors felt that the changes were regulated by enzyme synthesis rather than by the presence of more than

one enzyme. Aside from these studies, however, very little research has been done on the digestive enzymes of other crustacean larvae.

Vacquier (1971) followed the synthesis of laminarinase in embryos and pluteus larvae of the starfish Dendraster excentricus from fertilization to the point of yolk exhaustion (about 42 h). Laminarinase activity was first detected at 22.5 h, which coincided with the onset of gut differentiation, and continued to increase linearly until the plutei died of starvation. The larvae were not given food during the experiment but would normally have fed on phytoplankton. Similarly, α-amylase activity also appeared during gut differentiation of the pluteus larvae (Vacquier et al., 1971). Thus, digestive enzyme synthesis was associated with development of the gut in these starfish larvae.

This correlation of gut development with an increase in digestive enzyme activities has been noted in some planktonic marine larval fishes. At hatching, the digestive enzymes of fish larvae, if present, are at low levels (Blaxter, 1969). The gut is unspecialized and digestive enzymes are secreted along its length rather than by a gland. Tanaka (1969) and Bogdanova (1980) have shown that larval marine fish lack a true stomach and that digestion must occur under alkaline conditions. Generally, fish larvae begin to ingest food before the yolk sac is completely resorbed but they retain the potential to feed for some days after resorption. However, if the larvae deplete their energy reserves to a critical low level, the point of no return (PNR), they are unable to make up the loss by feeding (Blaxter and Hempel, 1963). There is thus a critical transitional period of mixed feeding in marine larval fishes: a dependence both on yolk metabolism and exogenous food (Braum,

1967). During this "critical period" (Fabre-Domerque and Bietrix, 1897, cited by Braum, 1967) fish larvae are threatened by starvation if they do not develop the proper feeding behavior. There is evidence that digestive enzymes of marine fish larvae become active during the critical period. Bogdanova (1980) was unable to detect any development in the digestive system of larval whitefish Coregonus lavaretus pallasi when it fed on an artificial ration. Control fish given live food developed secretory cells in the intestine and showed development of the musculature. Thus, increased levels of digestive enzymes were presumed to be induced by feeding on living organisms. Dabrowski and Glogowski (1977) have suggested that larval marine fishes require an exogenous source of digestive enzymes from a live, or at least natural, food in order for enzyme secretion to function properly since initially the gastrointestinal cells were not completely differentiated. Whether a similar induction of specific digestive enzymes by exogenous substrate or enzyme molecules occurs in invertebrate larvae is unknown.

Anger and Dawirs (1981) studied spider crab larvae Hyas araneus exposed to varying periods of starvation. They determined that crab larvae must feed from the beginning of each larval stage and for more than one-fifth of that stage in order to successfully molt to the next stage. The minimum time during which enough energy reserves have accumulated for successful molting to the next stage was called "point of reserve saturation" (PRS). If only this minimum period preceeded starvation, development time was increased and mortality was greater compared to the fed controls. If about 70% of the maximum survival time for the first stage elapsed without feeding, crab larvae became unable to

recover and molt to the second stage even when re-fed; this was termed the PNR after the phenomenon observed in larval marine fishes. During starvation, protein was the main metabolic substrate of crab larvae; lipid was used to a much lesser extent (Anger and Nair, 1979).

Anger et al. (1985) examined the effect of starvation on the molt cycle and midgut gland of stage I American lobster larvae. They concluded that the PNR in this decapod crustacean was caused by an irreversible loss of the ability to store lipids in the midgut gland. It is not known what effect starvation has on the production of digestive enzymes in crustacean larvae or what role, if any, digestive enzyme activities play in relation to PNR.

Previous investigation (Chapter 3 of the present study) has determined that F- and B-cells, the digestive enzyme synthesizing cells and enzyme secreting cells, respectively, are present in the midgut gland of the hatching American lobster. Presumably, digestive capabilities may exist at this stage. It is not known, however, if a suite of digestive enzymes are produced or how active these enzymes might be. Furthermore, R-cell morphology changed during early lobster development which suggested increased capacity for lipid storage among the postmetamorphic stages. It is not known whether levels of digestive enzymes also change during the course of larval development and metamorphosis. Therefore, the present research was undertaken to measure activities of digestive protease, lipase, and amylase among early developmental stages of the American lobster _Homarus_ _americanus_ in order to determine whether there is a correlation with the morphological changes observed in the midgut gland. The life history stages examined in these studies included:

well-advanced gold embryos approximately three days prior to hatching, newly-hatched and intermolt stage I larvae, intermolt stage II, III, and IV larvae, and the stage V and VI postlarvae. Sasaki (1984) has demonstrated that larvae which are induced to hatch in the winter retain larger than normal yolk reserves which may delay the onset of first feeding. Therefore, the enzyme activities of summer-hatch lobsters were compared to those of winter-hatch larvae. A time course study was also conducted to determine the pattern of enzyme induction, if present, in relation to the first feeding. Finally, levels of enzymes were measured in relation to the molt cycle.

BACKGROUND INFORMATION

Digestive enzymes of the adult American lobster

Brockerhoff et al. (1970) surveyed the digestive enzymes present in the adult American lobster. Although this has been the only extensive survey of enzymes in the American lobster, there are some problems associated with the experimental design and the analyses which, in hindsight, led to confusing results. The lobsters were fed chopped mackerel; five to seven days after feeding the gastric juice was collected by suction, filtered, adjusted to pH 8, and lyophilized. The lyophilized powder was rehydrated and desalted by gel filtration on Sephadex G-25. Desalted gastric juice proteins were separated on Sephadex G-100; protein concentration was monitored at 280 mu and principal enzyme activities measured. Enzymes were separated by chromatography in a DEAE-cellulose column. The "surprising results of the survey" were the apparent absence (or very low activity) of some enzymes that were expected to be present, the low activity in general of

those enzymes which were present, the presence of large quantities of non-enzymatic protein, and the finding that the pH optima of most enzymes was not near pH 5, the pH of the gastric juice.

The authors (Brockerhoff et al., 1970) stated that much of the gastric protein was not associated with known enzymatic activities, even in fed lobsters. Assuming that the pure enzymes had specific activities comparable to those of mammals, they determined that enzymes made up less than 5% of the total protein in gastric juice. However, in order to obtain gastric juice free from half-digested food residue, they starved the lobsters for five to seven days; it is questionable how well-fed the "fed lobsters" were. The week-long fast may have affected enzyme secretion. Rosemark et al. (1980) demonstrated that stage VII juvenile H. americanus showed slight atrophy of the lobes of the midgut gland after only four days of starvation and showed 50% atrophy by day twelve; reduction in size and number of both the enzyme secreting cells and the storage cells was observed as early as day four. Thus, the absence or low activity of some enzymes expected to be present in the gastric juice may have been related to the fast. Brockerhoff et al. (1970) showed that the dominant protein fraction in the gastric juice of adult lobsters starved four weeks was almost completely devoid of enzymatic activity.

No evidence was found that zymogens were present in the nonactive protein fraction and the authors stated that to their knowledge zymogens had not yet been detected in invertebrates. More recently zymogen forms of trypsin- and chymotrypsin-like proteases have been found in the sea anemone Actinia equina (van Praet, 1982). Al-Mohanna et al. (1985) reported the synthesis and secretion of zymogen granules by F-cells of

the shrimp Penaeus semisulcatus, making this the first report of zymogens in crustaceans. Their claim is based upon the appearance of dense inclusions in the distal cytoplasm of the F-cells but they give no histochemical or immunocytochemical evidence that these bodies are true zymogens.

The apparent absence of α-amylase, a carbohydrase which is normally present in most invertebrates, reported in the Brockerhoff et al. (1970) study was resolved in a later experiment. By using a soluble form of starch rather than the original insoluble starch substrate, Wojtowicz and Brockerhoff (1972) were able to show appreciable amylase activity with optimum activity at pH 5.2. With regard to the pH optima of the enzymes not being related to the pH of the gut, Hoyle (1973) did not adjust the gastric fluid to pH 8 as done by Brockerhoff et al. (1970). Proteolytic activity had optima at pH 5.5 and 7.5 in the former study rather than at pH 4 and 8 as reported in the latter.

Brockerhoff et al. (1970) speculated that small peptide chains might be digested intracellularly in the midgut gland since they were unable to demonstrate dipeptidase or aminopeptidase activity in the gastric juice. Barker and Gibson (1977) have claimed to demonstrate the presence of an intracellular phase of protein and lipid digestion in the European lobster H. gammarus which occurred within a few hours of feeding.

Localization of digestive enzymes in the adult lobster

Barker and Gibson (1977) used histochemical techniques to localize the digestive enzymes of adult H. gammarus. Esterases demonstrable by the α-naphthyl acetate technique (NA-esterases) were present at the epithelium/chitin interface in both the foregut and hindgut. Strong acid

phosphatase and moderate ATPase activity was seen in the cytoplasm of the tegmental glands of both gut regions; these, however, are not digestive enzymes and are presumed to be involved in some aspects of glandular physiology.

In the midgut gland, enzymes were present in all but E-cells. Enzymes in F-cells were confined to the supranuclear vacuole and showed intense acid and alkaline phosphatase and indole acetate esterase (IA-esterase) activity. F-cell enzyme activity was displayed until 2 - 2.5 h after feeding. Both IA- and NA-esterases were found in the vacuole and apical cytoplasm of B-cells. Acid and alkaline phosphatases were distributed throughout the cytoplasm of B-cells but only acid phosphatases were also found in the vacuole. Other B-cell enzymes included cytoplasmic ATPase, demonstrable at 2 h and 9 h post-feeding, and vacuolar lipase, demonstrable 5 h after feeding. Enzymic activity in B-cells decreased within 15 min of feeding. After restitution of their contents (which took about 1 h), B-cells discharged again. The brush border of R-cells showed strong IA- and NA-esterase and acid and alkaline phosphatase activity. Phosphatase activity is associated with absorption or transport of metabolites across membranes in crustaceans. Weak NA-esterase could be shown in the distal R-cell cytoplasm between the large lipid droplets. IA-esterases and phosphatases were also present in the same region but only during certain stages in the digestive sequence (strongest activity 2-4 h post-feeding). Acid phosphatase appeared before alkaline phosphatase; IA-esterases were not demonstrable immediately after feeding or after about the 9 h stage. The PAS-negative granules in the distal cytoplasm of the R-cells were the loci for

moderate acid phosphatase and strong ATPase activity, irrespective of the nutritive state. In lobsters fed on high fat diets the granular ATPase activity was enhanced and lipolytic enzymes at 5 h post-feeding could be visualized in the granules, throughout the R-cell cytoplasm, and in the tubule lumen contents.

No proteolytic enzymes were detected with certainty in any part of the gut. Weak cathepsin C-like endopeptidases appeared in the distal cytoplasm of R-cells in sections which had been pre-incubated with ascorbic acid (a known activator of cathepsin C-type proteases). Traces of exopeptic enzymes were distinguished in B-cell vacuoles 5 h after a meal and in the distal cytoplasm of R-cells 7-9 h after feeding. Barker and Gibson (1977) hypothesized that although B-cells secrete an exopeptidase during the latter part of extracellular digestion, proteolysis is completed intracellularly in R-cells.

