A PRELIMINARY INVESTIGATION OF THE MYOGENS OF ADULT COMMERCIAL SHRIMP

A Thesis

Presented to

the Faculty of the Department of Biophysical Sciences

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Alden Van Vechten Rodgers

August 1968

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ABSTRACT

A preliminary study was made of myogens from juvenile and adult shrimp, Penaeus setiferus Linnaeus ("white"), P. Aztecus Ives ("brown") and P. duoranum Burkenroad ("pink") to determine whether the electrophoretic behavior of these proteins can be used to identify these species. When examined by acrylamide gel and cellulose-acetate electrophoresis, it was found that these species can be identified by the electrophoretic behavior of their myogens. The sizes of white and brown shrimp myogens are similar as shown by their chromatographic behavior on Sephadex G-75. On the basis of calibration of the gel with known proteins, the three peaks observed were estimated to consist of molecules of molecular weight greater than 69,000, 30,000, and less than 3,000 daltons. The peak with low molecular weight components contained nucleotides, nucleosides, and ninhydrin positive molecules. Two nucleotides were identified tentatively as 5' IMP (inosine-5'-monophosphate) and 5' CMP (cytidine-5'-monophosphate) and one nucleoside as inosine. One of the ninhydrin positive molecules has been identified tentatively as proline or hydroxyproline.

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I.

INTRODUCTION

INTRODUCTION

One of our most important resources of the sea is food. If we are to realize its potential to help alleviate the world's food problem, then fundamental biological questions about marine organisms which show the greatest potential as food resources must be answered. The species to be studied and the biological questions to be asked, therefore, are easily determined; however, the answers are difficult to obtain in the marine environment when well known approaches are taken.

A basic objective of marine biologists interested in commercial species of marine organisms is to readily and accurately identify breeding populations. A new and powerful approach to this task is based upon the fact that these population units constitute "gene pools" which now can be measured precisely in biochemical terms, in which proteins are considered to be the direct and simple products of genes. The inherent advantages of biochemical and immunochemical means for identification and "tagging" of marine organisms in migration and breeding studies have been initiated in the North Atlantic and Pacific regions, but not, to our knowledge, in the Gulf of Mexico (Cushing, 1964; Leone, 1964). Although the Gulf species are known to migrate annually, especially into estuarine nursery areas, essentially nothing is known about their breeding populations (Circular 230, 1964).

The difficulty of identifying the larval and postlarval stages of Gulf shrimp creates a real barrier to understanding the biology of

this important source of food. It is a major problem for personnel of the Bureau of Commercial Fisheries at Galveston, Texas. Therefore, upon their recommendation, species of commercial shrimp were chosen as one of the first Gulf species to be studied.

This thesis is an initial study to show that biochemical markers can be used to identify species of commercial shrimp for which the larvae are difficult to identify on a morphological basis. Furthermore, it is apparent that little is known about the comparative protein chemistry of crustaceans. If relevant markers can be identified then it would be possible not only to identify species in a sample, but also to study the breeding and migration patterns of shrimp.

This approach has been possible by the recent success of the Biological Laboratory at the Bureau of Commercial Fisheries in raising shrimp in artificial cultures. It is now possible to obtain larval and postlarval shrimp of known identity. However, the quantities available during the time of this research were not sufficient for initial feasibility studies of this approach, therefore, juvenile and adult shrimp have been used to show that biochemical markers do occur and to carry out initial fractionation of the myogens.

The species studied in this work are Penaeus aztecus Ives, the brown shrimp; P. setiferus (Linnaeus), the white shrimp; and P. duoranum, the pink shrimp. In this thesis, the species will be referred to by their common names. The adults are easily identified by their morphology,

(Burkenroad, cf. Leone and Pryor, 1952). The brown and pink shrimp have a groove alongside the middorsal carina while the white shrimp does not. The brown and pink shrimp have a pink spot on the side, white shrimp does not; the brown shrimp has a smaller spot than the pink shrimp.

Commercial shrimp spawn in the Gulf of Mexico and are believed to undergo several stages of early development: nauplii (five stages), protozoea (three stages), and mysis (three stages) (Moffett, 1967). After the last mysis stage, the shrimp are called postlarva, which migrate into the coastal estuaries (nursery areas). The postlarvae grow in the nursery areas until they reach the juvenile and adult stages, at which time they begin to migrate to the open Gulf to complete their life cycle.