Acid phosphatases and IA-esterases were identified in the midgut; the former also appeared in the epithelia of both diverticula. Epithelial enzyme activity was always confined to the distal cytoplasmic regions, never in the brush border. NA-esterases were found in and around the "wandering cells" of the connective tissue.

MATERIALS AND METHODS

Experimental animals

The lobsters used for these assays were reared as described in the general methods section (Chapter 2). Details of sample collection and storage were also given previously. In most instances it was necessary to pool several lobsters in order to obtain sufficient tissue for the digestive enzyme analyses. Numbers of lobsters pooled per sample

included: 12 gold eggs, 5-6 blue eggs, 5 prelarvae, 5-6 stage I larvae, 5 stage II larvae, 4 stage III larvae, 2 stage IV larvae, and 1 stage V postlarva.

Homogenates of digestive tract tissues were made in 0.1 M citrate phosphate buffer, pH 5.5, as described in Chapter 4 and used as the enzyme source for the assays.

Digestive enzyme activities during lobster development

Sibling lobsters from a winter-hatch were used in these experiments. Replicate samples of gold and blue eggs, prelarvae, the four larval stages, and the fifth (postlarval) stage were taken. Stage I larvae were sampled at molt stages A and C; both unfed and fed stage C larvae were sampled. All the older lobsters were well fed and in intermolt.

Enzyme activities of summer-hatch and winter-hatch larvae

Digestive enzyme activities were determined for intermolt lobster larvae stages I through IV which hatched at two different seasons. Although the two groups of lobsters underwent embryonic development under different thermal regimes, ambient fluctuating water temperature (summer-hatch larvae) versus constant temperature (winter-hatch larvae), the larvae were reared at the same temperature 20-22°C.

The enzyme activities of stage IV larvae which had been collected from the wild (summer-hatch) were also determined and compared to the activities measured for the laboratory reared stage IV larvae.

Enzyme activities in relation to first feeding of lobster larvae

First feeding experiments were conducted using both summer- and winter-hatch stage I lobster larve. For each experiment, larvae from a

single hatch were put in individual containers within two to four hours after the hatching event. Summer-hatch larvae were presented with food either 12 or 18 h after hatching; winter-hatch larvae were fed 24 or 48 h after hatching.

The larvae were fed adult Artemia sp. which had been cooked in a microwave oven for 5 min to denature any digestive enzymes. Each larva was individually given food to insure that ingestion took place; one whole Artemia was gently placed against the periopods with forceps until the lobster grasped its prey. Replicate time course samples were taken at 30 min and 1, 2, 4, 6, 8, and 12 h after the larvae were fed. Six larvae were pooled per sample.

Enzyme activities in relation to molt stage

Wild stage IV lobster larvae were collected from Buzzards Bay, MA during the summer of 1985. Molt stage was determined by the method described in Sasaki (1984). Digestive enzyme activities were determined for larvae in stages C, D_0, D_1', D_1'', and D_1'''. Digestive tract tissues from two larvae were pooled per sample.

Protease analysis

Azocoll was washed for 2 h in 0.1 M citrate phosphate buffer, pH 5.5, at a concentration of 10 mg/ml, filtered through Whatman No. 1 filter paper, and resuspended in buffer just before use to give a final substrate concentration of 10 mg/ml.

The washed, resuspended substrate was stirred continuously to keep the particles in suspension while 2.8 ml aliquots were transferred to screw-top test tubes. The tubes were pre-incubated at 37°C for 10 min in a shaking water bath; agitation was 280 cycles per min. Then, 200 ul

of lobster digestive tract supernate were added to start the reaction. The reaction tubes were mixed on a vortex mixer, capped, and returned to the water bath. Incubation time was 60 min. The reaction was stopped by putting the tubes in an icewater bath. The tubes were centrifiged for 2 min and the supernates transferred to clean test tubes with disposable Pasteur pipets. Absorbance was read at 520 nm and protease activity reported as A_{520} per mg protein per h. Duplicate assays were performed for each sample and the mean value used in the calculations.

The reaction blank was prepared by incubating Azocoll with buffer. At 37°C the A_{520} of the blank was about 0.03-0.04 after 60 min at the substrate concentration 10 mg/ml. A supernate blank was prepared by adding 200 ul of supernate to 2.8 ml buffer. The A_{520} of supernate produced from homogenizing six larvae per ml was about 0.01 to 0.02; this blank value was subtracted from the sample absorbance before protease activity was calculated.

Lipase analysis

A 2% solution of gum arabic was made with 0.2 M citrate phosphate buffer, pH 5.5; 0.56 M NaCl and 0.04 M $MgCl_2$ were added to the buffer before the acacia was mixed. Purified triolein (Sigma) was used as the substrate. It was stored under nitrogen at -20°C but warmed to room temperature before being weighed. The triolein was added to the acacia solution at a concentration of 1.0 umole/ml and emulsified for 5 min using a Virtis mixer. Fresh substrate emulsion was prepared each day immediately before use.

Disposable screw-top test tubes were used for reaction vessels. 0.5 ml of the substrate emulsion and 0.1 ml of BSA (3.5 mg/ml) were pipetted

into each tube. Final concentrations of the reaction components were 1% acacia, 0.5 umole/ml triolein, 0.1 M phosphate buffer, 0.28 M NaCl, 0.02 M $MgCl_2$, and 0.35 mg/ml BSA. The tubes were pre-incubated for 10 min at 37°C in a shaking water bath at 190 cycles per second. The reaction was initiated by the addition of 400 ul of lobster digestive tract supernate. The reaction proceeded for 60 min and was stopped by the addition of 3.75 ml C:M (1:2). The extraction of lipids, storage of samples, and subsequent Iatroscan analysis were performed exactly as described previously in Chapter 4.

Amylase analysis

A 30 mg/ml solution of soluble potato starch was made each day with 0.2 M phosphate buffer, pH 6.5, which had 0.10 M NaCl added. The solution was boiled for 5 min, cooled to room temperature, and brought back up to volume with distilled water. One ml of starch solution and 0.8 ml of distilled water were pipetted into 16 x 100 mm screw-top test tubes and pre-incubated in a water bath at 37°C for 10 min. Then 200 ul of lobster digestive tract supernate were added to the mixture to start the reaction. Final concentration of the reactants were 15 mg/ml starch and 0.05 M NaCl. The tubes were mixed on a vortex mixer and returned to the water bath. Duplicate assays were run for each sample. The reaction was stopped after 30 min by adding 2.0 ml of the alkaline dinitrosalicylic acid reagent. All the tubes were capped, heated for 10 min in boiling water, and cooled to room temperature in an ice water bath. Absorbance was read at 540 nm and converted to micro-equivalents of maltose using a standard curve. The blank was 1.0 ml of starch solution and 1.0 ml of distilled water. A supernate blank for each

sample was made by adding 200 ul of supernate to 1.0 ml starch and 0.8 ml distilled water after 2.0 ml of DNSA was added; the absorbance of the blank was subtracted from the sample absorbance before amylase activity was calculated.

Protein measurement

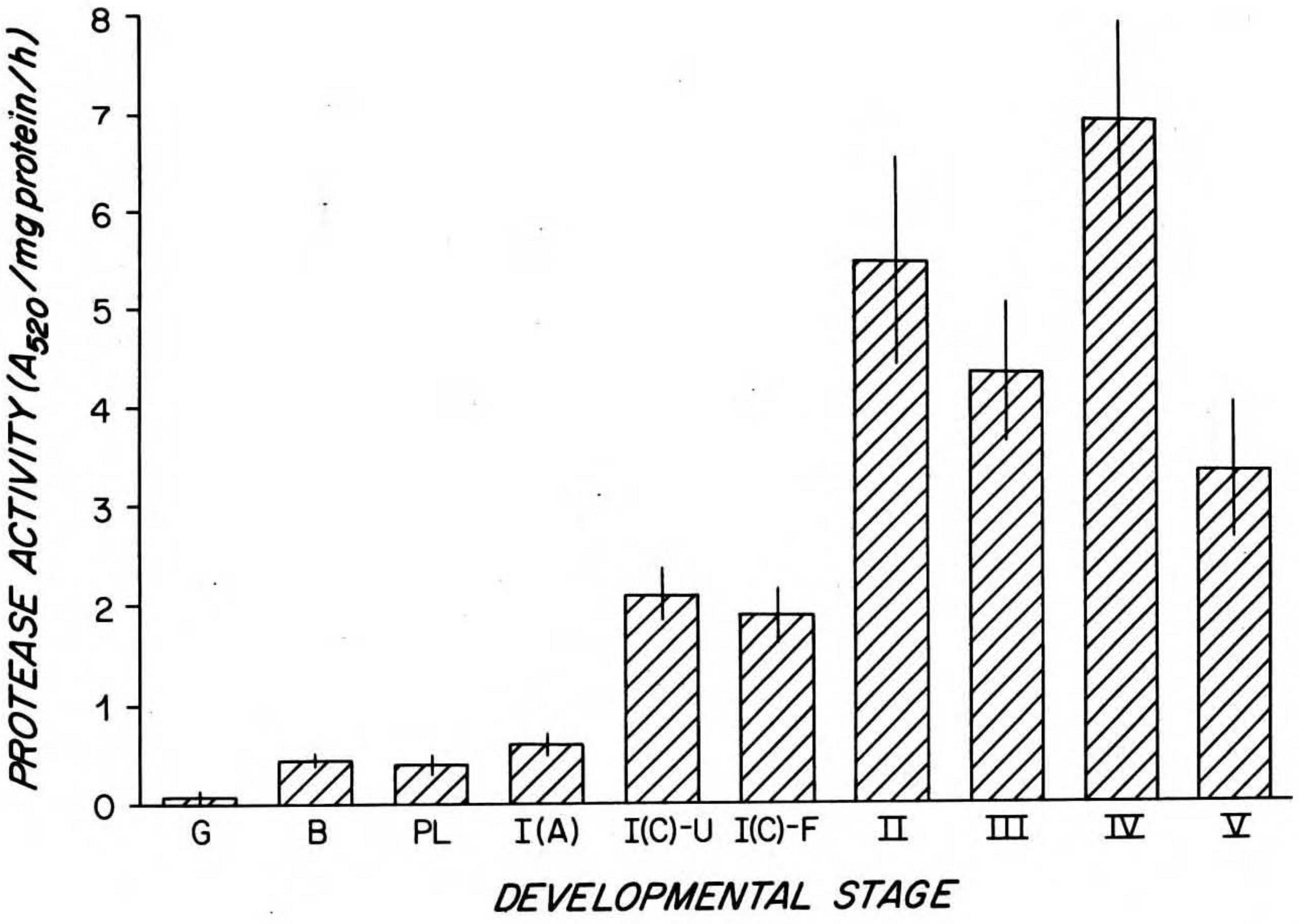
Protein was determined using the Hartree (1972) modification of the Lowry et al. (1951) assay. For this analysis, 100 ul of lobster digestive tract supernate were diluted with 0.9 ml of distilled water. Bovine serum albumin (BSA) was used as the standard. All samples were run in duplicate and the mean value used for calculations.

RESULTS

The levels of protease activity throughout early lobster development are presented in Fig. 5.1. Activity was very low but detectable among the gold embryos which had been sampled approximately three days before their siblings started to hatch. Protease activity increased by the blue egg stage but did not change from this level during the prelarval stage. These two latter developmental stages represent arbitrarily chosen sampling points during the continuum of the hatching process, the blue egg at the beginning and the prelarvae comprising the middle to late portion. Thus, it is not unexpected that no change in enzyme activities occurred during this short period of time.