Other reports relevant to a comparative biochemical study of crustacea include serological studies of body fluid proteins and starch gel electrophoretic analysis of body fluids or the entire organism's proteins. Studies of the comparative serology of crustaceans are: Clark and Burnet (1942), Tyler (1945), Tyler and Metz (1945), Tyler and Scheer (1945), Leone (1949 and 1954), Leone and Pyror (1952), and Deñuce and Kühn (1963). These studies show that body fluid proteins of different crustaceans show different antigenic specifities, in which the differences correspond to phylogenetic relationships. Leone and Pryor have done the only previous work in comparing commercial shrimp in our knowledge. They found the serological differences in body fluid proteins between brown, white, and pink shrimp were significant, but they are closely related to each other.

Woods, et al. (1958), Cowden and Coleman (1962), and Manwell and Baker (1962) compared body fluid proteins by starch gel electrophoresis. The advantages of working with body fluid proteins is that they are already in solution and hemocyanin is usually the major protein in the body fluid; thus, these workers are working with homologous proteins between species. The disadvantages of using body fluid proteins is that the amount of body fluid obtained per individual is small and this method is not easily adapted to larval forms or individual variation studies. Therefore, if myogens can serve as genetic markers, their ease of extraction would make it the method of choice.

The basis of this research has been the excellent article of Manwell, et al., (1967), in which an extract of copepods (Calanus finmachicus or C. helgolandicus) was analyzed by starch gel electrophoresis. The advantages of this approach are that a wide variety of proteins can be studied and enzymes can be identified.

We felt, therefore, that the best approach to try first was an electrophoretic analysis of the myogens of each species of shrimp. Starch gel electrophoresis is difficult to use, so acrylamide gel electrophoresis, with its excellent resolution, was used. Sephadex chromatography was also used to estimate the molecular weight range of these proteins.

II.

EXPERIMENTAL PROCEDURES

EXPERIMENTAL PROCEDURES

The shrimp were identified and collected from Galveston Bay, Texas with the help of personnel and facilities of the Biological Laboratory, Bureau of Commercial Fisheries, Galveston, Texas. The times of collection corresponded to their seasonal occurrence in the Bay. If the shrimp were not used immediately for chemical analysis, they were kept in a freezer until needed. The shrimp were peeled before the abdominal muscles were used for extraction.

A number of extraction procedures were used to find the one which gives the largest number of bands in acrylamide gel electrophoresis. Initial solutions examined were those used by Manwell, et al., (1967) for their extracts of copepods (crustaceans). That is, 0.01 M K_3PO_4 with toluene; 0.01 M K_3PO_4 saturated with n-butanol; 0.01 M EDTA; 0.05 M K_2HPO_4; distilled water; 10% NaCl; and 1% NaCl were tried. The proteins were extracted by homogenizing or grinding the shrimp in a mortar and pestle with alumina and the appropriate solutions. Although good results were obtained with distilled water or 10% NaCl, it was found that better extraction media were ones which have been used to extract mammalian myogens (Czok and Büchner, 1960). The solution finally chosen and used for this study was a potassium phosphate buffer with added KCl (9 mM K_2HPO_4, 6 mM KH_2PO_4, and 0.05 M KCl; pH 6.85-7.00; ionic strength 0.08). The alumina used was neutral, 80-200 mesh and had a Brockman Activity 1; it was obtained from Fisher Scientific Company. Weighted samples in the range of ten to twenty grams of

peeled, shrimp abdomens were ground in two parts of precooled buffer and one-fourth part alumina; the quantities of alumina and buffer were determined by the weight of the muscles. This mixture was homogenized with a Waring blender for 30 seconds at room temperature. Subsequent grinding in a mortar would increase the protein concentration of the extract, but did not appear to release any new relevant myogens.

The mixture was centrifuged in three steps: 8,000 g in an International centrifuge for 30 minutes at 4° C; 54, 500 g in a Beckman Model L preparative ultracentrifuge for 30 minutes at 4° C; and 99, 500 g in a Beckman Model L preparative ultracentrifuge for 60 minutes at 4° C. The final supernants are stable for up to two weeks when kept at 4° C.

Determination of the Concentration of the Proteins

The protein concentration of the extracts was estimated by the Biuret test (Campbell, et al., 1963), standardized with bovine plasma albumin obtained from the Armour Pharmaceutical Company, lot #V68802. The absorbancy of 5 mg/ml was 0.40 at 520 mμ using a Beckman DB-G spectrophotometer. On this basis, the average extract contained about 5 mg/ml of protein.

Acrylamide Gel Electrophoresis

Acrylamide Gel Electrophoresis was used as the basic analytical tool to study the electrophoretic behavior of the myogens. The advan-

tages of this method are the mechanical stability of the gels and its resolution. The procedure of Davis (1964) was followed with the modification that the tubes were cleaned with a chromic-sulfuric acid solution and were not coated with Kodak Photo-Flo solution as Davis suggests.