Protease activity increased slightly, although not significantly, among the newly hatched stage I larvae. By molt stage C, protease activity of the stage I larvae increased by a factor of three, regardless of whether the larvae were fed or fasted. The older larval stages (II through IV) exhibited increased protease activity, which was two to three

Figure 5.1. Protease activity in relation to developmental stage of the American lobster H. americanus ($\bar{X} \pm SD$). G, gold eggs sampled three days prior to hatching, n=3; B, blue eggs, n=3; PL, prelarvae, n=3; I(A), newly hatched stage I larvae in molt stage A, n=4; I(C)-U, unfed intermolt stage I larvae, n=5; I(C)-F, fed intermolt stage I larvae, n=5; II, intermolt stage II larvae, n=5; III, intermolt stage III larvae, n=5; IV, intermolt stage IV larvae, n=5; V, intermolt stage V postlarvae, n=3.



PROTEASE ACTIVITY (A_{520} / mg protein/h)
8
7
6
5
4
3
2
1
0
G
B
PL
I(A)
I(C)-U
I(C)-F
II
III
IV
V
DEVELOPMENTAL STAGE

times the level seen among stage I larvae, but which did not increase consistently or significantly. Protease activity declined among the stage V postlarvae.

The levels of lipase activity measured during the course of lobster development are given in Fig. 5.2. Activity was low, and not significantly different, among gold and blue eggs, prelarvae, and newly hatched stage I larvae. Lipase activity increased significantly by intermolt among stage I larvae, regardless of feeding regime. Although the lipase activity was greatest in stage II and V lobsters, the activity of this enzyme was not significantly different among any of the larval stages or the postlarvae.

Amylase activity levels are presented in Fig. 5.3. As was the case with the other enzymes measured, activity was lowest in the gold eggs. Activity doubled by the blue egg stage and remained at that level during the prelarval stage. There was a slight but not significant increase in amylase activity among stage I larvae but there was no effect on activity due to molt stage or feeding regime. The amylase activity approximately doubled by stage II. There was no significant difference in activity during any of the older stages (II through V).

In general, the temperature regime experienced during embryonic development of the lobster appeared to have no consistent marked effect on the digestive enzyme activities subsequently measured among the larvae. There were, however, some specific exceptions to this finding.

Protease activity (Fig. 5.4) was not significantly different for summer-hatch or winter-hatch larvae. Summer-hatch stage II larvae had somewhat reduced activity compared to winter-hatch stage II larvae but it

Figure 5.2. Lipase activity in relation to developmental stage of the American lobster H. americanus ($\bar{X} \pm SD$). G, gold eggs sampled three days prior to hatching, n=3; B, blue eggs, n=3; PL, prelarvae, n=3; I(A), newly hatched stage I larvae in molt stage A, n=4; I(C)-U, unfed intermolt stage I larvae, n=5; I(C)-F, fed intermolt stage I larvae, n=5; II, intermolt stage II larvae, n=4; III, intermolt stage III larvae, n=5; IV, intermolt stage IV larvae, n=5; V, intermolt stage V postlarvae, n=3.

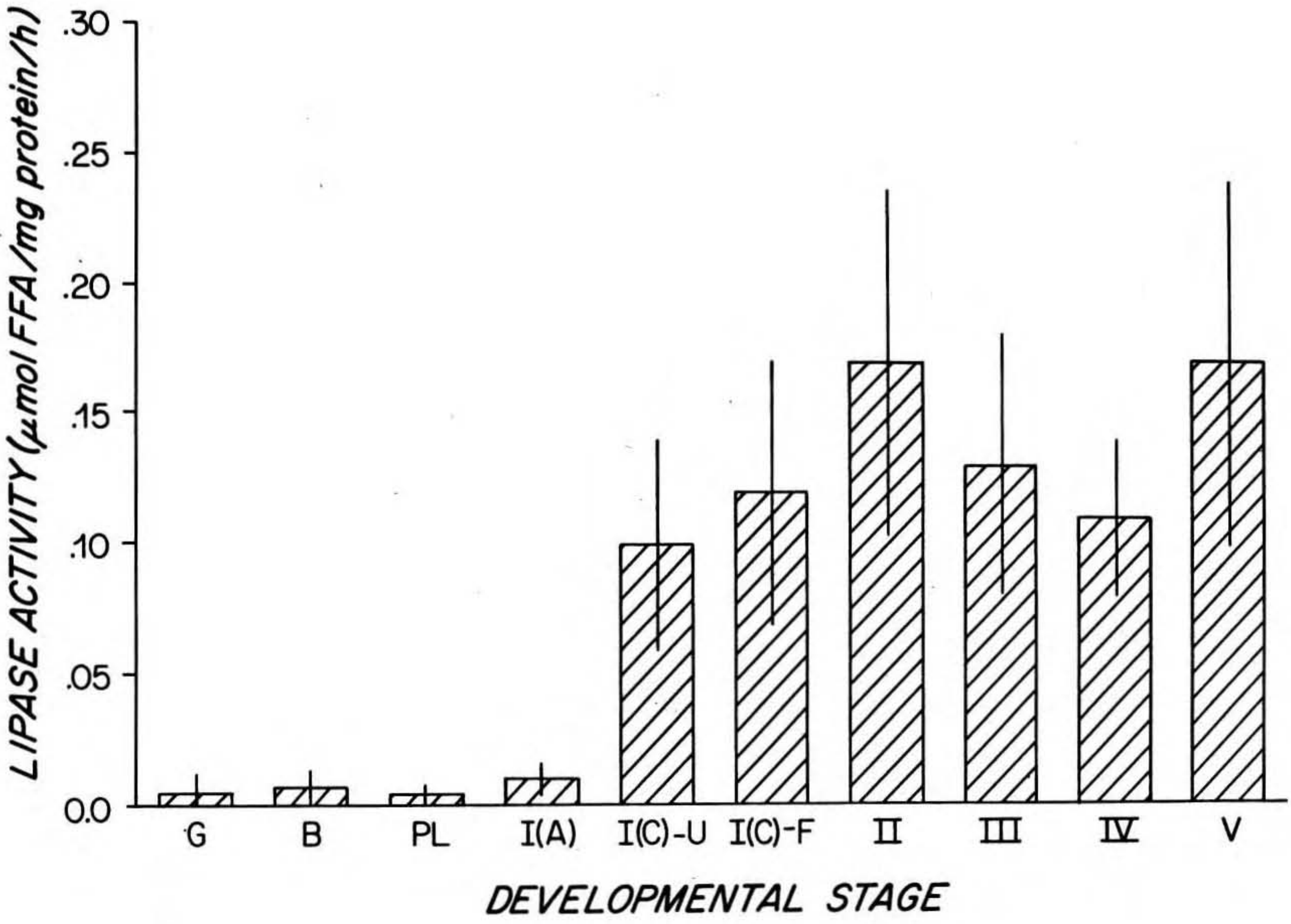
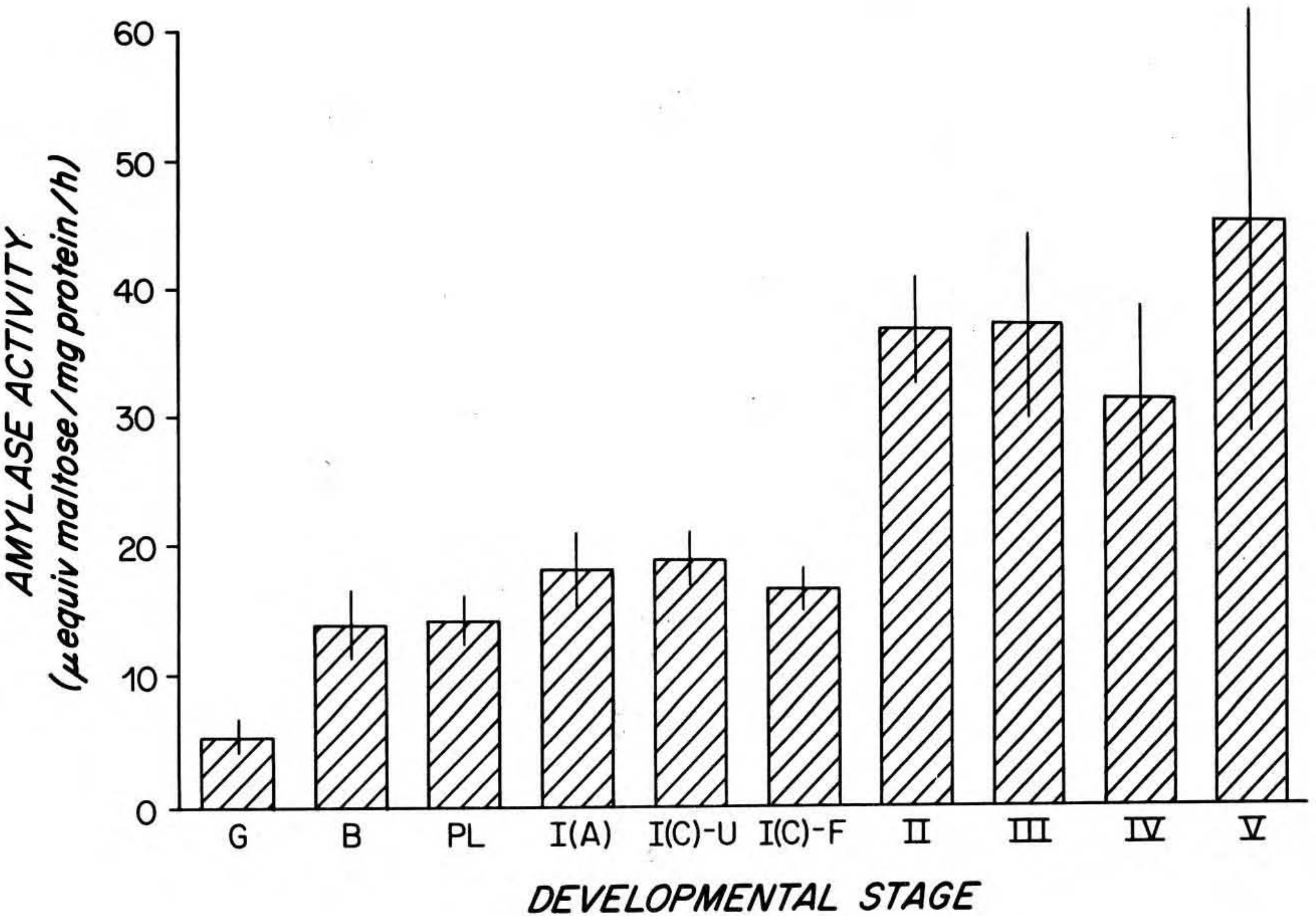
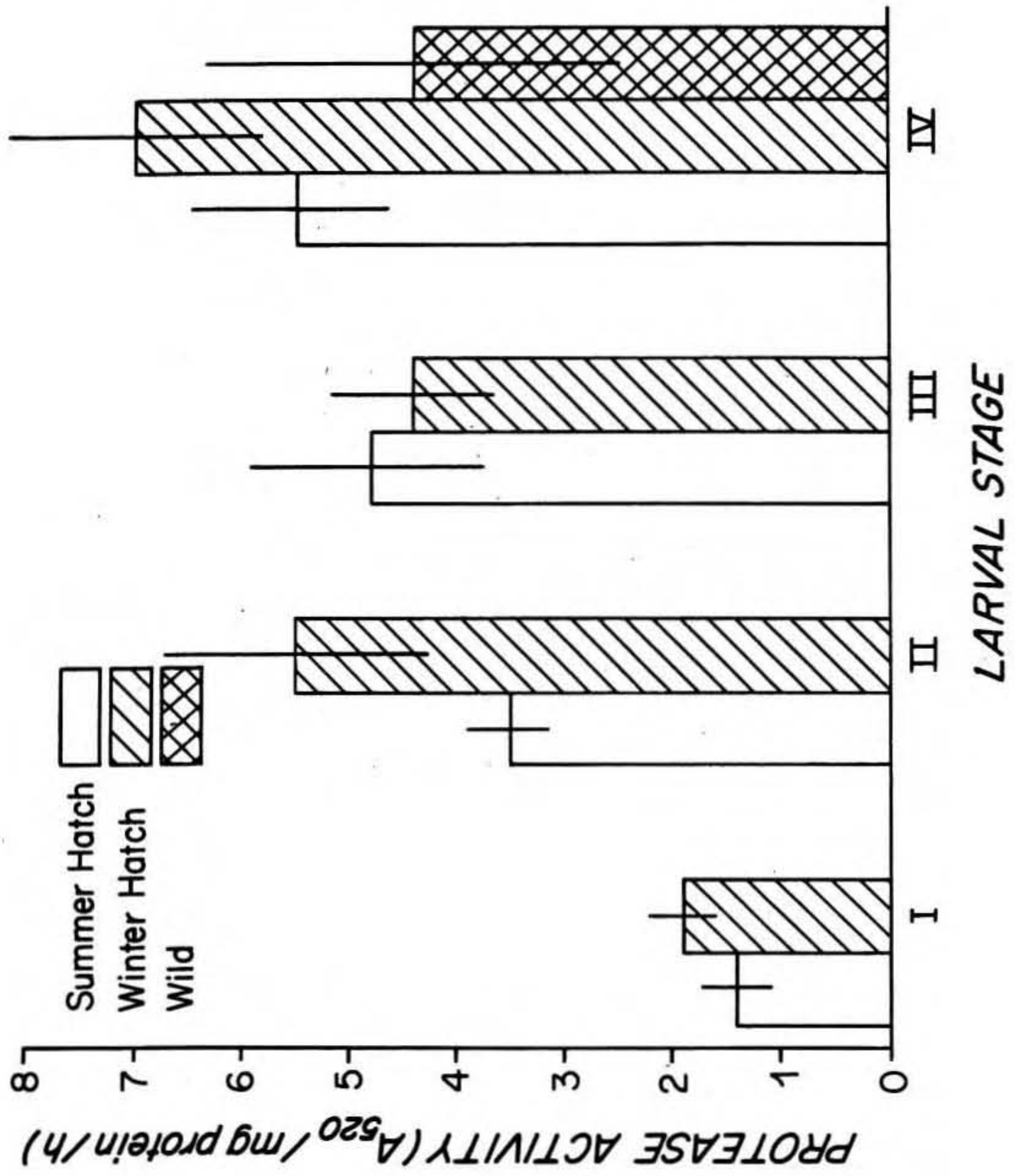

LIPASE ACTIVITY (µmol FFA/mg protein/h)
.30
.25
.20
.15
.10
.05
0.0
G
B
PL
I(A)
I(C)-U
I(C)-F
II
III
IV
V
DEVELOPMENTAL STAGE

Figure 5.3. Amylase activity in relation to developmental stage of the American lobster H. americanus ($\bar{X} \pm SD$). G, gold eggs sampled three days prior to hatching, n=3; B, blue eggs, n=3; PL, prelarvae, n=3; I(A), newly hatched stage I larvae in molt stage A, n=4; I(C)-U, unfed intermolt stage I larvae, n=5; I(C)-F, fed intermolt stage I larvae, n=5; II, intermolt stage II larvae, n=5; III, intermolt stage III larvae, n=5; IV, intermolt stage IV larvae, n=5; V, intermolt stage V postlarvae, n=3.



60
50
40
30
20
10
0
AMYLASE ACTIVITY
(μequiv maltose/mg protein/h)
G
B
PL
I(A)
I(C)-U
I(C)-F
II
III
IV
V
DEVELOPMENTAL STAGE

Figure 5.4. Protease activity during larval development of the American lobster _H_. _americanus_ in relation to season of hatch ($\bar{X} \pm SD$, n=5 for summer hatch, n=6 for winter hatch, and n=11 for wild).


Summer Hatch
Winter Hatch
Wild
PROTEASE ACTIVITY (A_{520} / mg protein / h)
0
1
2
3
4
5
6
7
8
I
II
III
IV
LARVAL STAGE

was not significant. Although the protease activity among wild stage IV larvae was less than that measured among winter-hatch stage IV larvae, the difference was not significant.

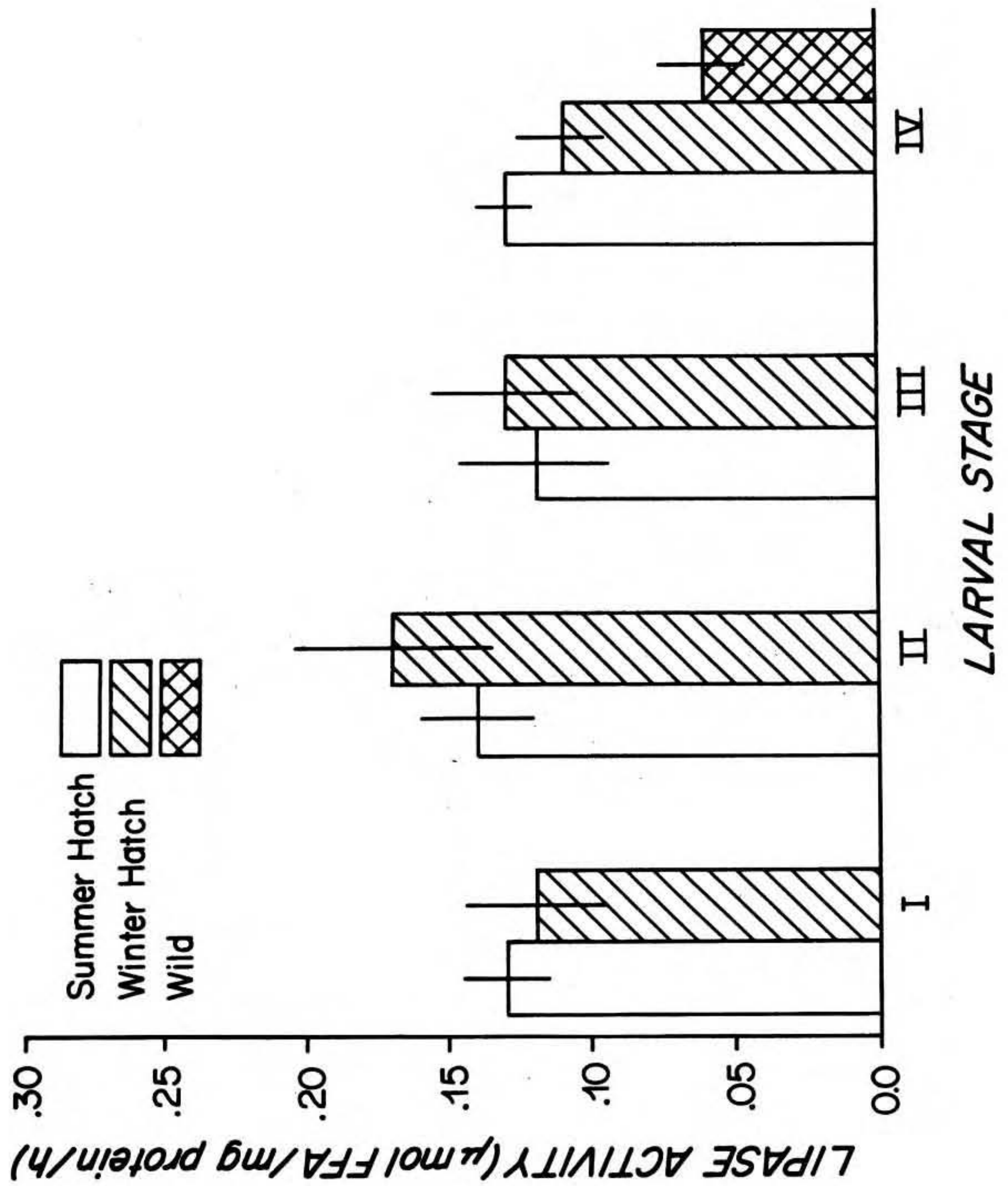
The activity of digestive lipase among laboratory reared lobster larvae was not significantly affected by season of hatch (Fig. 5.5). Stage II larvae had the highest lipase activity regardless of season but it was not significantly greater than activity measured among the other larval stages. Thus, the lipase activity of laboratory reared lobsters was relatively constant throughout larval development. The activity measured for wild stage IV larvae, however, was about half the value determined for their laboratory reared counterparts.

Amylase activity measured for larval stages I, II, and IV was not affected by the temperature experienced during embryonic development (Fig. 5.6). Summer-hatch stage III larvae, however, showed activity almost twice that determined for the winter hatch stage III larvae. Amylase activity of wild stage IV larvae was not significantly different from that of the laboratory reared stage IV larvae.

Feeding did not appear to affect the production of digestive enzymes in stage I lobster larvae. The results of first feeding experiments conducted with both summer-hatch and winter-hatch larvae were identical. Therefore, only the results obtained with summer-hatch stage I larvae will be reported here.