As the shrimp extracts contained about 0.5% protein, 0.3 ml of 0.25% protein solution (750 μg) was added to the gels. The electrophoresis was carried out at room temperature with the current held constant, at 3 to 5 mA per tube (100-200 volts). The average time of the run was 3 to 4 hours.

Cellulose-Acetate Electrophoresis

Cellulose-acetate electrophoresis was used when a rapid and easy method was desired to follow the extraction procedures. However, it lacks the sensitivity and resolving power of acrylamide gel electrophoresis. The Sepraphore III cellulose-acetate strips were soaked in buffer and excess buffer was removed by blotting before the sample application.

Cold barbital buffer (Gelman high resolution buffer, pH 8.6) was used for electrophoresis. The electrophoresis was performed with 1.5 mA per strip (about 250 volts) for 45 minutes. The strips were stained with 500 mg of Ponceau S stain (Gelman Instrument Company) dissolved in 5% trichloroacetic acid for ten minutes and destained with 5% acetic acid.

Sephadex Chromatography

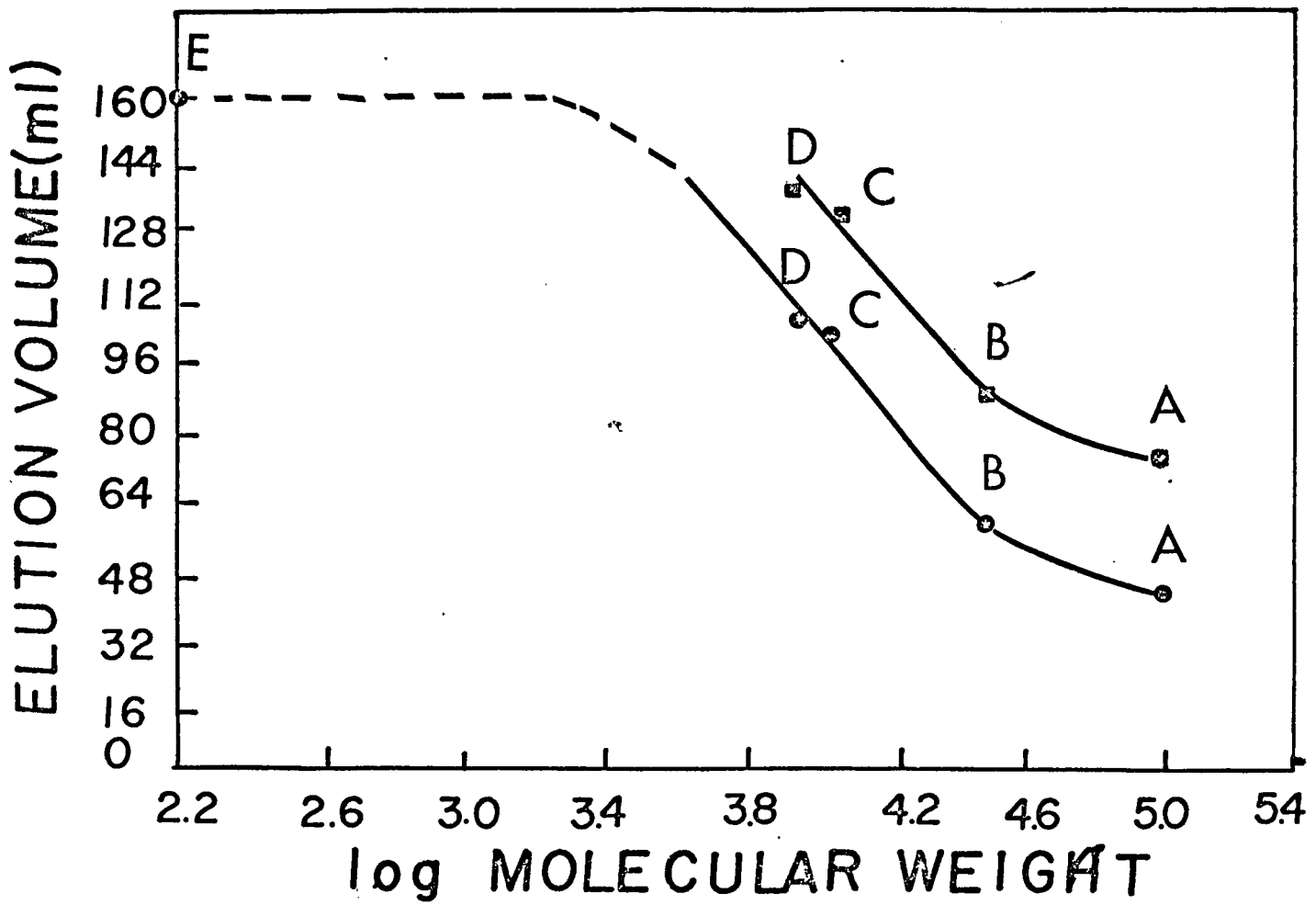
Sephadex G-75 (Pharmacia Fine Chemicals, Inc., lot #JO-5079) was prepared according to the manufacturer's specifications and deaerated with low vacuum. Descending chromatography with 1 x 30 cm or 2.5 x 45 cm columns was used. KCl-potassium phosphate buffer (pH 6.85, ionic strength 0.08) was used as the eluant. The homogeneity of the packing was tested by Blue Dextran. Void volumes of 13-18 ml for the 1 x 30 cm column and 36-48 ml for the 2.5 x 45 cm column were found.

The Sephadex G-75 in a 2.5 x 45 cm column was calibrated for molecular weight by measuring the elution volumes of known molecules according to the method of Andrews (1964), with a KCl-potassium phosphate solution of bovine plasma albumin (0.026 g/10 ml), Armour Pharmaceutical Company, lot #V68802; ribonuclease (0.031 g/10 ml), Worthington Biochemical Company, R637; myoglobin (0.025 g/10 ml, Mann Research Laboratories, lot #L2592; and tryosine (3 mg/10 ml), Calbiochem, lot #36337. Two ml samples were used and three ml fractions were collected. These results are given in Figure 1 with Andrew's results included for comparison.

Sephadex chromatography of the extracts was performed by applying either a two ml sample on the 1 x 30 cm column or a ten ml sample on the 2.5 x 45 cm column. Three ml fractions were collected and analyzed at 260 and 280 mμ. The flow rate of the Sephadex G-75 column was 1 ml/4 min with a pressure head of 42 cm.

FIGURE 1

Calibration of Sephadex G-75 (circles) in a 2.5 x 45 cm column and Andrew's calibration (squares); A-BSA dimer, B-BSA, myoglobin, D-RNase, and E-tryosine.


ELUTION VOLUME(ml)
160
144
128
112
96
80
64
48
32
16
0
E
D
C
B
A
D
C
B
A
2.2
2.6
3.0
3.4
3.8
4.2
4.6
5.0
5.4
log MOLECULAR WEIGHT

Detection and Identification of Small Molecules

Preliminary identification of nucleotides and nucleosides was made by determining their behavior on paper chromatography and comparison of their ultraviolet spectra with known nucleotides and nucleosides (see Table 1).

The Sephadex fractions (see Results), believed to have nucleotides and nucleosides, were concentrated ten times by lyophilization before the chromatography. Whatman #1 chromatographic paper and descending chromatography was used. The first solvent system used was n-propanol: NH_4OH:water (50:30:10) (Berkvist and Deutsch, 1955). A 0.2 ml sample was applied in a 16 cm band. The sample was chromatographed overnight (solvent moved 44 to 45 cm from the origin). After drying, the bands were detected by ultraviolet absorption and their Rf values were determined. The bands were extracted overnight in 3 M NH_4OH. The spectra of the nucleotides were determined with a Beckman DB spectrophotometer.

The above solutions were then concentrated to dryness and redissolved in water immediately before another chromatographic analysis with n-butanol:acetic acid: water (50:25:25) solvent system (Manutt, 1952). Chromatography was carried out overnight (the solvent moved 44 to 45 cm from the origin), and after detecting the spots by ultraviolet absorbation, their Rf values were determined. The chromatograms were further analyzed with ninhydrin (Sigma Chemical Company; 55 parts, 0.3% ninhydrin in ethanol, 45 parts fluorinated hydrocarbons). The

nucleotide used for a control was obtained from Sigma Chemical Company, 5' CMP (cytidine-5'-monophosphate), lot #124B-0260.

The extracts also were examined by paper electrophoresis. The electrophoresis was performed on 22.5 x 13 cm strips of Whatman #1 filter paper in potassium phosphate buffer (pH 6.03 and ionic strength 0.2) for two hours at 200 volts and 11 to 12 mA. After the strip was dry, it was sprayed with ninhydrin.