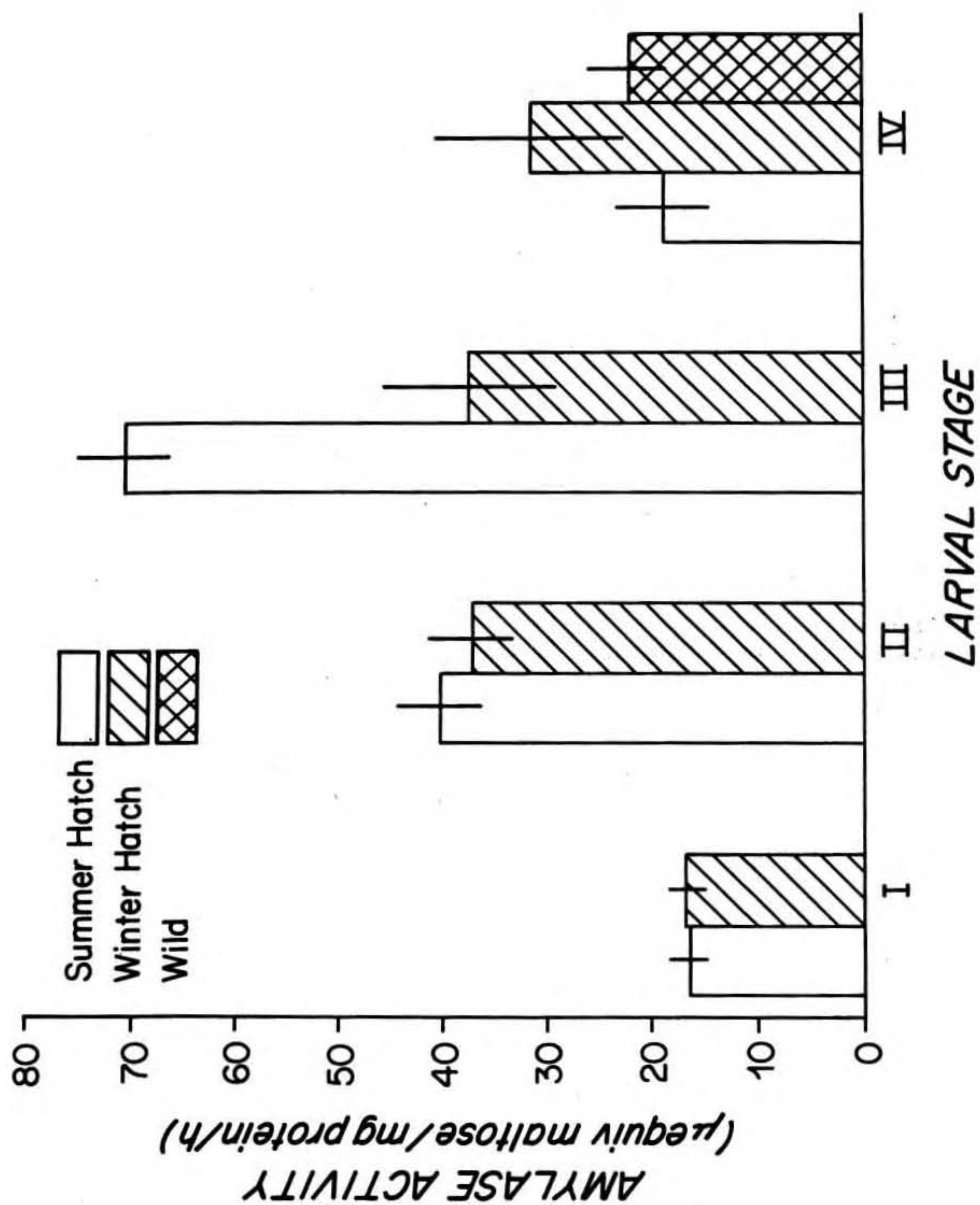
Protease activity increased slightly over time, from age 6 h to 30 h (Fig. 5.7), but the increase was seen among both fed and fasted larvae. It can be seen that when the larvae were first given food, the protease activity declined slightly for 30 to 60 min before rising back to the

Figure 5.5. Lipase activity during larval development of the American lobster H. americanus in relation to season of hatch ($\bar{X} \pm SD$, n=5 for summer hatch, n=6 for winter hatch, and n=11 for wild).



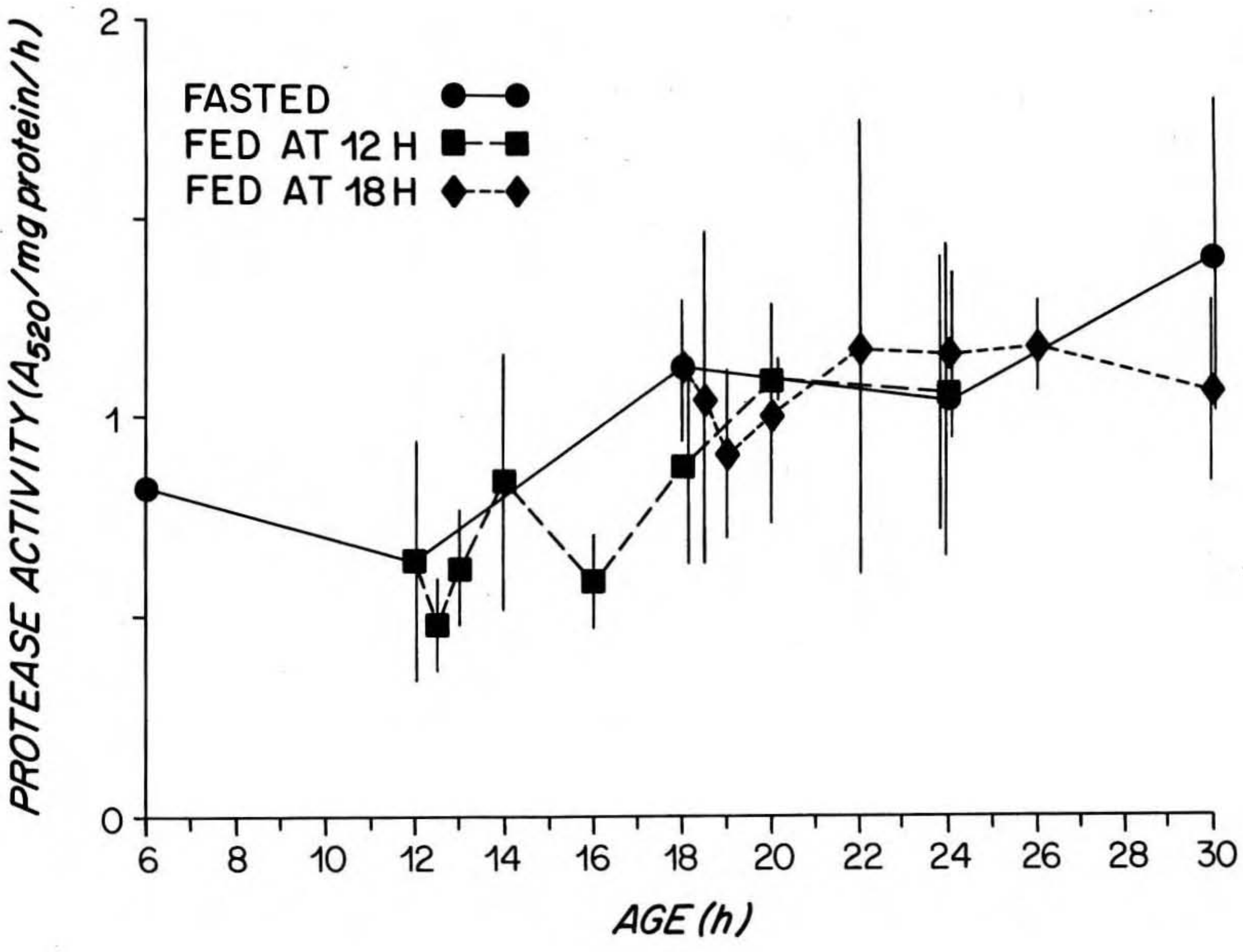
Summer Hatch
Winter Hatch
Wild
LIPASE ACTIVITY (μ mol FFA/mg protein/h)
.30
.25
.20
.15
.10
.05
0.0
I
II
III
IV
LARVAL STAGE

Figure 5.6. Amylase activity during larval development of the American lobster H. americanus in relation to season of hatch ($\bar{X} \pm SD$, n=5 for summer hatch, n=6 for winter hatch, and n=11 for wild).



Summer Hatch
Winter Hatch
Wild
AMYLASE ACTIVITY
(μequiv maltose/mg protein/h)
80
70
60
50
40
30
20
10
0
I
II
III
IV
LARVAL STAGE

Figure 5.7. Time course of protease activity among fed and fasted stage I larvae of the American lobster *H. americanus* ($\bar{X} \pm SD$, n=5).



FASTED
FED AT 12 H
FED AT 18 H
PROTEASE ACTIVITY (A_{520}/mg protein/h)
0
1
2
AGE (h)
6
8
10
12
14
16
18
20
22
24
26
28
30

baseline level. This decrease in activity although consistently seen was not significant.

Lipase activity was unaffected by feeding among stage I larvae (Fig. 5.8). The enzyme levels remained constant throughout the duration of the test. The slight increase in lipase activity seen among larvae fed 18 h after hatching was not significantly different from that measured among larvae fed 12 h after hatching.

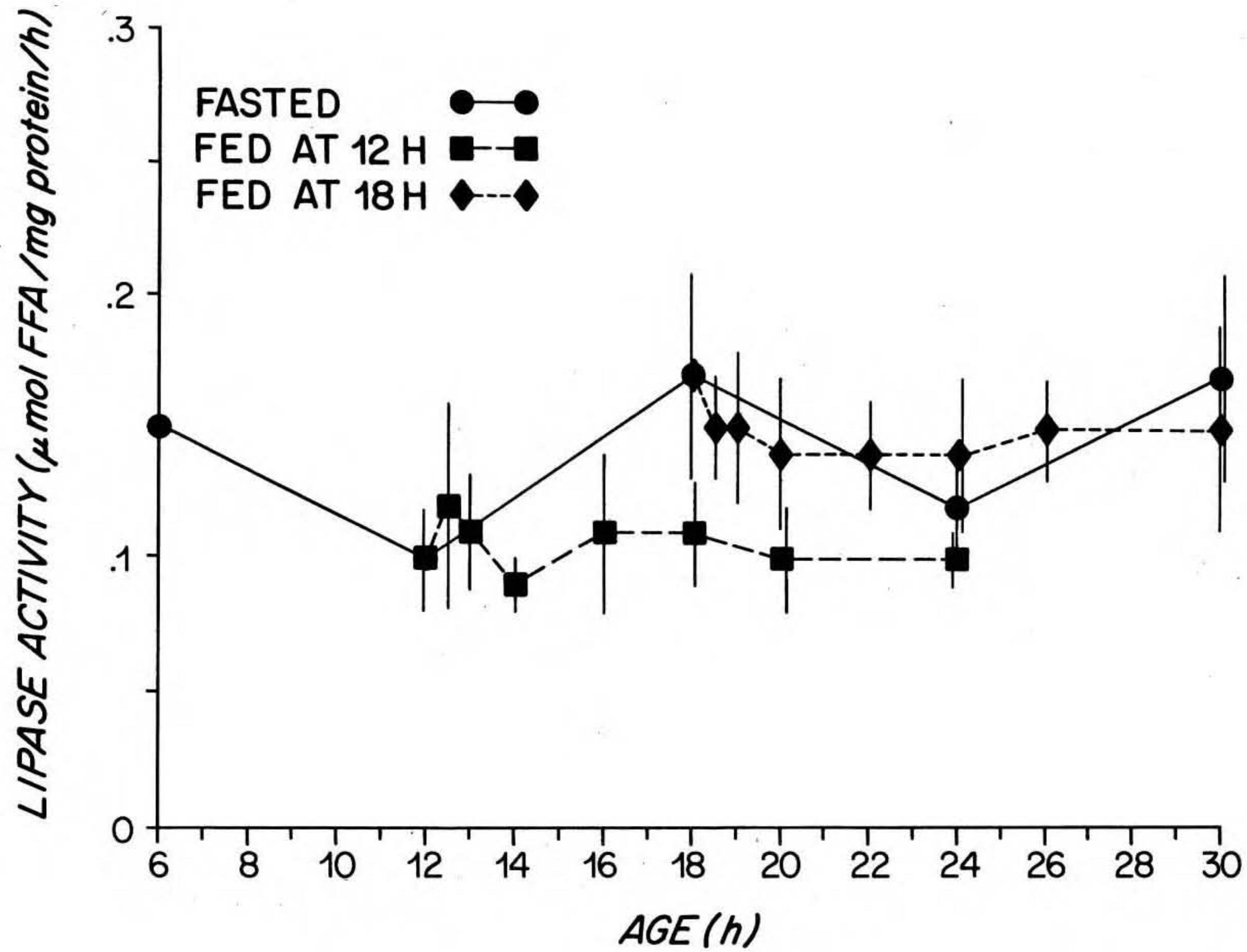
Amylase activity increased slightly during the course of the experiment (Fig. 5.9) but the increase was irrespective of feeding regime. As was the case with protease activity, there was a trend toward slightly reduced enzyme activity for 1 to 2 h immediately after feeding but the decrease was not significant.

Minor variations in the digestive enzyme activities of wild caught stage IV larvae were detected in relation to molt stage (Fig. 5.10). There appeared to be a slight reduction in amylase activity during D_1'' and D_1''' but these changes in activity were not significantly different from amylase activity measured earlier in the molt cycle. Protease activity showed more variability. There was a significant increase in activity at stage D_0 which was not significantly different from activity measured in D_1' or D_1''. Protease activity was low at D_1''. Lipase activity declined during the D_1 stages but it did not seem to be significantly different from activity earlier in the molt cycle.

DISCUSSION

The increase of digestive protease, lipase, and amylase during early lobster development coincided with differentiation of the midgut gland,

Figure 5.8. Time course of lipase activity among fed and fasted stage I larvae of the American lobster H. americanus (X+SD, n=5).



LIPASE ACTIVITY (μ mol FFA/mg protein/h)
.3
.2
.1
0
FASTED
FED AT 12 H
FED AT 18 H
6
8
10
12
14
16
18
20
22
24
26
28
30
AGE (h)

Figure 5.9. Time course of amylase activity among fed and fasted stage I larvae of the American lobster H. americanus ($\bar{X} \pm SD$, n=5).

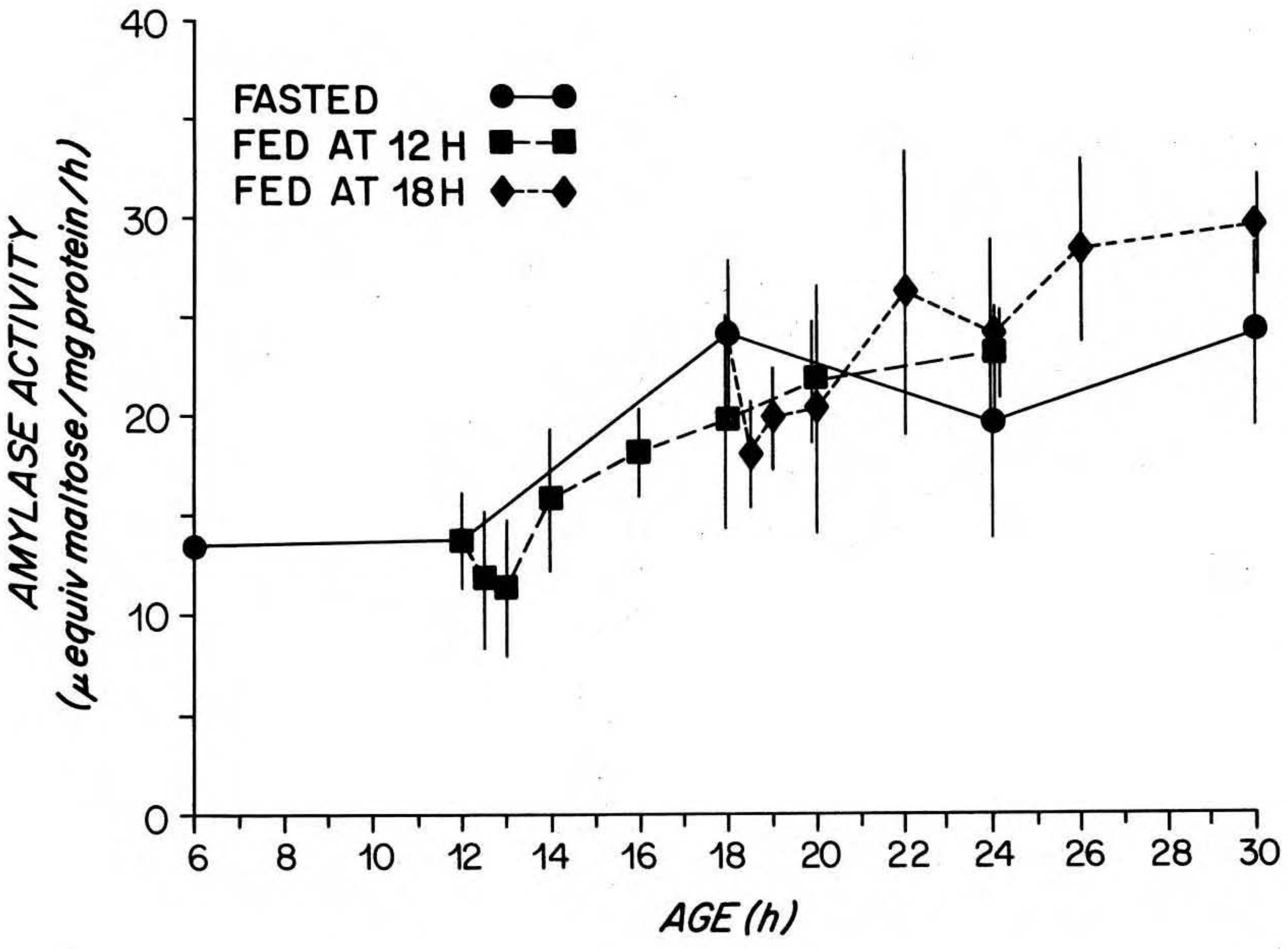
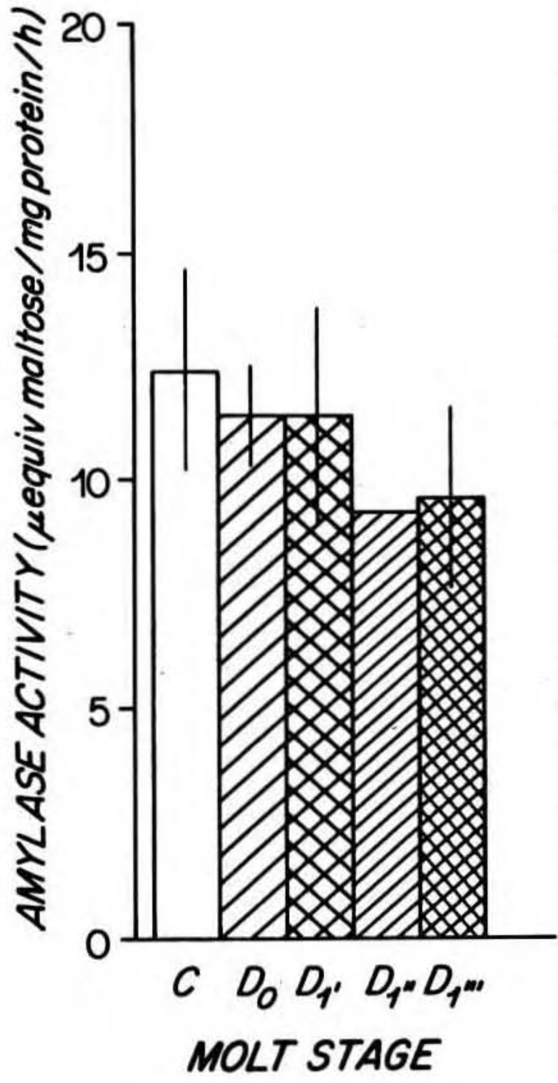
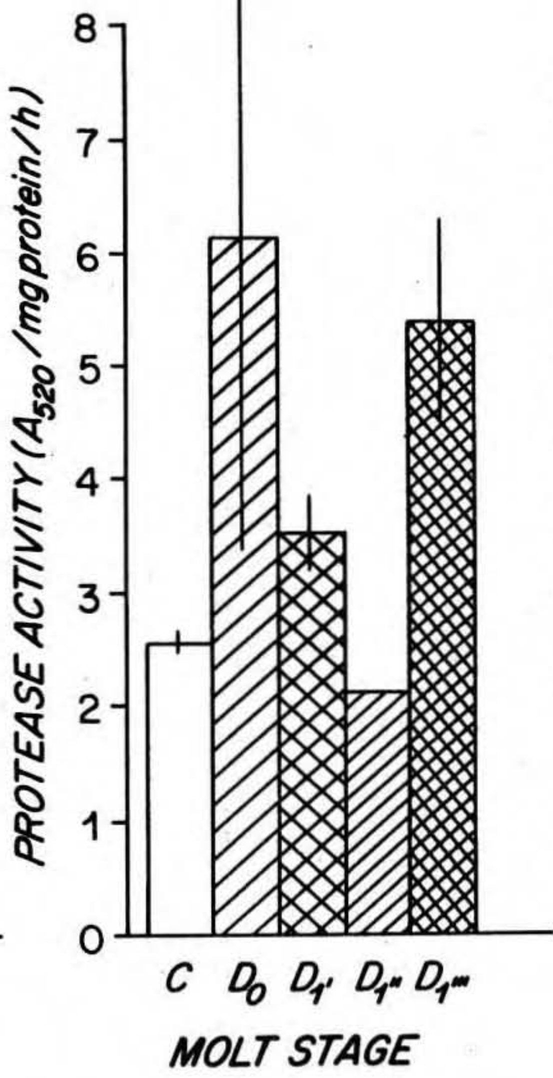

FASTED
FED AT 12 H
FED AT 18 H
AMYLASE ACTIVITY
(μ equiv maltose / mg protein / h)
0
10
20
30
40
6
8
10
12
14
16
18
20
22
24
26
28
30
AGE (h)

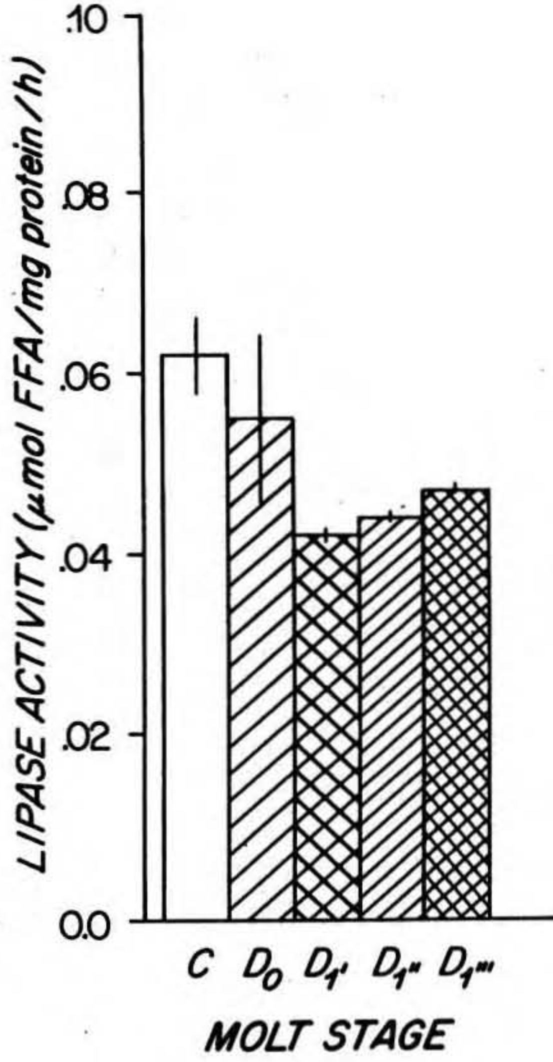
Figure 5.10. Digestive enzyme activities during the molt cycle of wild caught stage IV larval lobster H. americanus ($\bar{X} \pm SD$, n=3 for C, n=3 for D_0, n=2 for $D_1\cdot$, n=1 for $D_1\cdot\cdot$, and n=3 for $D_1\cdot\cdot\cdot$).