Ion Exchange Chromatography

Ion exchange chromatography was performed using DEAE cellulose (Cellex-D, Bio-Rad Laboratories) with an exchange capacity of 0.65 meq/g. The cellulose was packed under 14 to 20 pounds pressure in a 0.9 x 30 cm column. A linear ionic strength gradient was generated with 0.05 M Tris-HCl and 2 M NaCl in 0.05 M Tris-HCl (pH 8.6). Two ml samples were added to the column. The eluant was monitored by a Beckman DB-G spectrophotometer using a flow cell at 280 mμ.

III.

RESULTS

RESULTS

Electrophoresis

Electrophoresis is one of the easiest means to compare the myogens of shrimp. Acrylamide gel electrophoretic patterns of white, brown, and pink shrimp myogens are significantly different (Figure 2). The electrophoretic studies of pink shrimp are based on only a few samples and are included for comparison to support the belief that pink and brown represent two distinct species.

Since there are slight variations between the individual gels, the method used to compare the gels was the distance which the bands moved from the large pore/small pore interface. "Average" electrophoretic patterns of the species of shrimp are given in Figure 3.

Typical cellulose-acetate electrophoretic patterns of white and brown shrimp myogens are given in Figure 4. There are five and four bands observed respectively, as compared to eleven and twelve observed on acrylamide gel electrophoresis. The greater separation of the components of white shrimp seen in acrylamide gel probably accounts for the presence of more bands with cellulose acetate.

Sephadex Chromatography

Sephadex chromatography fractionates molecules according to their size. With Sephadex G-75, both white and brown shrimp extracts are separated into three main fractions (Figure 5). The size range of

FIGURE 2

Photographs of acrylamide gel electrophoretic patterns; brown shrimp (left), white shrimp (middle) and pink shrimp (right), the protein concentration in this gel is dilute; thus, some of the minor bands cannot be seen.

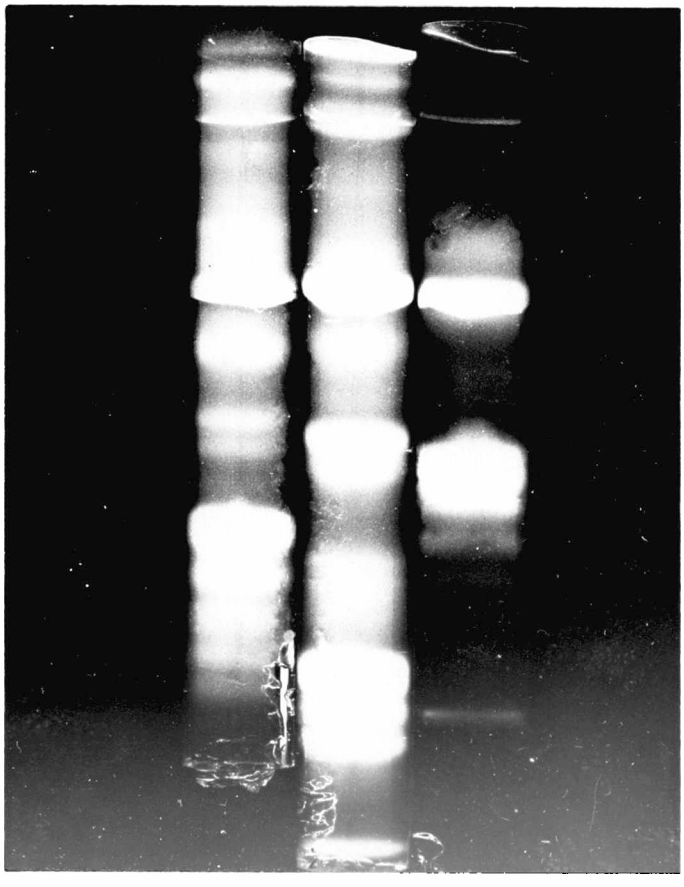
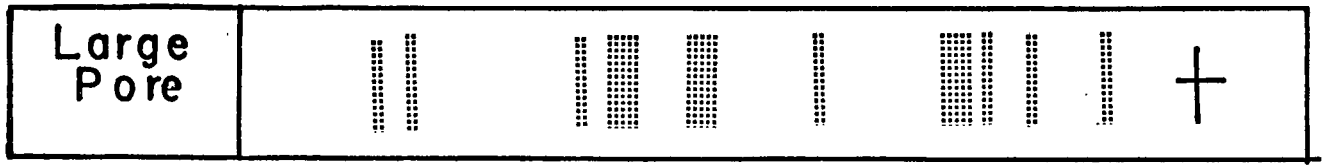
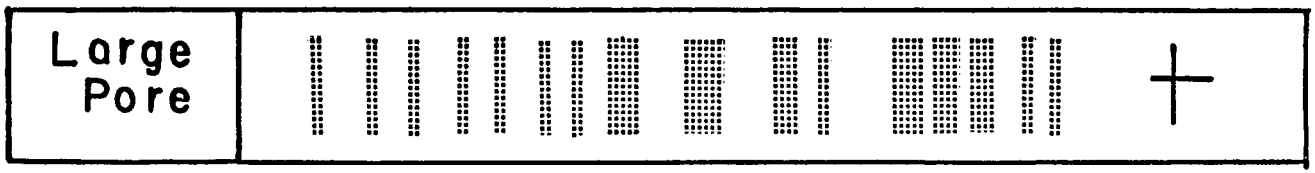