AMYLASE ACTIVITY (μequiv maltose/mg protein/h)
0
5
10
15
20
C D_0 D_1' D_1'' D_1'''
MOLT STAGE



PROTEASE ACTIVITY (A_{520}/mg protein/h)
0
1
2
3
4
5
6
7
8
C D_0 D_1' D_1'' D_1'''
MOLT STAGE



LIPASE ACTIVITY (μmol FFA/mg protein/h)
0.0
.02
.04
.06
.08
.10
C D_0 D_1' D_1'' D_1'''
MOLT STAGE

much like the situation reported for starfish pluteus larvae (Vacquier, 1971; Vacquier et al., 1971). Levels of activity of the three enzymes were very low in gold lobster embryos, which contained primarily R-cells in the midgut gland. The activity of protease and amylase doubled by the blue egg and prelarval stages at which point F- and B-cells had developed. Lipase activity, however, did not show a similar initial increase until the stage I larva had emerged.

By attainment of intermolt in the stage I larva, both protease and lipase activity had significantly increased over levels measured in the hatching stage although amylase levels were unchanged. This change was paralleled by an increase in the number of B-cells in the midgut gland. Ingestion of food was not responsible for these changes since B-cell number increased in both fed and fasted larvae and there was no difference in the activity of any of the enzymes between fed or fasted larvae. Hence, differentiation of the midgut gland and initiation of digestive enzyme synthesis are regulated by some other factor(s).

Protease and amylase activity levels doubled by the second larval stage and remained essentially unchanged throughout the remainder of larval development. Lipase activity did not change significantly during this period. It may be that ingestion of food was responsible for the increase in protease and amylase activity but these data cannot be used to support such an hypothesis, only to suggest it.

The possiblity exists that a series of different hydrolases, proteases for example, were expressed at different times during development and that the activities measured herein are the result of different proteolytic enzymes active in the different stages. However,

throughout this study it has been assumed that the same enzyme has been responsible for a specific hydrolytic activity in each of the developmental stages sampled.

There did not appear to be any consistent pattern of digestive enzyme activity in relation to the molting cycle of stage IV larval lobsters. Activity of amylase and lipase showed no change over the intermolt and premolt period. The changes observed in protease activity were ambiguous and may have been related to the small sample size and to large individual variation. Adult lobster do not feed for some time during the immediate premolt condition. Larval lobsters, on the other hand, appear to feed up to the time of molting; food was seen in the guts of the lobsters assayed. If ingestion of food is a cue for continued digestive enzyme output by the midgut gland, then the results of the this portion of the present research are not surprising.

Logan and Epifanio (1978) constructed an energy budget for larval and postlarval H. americanus which were reared on Artemia. Efficiency of digestion [(consumption - egestion) ÷ consumption] was reported to be the same (82%) for all the stages. However, since their discussion did not agree with the numbers reported in the tables, I recalculated the efficiency of digestion for each stage and obtained different numbers: efficiency ranged from 86-88% for stages I through III but was 68% for stage IV and 79% for stage V. Since the present study has shown that digestive enzyme activities do not change at these latter stages, the results for efficiency of digestion may be related to changes in feeding behavior or to physiological factors associated with the morphological change at stage IV and/or to the habitat change at stage V.

Aside from the fact that an increase in digestive enzyme synthesis and secretion accompanied differentiation of the midgut gland in the prelarva and the stage I larva, there is little unqualified evidence that enzyme activities are affected by later developmental changes in the lobster larvae and postlarvae. Lipase and amylase activities were not significantly different between the premetamorphic (II and III) and postmetamorphic (IV and V) stages. Nor were there consistent significant differences in the activities of these enzymes after the young lobsters left the plankton (stage IV) and settled to the benthos (stage V). Only protease activity appeared to show any variations which could be attributed to developmental causes. There was a significant increase in protease activity between stages III and IV and a significant decrease in activity between stages IV and V which may be related to the alteration in body form and to the new benthic habitat, respectively. Since the other digestive enzymes did not show a similar pattern, these results remain somewhat ambiguous and may be due to the small sample size. Unlike the change in preferred metabolic substrate (from lipid to protein) which occurred among postmetamorphic lobsters (stage IV), there appear to be no physiological changes in digestive function associated with development to the postlarval stage (stage V).

When lobster embryos are exposed to constant elevated temperature (16 to 20°C), the duration of embryonic development is shortened. Sasaki (1984) has reported that larger than normal yolk reserves are retained by the stage I larvae and that as a consequence first-feeding may be delayed. The present study has demonstrated that the extra yolk reserves had no effect on the increase in activities of digestive enzymes.

Although feeding may or may not have been delayed among winter-hatch larvae, the timing of first feeding had no effect on the activity of the digestive enzymes measured. There were no signficant differences in enzyme activities of intermolt stage I larvae in relation to season of hatch nor were there consistent differences noted for the older larval stages from the two groups. This is further evidence that access to exogenous nutrients in the first larval stage is not a factor regulating the initiation of digestive enzyme synthesis.

The results of the first feeding studies indicate that there is a steady increase in activity of amylase and protease over time which occurs irrespective of the previous feeding history of the stage I larva. Although the results presented here were obtained from summer-hatch animals, the pattern was the same for winter-hatch larvae. The increase could be due either to activation of enzymes already present or to an increase in the rate of enzyme synthesis.

The results of the first feeding studies consistently indicated a small drop in mean enzyme activity for 30 to 60 min after ingestion. This may have been due to a dilution effect by the ingested food but it seems unlikely since the amount of protein ingested per larva was miniscule compared to the total protein in the crude homogenate of digestive tract tissues. However, if that was the cause, enzyme secretion may have occurred to bring the levels back to baseline within a very short period.

Kelly (1985) reviewed the mechanism of protein secretion in eukaryotes. Secretion is constitutive if proteins are secreted as fast as they are synthesized. Alteration of the rate of protein secretion by

constitutive secretory cells is effected by altering the rate of protein synthesis. In regulated secretion, newly synthesized proteins are stored at high concentration in secretory vesicles until the cell receives the appropriate stimulus. They are specialized to release, for a brief period, large amounts of protein at a rate much higher than the synthetic rate. In vertebrates the formation of zymogen granules is a mechanism for concentration and storage of inactive digestive enzymes. The material inside the mature secretory vesicle is condensed and appears as a dense core in electron micrographs. Secretory cells are also polarized or non-polarized. Polarized cells secrete their products through a specialized portion of the plasma membrane while non-polarized cells can secrete anywhere on their cell surface. Mammalian exocrine cells, for example, are regulated and polarized.

It is possible that digestive enzyme synthesis and secretion by crustaceans may be constitutive. These processes were not induced by ingestion in stage I lobster larvae but rather occurred whether or not the larvae fed. Further, zymogens have not unequivocally been proven to be formed in crustaceans and there is a fair body of literature which suggests that they are not formed. B-cells, therefore, may be polarized, constitutive secretory cells. Factors which cause an alteration in crustacean enzyme activities would do so by changing the rate of enzyme synthesis rather than by changing the secretion rate of previously synthesized but inactive enzymes.

The activities of the three enzymes measured in the present study do not change in parallel. That is, protease activity appears to double at hatching, double in the stage I larva, and double again in stage II but

it does not change signficantly until stage V when activity decreases. Amylase activity doubles between the gold and blue egg stages and again in stage II with activity then remaining essentially unchanged through stage V. Lipase activity, on the other hand, only increases significantly between the newly hatched and intermolt stage I larva. It has been generally assumed that each F-cell synthesizes the whole complement of digestive enzymes present in a particular crustacean species and that each B-cell secretes the entire vacuolar contents. If the preceeding statement is true, then the rate of production of each enzyme must in some manner be regulated individually.

The exocytosis hypothesis (Palade, 1975) was formulated to explain the mechanism underlying the secretion of digestive enzymes by the mammalian pancreas. According to this hypothesis, distinct digestive enzymes are synthesized, processed, segregated from the cytosol, and stored in zymogen granules, in parallel with each other, within 45 to 60 minutes. Nonparallel or enzyme-specific secretion has also been documented, however, and Rothman (1975) offered an alternative model, the equilibrium hypothesis, in which secreted enzymes cross through the membranes of a cell in a regulated fashion rather than being totally segregated from the cytosol and stored within the membranes. Adelson and Miller (1985) have recently shown that pancreatic enzymes are secreted in groups, in a linked fashion from prepackaged organelles, as expected from exocytosis theory. However, they also showed clear evidence of nonparallel secretion which meant that the pancreas secreted groups of enzymes from several distinct sources within the gland. These different pools packaged the enzymes in different ratios. Their results indicated

that regulation of the composition of the digestive enzyme mixture was independent of the regulation of the rate of digestive enzyme secretion. Similar work has not been attempted in crustaceans but the exocytosis hypothesis may have general application.

The lipase activities measured among early developmental stages of the American lobster in the present study were an order of magnitude lower compared to the lipase activity measured for the adult lobster by Brockerhoff et al. (1970). The enzyme preparation in the latter study was slightly purified whereas the former used a crude homogenate. Since a series of control tests had been conducted to optimize the assay conditions for lipase activity measurement in the present study, it may be assumed either that Brockerhoff et al. (1970) used much cleaner enzyme preparations or else that weight-specific lipase activity increased sometime later in development. A number of other workers have attempted to measure lipase activity in marine invertebrates, including crustaceans, but either found weak activity or none at all. These studies include Ceccaldi and Trellu (1975) for embryonic shrimp _Palaemon serratus_, Trellu and Ceccaldi (1976) for adult _P. serratus_, Lee et al., (1980) for prawn _Macrobrachium rosenbergii_, Hoeger and Mommsen (1984) for ctenophores _Pleurobrachia pileus_ and _Beroe gracilis_, and Kuhle and Kleinow (1985) for rotifer _Brachionus plicatilis_. The absence of lipase activity reported in those studies, however, could simply mean that the conditions were not optimal for measurement or that the methods used were not very sensitive. It is also possible that these organisms did in fact lack true lipase. Kruger (1929, cited by Yonge, 1937) wrote about the esterase nature of the digestive enzymes of many invertebrates although

he did find true lipase in the lobsters H. vulgaris (=gammarus) and Palinurus vulgaris. Berner and Hammond (1970) assayed for digestive lipase specificity using a number of marine invertebrate species representing several phyla. They demonstrated true lipase activity in only about half of the invertebrates, including crayfish Cambarus virlis, whereas esterase activity was demonstrated for all species. The invertebrate lipase(s) tended to lack the 1,3-positional specificity for fatty acids which is a characteristic of vertebrate pancreatic lipase. In this, marine crustaceans lipase resembled the lipase found in marine fishes which also lacks positional specificity (cf. Cowey and Sargent, 1979 for review). Brockerhoff et al. (1967, 1970) reported that adult American lobsters possess lipase and the results of the present study indicate the presence of lipase among the early developmental stages of the lobster.