FIGURE 3

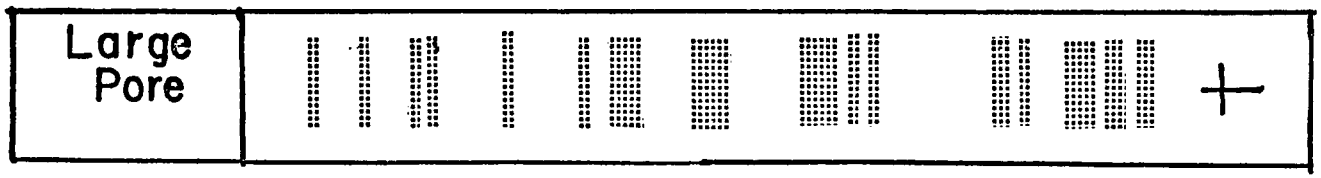
Diagrammatic electrophoretic patterns for white, brown, and pink shrimp.



PINK SHRIMP



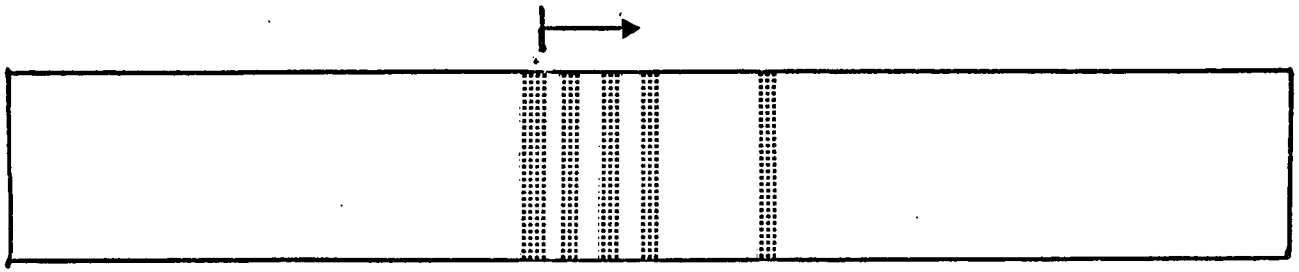
BROWN SHRIMP



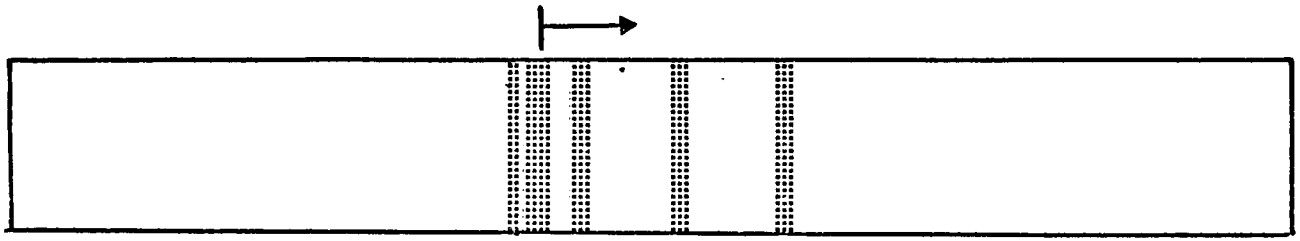
WHITE SHRIMP

FIGURE 4

Cellulose-acetate electrophoretic patterns of white and brown shrimp myogens.



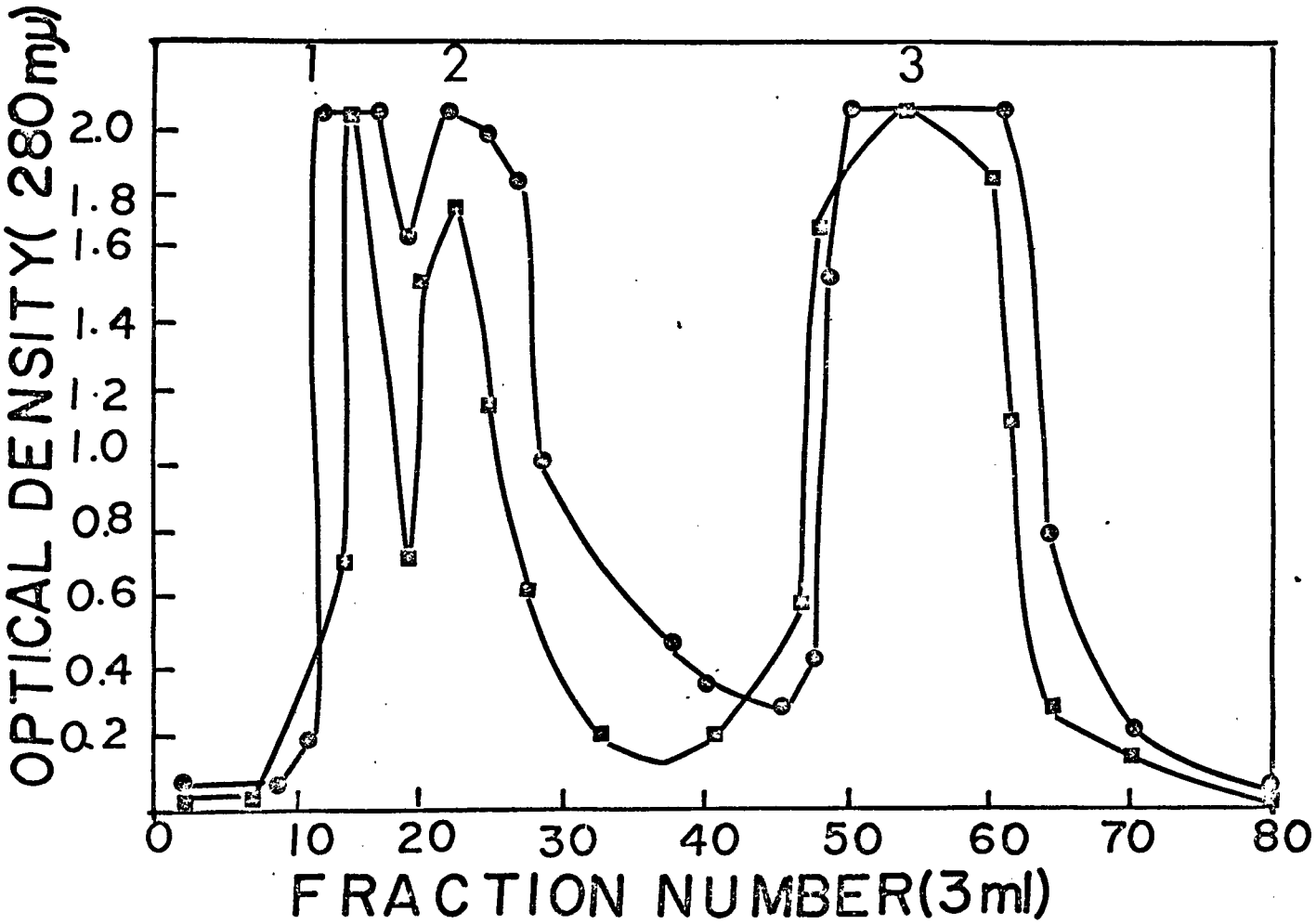
WHITE SHRIMP



BROWN SHRIMP

FIGURE 5

Sephadex G-75 chromatography in a 2.5 x 45 cm column of myogens from white shrimp (circles) and brown shrimp (squares) extracts.


OPTICAL DENSITY(280mμ)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1
2
3
0
10
20
30
40
50
60
70
80
FRACTION NUMBER(3ml)

the molecules in the extracts of different shrimp are similar, therefore, as indicated by the similarity of the elution volumes of the three major peaks. However, extracts of white shrimp show greater heterogeneity in size than do brown shrimp extracts.

The size of the molecules in these peaks can be estimated from the calibration of Sephadex G-75 (Figure 1). On this basis Peak 1 (see Figure 5) contains molecules of molecular weight greater than 69,000, Peak 2 contains molecules in the range of 30,000, and Peak 3 consists of molecules of less than 3,000 in molecular weight.

The ultraviolet spectra of the three main fractions obtained from Sephadex G-75 chromatography are given in Figure 6. Comparison of these spectra with the spectra of yeast ribonucleic acid and bovine serum albumin, Figure 7, indicates Peaks 1 and 2 consist predominantly of proteins; whereas Peak 3 contains sizeable amounts of nucleic acid derivatives.