CHAPTER 6: SUMMARY AND CONCLUSIONS

The development of digestive processes by planktotrophic crustacean larvae is an important aspect of their feeding ecology and nutritional physiology and as such has an impact on their survival in the plankton. This study on the development of digestion in early life history stages of the American lobster Homarus americanus has added to our understanding of crustacean digestive physiology. In addition, it has provided information on some of the characteristics of crustacean digestive enzymes.

Digestive capabilities are marginally functional in the newly hatched stage I lobster larva. There is histological evidence that the enzyme synthesizing F-cells occur in the midgut gland late in embryonic development and that they differentiate to produce the enzyme secreting B-cells soon before the lobster hatches. The biochemical data corroborate this finding as activities of digestive enzymes were low before the B-cells were formed but increased during the time that B-cells were differentiating.

Digestive capabilities increased after hatching in the stage I lobster larva regardless of whether or not it ingested food. Again, the histological and the biochemical data provide supportive evidence. Numbers of B-cells increased and digestive enzyme activities increased in both fed and fasted intermolt first stage larvae. This observation provides indirect evidence that Hopkin and Nott (1980) and Al-Mohanna et al. (1985) are probably incorrect in their view that B-cells are excretory or absorptive, respectively,

rather than secretory in nature. Their basic argument has been that F-cells synthesize and then secrete digestive enzymes and that B-cells develop from F-cells but function either to store metabolic wastes generated during the process of digestion or to absorb digested nutrients. In the present study, the results obtained with fasted stage I larvae indicate that B-cells are produced before digestion has proceeded and not afterwards. Therefore, neither of the proposed alternative B-cell functions (Hopkin and Nott, 1980; Al-Mohanna et al., 1985) are possible.

It is advantageous for planktotrophic larvae to have functional digestive capabilities when they start to feed. Successful degradation of nutrients after successful capture of food translates into efficient utilization of substrates for energy and growth. This capacity for digestion of the first meal did not occur in the larval whitefish Coregonus lavaretus pallasi (Bogdanova, 1980). However, marine fish larvae are usually lecithotrophic when they hatch, have undifferentiated guts, and normally do not (or can not) feed for several days. Lobster larvae are partially lecithotrophic since they hatch with some yolk remaining in the gut but they hatch at a more advanced stage of development than do fish larvae. The digestive tract is well-developed and lobster larvae are capable of feeding within hours of hatching. Thus, it seems appropriate that they also possess functional digestive ability.

The activities of amylase and protease increased among the older stages (II to V) compared to the stage I larva whereas lipase activity remained constant throughout early development. Since lobsters do not

normally molt to the second stage without feeding, it is not possible to evaluate the effect of feeding on this increase in enzyme activity.

Metamorphosis from the stage III larva to the stage IV larva can be considered a transition period in several contexts. Capuzzo and Lancaster (1979a,b) have shown that lipid was important in energy production during development of lobster larvae but that it tended to be replaced in importance by protein just prior to metamorphosis. Sasaki (1984) has demonstrated marked changes in biochemical composition of the postmetamorphic larva compared to earlier stages, including an increase in triacylglycerol content. It has been shown in the present study that dietary lipid is not stored in R-cell vacuoles of premetamorphic larvae but that a small number of lipid vacuoles occur in the stage IV larva. However, these changes in energetic patterns, biochemical composition, and energy reserve storage were not accompanied by changes in digestive enzyme activities.

It had been considered that the ontogenic migration from the plankton to the benthos which occurs between stages IV and V might be correlated with changes in digestive enzyme activities. However, that was not the case in the present study. There was an indication of decreased protease activity but lipase and amylase activity did not change significantly. In the field the change in habitat would have been accompanied by a different suite of prey items in the diet. In the laboratory the food type was kept constant over the experimental period with the intention of detecting potential changes in activity due to the developmental stage rather than to the diet. Although such

a change in enzyme activities did not occur in the present study, it cannot be concluded that such a change might not occur in nature.

A changing pattern of lipid storage in the R-cells of the developing lobster was shown. Late in embryonic development the R-cells of the midgut gland contained one to a few large lipid vacuoles, presumably resulting from the storage of yolk lipid. The lipid droplets persisted to some extent in the first larval stage but were apparently metabolized during development, perhaps through the molt to the stage II larva. The R-cells of the intermolt stage II larva did not contain large lipid vacuoles nor did those of the stage III. There was an increase in storage of lipids derived from the diet in R-cells of the stage IV larva but these cells did not truly resemble those of the adult until stage VI when large numbers of lipid vacuoles were present. The histological data were corroborated by the biochemical data of Sasaki (1984) which indicated an increase in lipid content, primarily due to triacylglycerol which is a storage lipid, among stage IV lobster larvae.

The present research is one of the few studies on digestion in marine invertebrates in which crustacean lipase activity was detected. This was probably due to the method of detection, which is very sensitive. Lipase activity was low and did not change much over the course of larval or postlarval development. It seems incongruous that such low activity is sufficient to digest the amount of lipid which must occur in the diet. It is possible that the conditions of the assay were such that the enzyme was partially inactivated or else that lobster larvae simply have very low lipase activity and that

esterase is responsible for lipid digestion. Esterase activity was not measured during these experiments but it has been shown to occur in other crustaceans. Brockerhoff et al. (1967, 1970), however, detected true lipase activity in the adult American lobster.

The present studies have further shown that the digestive enzymes of developing lobsters showed optimal activity at a pH close to that of the gastric fluid (pH 5.5). Both lobster protease and lipase were optimally active at that pH while lobster amylase was most active at pH 6.5. However, the activity of amylase was still quite high at pH 5.5 and the slight reduction in activity at the lower pH is probably not important in vivo.

APPENDIX A: THE pH OF JUVENILE LOBSTER GASTRIC FLUID

Gastric fluid in most crustaceans ranges from pH 5 to 7 and is generally higher in well-fed than fasted animals (van Weel, 1970). Brockerhoff et al. (1970) reported the gastric juice of adult American lobsters to be approximately pH 5. Their measurements, however, were made on lobsters starved for two weeks. Hoyle (1973) found the gastric juice of both fed and fasted adult Homarus to be between pH 4.6 and 4.8.

The pH of juvenile lobster gastric fluid was measured to corroborate these findings. Laboratory-reared juveniles (2 to 3 cm carapace length) were used. They were routinely fed three times a week. One group (n=5) was sampled 2 h after feeding and the other group (n=3) was sampled 24 h after feeding. The lobsters were blotted dry and a glass capillary tube gently inserted through the mouth and into the stomach. The gastric juice so collected (about 10 ul) was spotted onto each of four pieces of pH paper which indicated different ranges of pH: 2.9 to 5.2, 4.9 to 6.9, 5.5 to 8.0, and 6.0 to 9.5.

The pH of gastric juice in all cases was greater than 5.1 and less than 6.0. Lobsters fed recently had a slightly higher gastric fluid pH (median = pH 5.7) than those starved for 24 h (about pH 5.3). The sample size, however, is too small to make much of this difference.

Brockerhoff et al. (1970) reported that pH 5 was not in the pH range for optimum activity of lobster digestive enzymes determined by their study. In contrast, the present study has shown that larval

lobster protease, lipase, and amylase are all active at or near pH 5.5 (cf. Chapter 4). Optimum activity for amylase was at pH 6.5, for protease at pH 5.3 and 6.4, and for lipase at pH 5.5.

APPENDIX B:
THE EFFECT OF FROZEN STORAGE ON THE ACTIVITY OF LARVAL LOBSTER PROTEASE

To determine the effect of freezing and thawing on the activity of lobster digestive enzymes, protease assays were performed on fresh samples and the results compared to assays done with replicate frozen samples. Sibling larvae from a winter-hatch were reared communally to the intermolt stage as described in Chapter 2. When the larvae were 36 h old, nine samples (composed of two larvae pooled per sample) were collected and assayed for protease activity according to the protocol described in Chapter 4. The rest of the larvae were frozen in liquid nitrogen and stored at -85°C. Protease activity of the frozen samples was determined at biweekly intervals for eight weeks ($n=9$; two larvae pooled per sample).

The results of the assays are presented in Table B.1. According to the Kruskal-Wallis non-parametric ANOVA (Zar, 1984), there was no significant difference in protease activity over time ($\alpha=0.05$).

Table B.1.

Protease activity in stage I larval lobsters.

Storage time (days)	Protease activity (A_{520}/mg protein/h)
0	1.30±0.19[a]
14	1.55±0.37[a]
28	1.32±0.17[a]
42	1.34±0.21[a]
56	1.26±0.17[a]

[a]No significant difference in protease activity at α=0.05 (Kruskal-Wallis ANOVA).

Values expressed are mean ± standard deviation (n=9).

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BIOGRAPHICAL NOTE

Patricia Marie Biesiot was born 23 March 1950 in St. Cloud, Minnesota and enjoyed a nomadic early life, her family moving thirteen times among several midwestern states in fewer than thirteen years. She was valedictorian of her class at Bowling Green Senior High School, Bowling Green, Ohio in June, 1968.

The author attended Bowling Green State University, Ohio as an Alumni Merit Scholar. She served as an undergraduate assistant in the Department of Biological Sciences, working one year for a microbiologist and another year for an entomologist. It was during her senior year that Ms. Biesiot was first introduced to the ocean world. Although BGSU is an inland school, it offers a marine biology program, albeit one which relies on artifical seawater. The highlight of the Marine Biology course is an annual field collecting trip to the Gulf Coast Research Laboratory, Ocean Springs, MS. With that occasion in 1972, the die was cast and Ms. Biesiot became committed to study of the marine environment.

The author graduated Magna Cum Laude from BGSU in 1972 and began study toward a Master's degree in aquatic biology. As part of this program, she took several summer courses at GCRL. In July 1973 Ms. Biesiot accepted a position in the Eco-physiology Section of GCRL and became involved in a long-term study of the ionic and osmotic regulation of juvenile brown shrimp _Penaeus aztecus_. During this period she also completed her thesis on the developing salinity tolerance of postlarval _P. aztecus_ during the time of their inshore migration. She graduated from BGSU with an MS in 1975.

By 1978 Ms. Biesiot was co-author of several papers dealing with basic problems in shrimp aquaculture and with the biochemical composition of some commercially important Gulf of Mexico fisheries species. She advanced to the position of Laboratory Supervisor and served as Co-Principal Investigator on a DOE project which was conducted to determine the toxicity of various components of an OTEC power plant. During her tenure at GCRL, the author became interested in the feeding ecology and nutritional physiology of decapod crustaceans.

Ms. Biesiot was admitted to the WHOI/MIT Joint Program in Biological Oceanography in June 1980. She was awarded the Tai-Ping Aquaculture Fellowship in 1984. During the fall of 1984, the author was co-instructor for the Introduction to Oceanography course at Bridgewater State College, MA.

The author has accepted a postdoctoral position in the laboratory of Dr. Dorothy M. Skinner at the Oak Ridge National Laboratory, TN.