Detection and Identification of Small Molecules

As indicated by the spectra of the extracts, Figure 8, nucleic acid derivatives were believed to be in the extracts. These molecules were observed consistently about two and a half hours after the initial extraction (the time it takes to clarify the extract). Therefore, they exist in the muscle, or they are formed rapidly in the extract.

Sephadex G-75 chromatography yields a fraction, Figure 5 (Peak 3), which has a spectrum similar to a nucleic acid (Figure 6). The

FIGURE 6

Spectra of Peak 1 (circles), Peak 2 (triangles), and Peak 3 (squares) obtained from Sephadex G-75 chromatography of brown shrimp extract (pH 6.85, KCl-phosphate buffer).

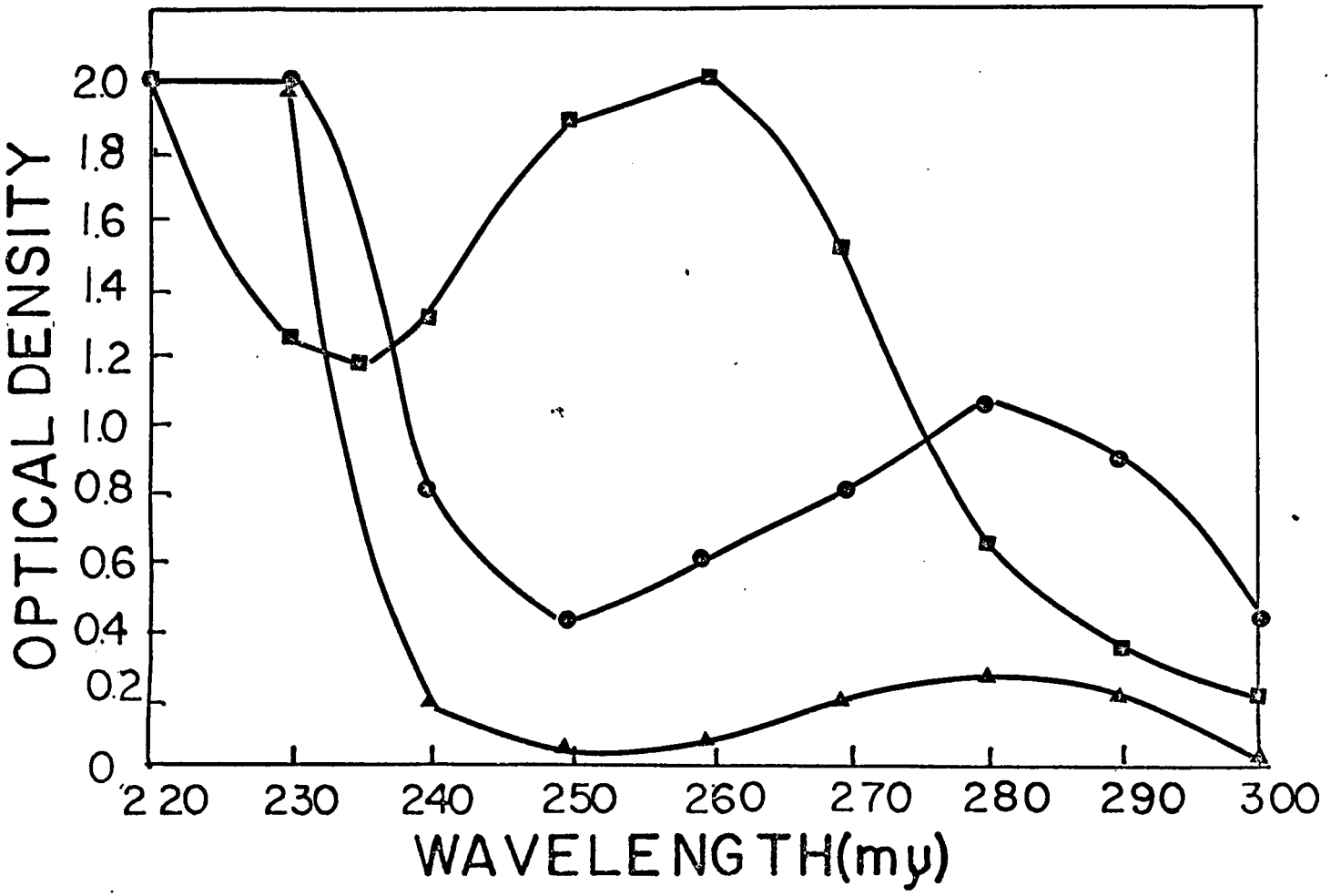
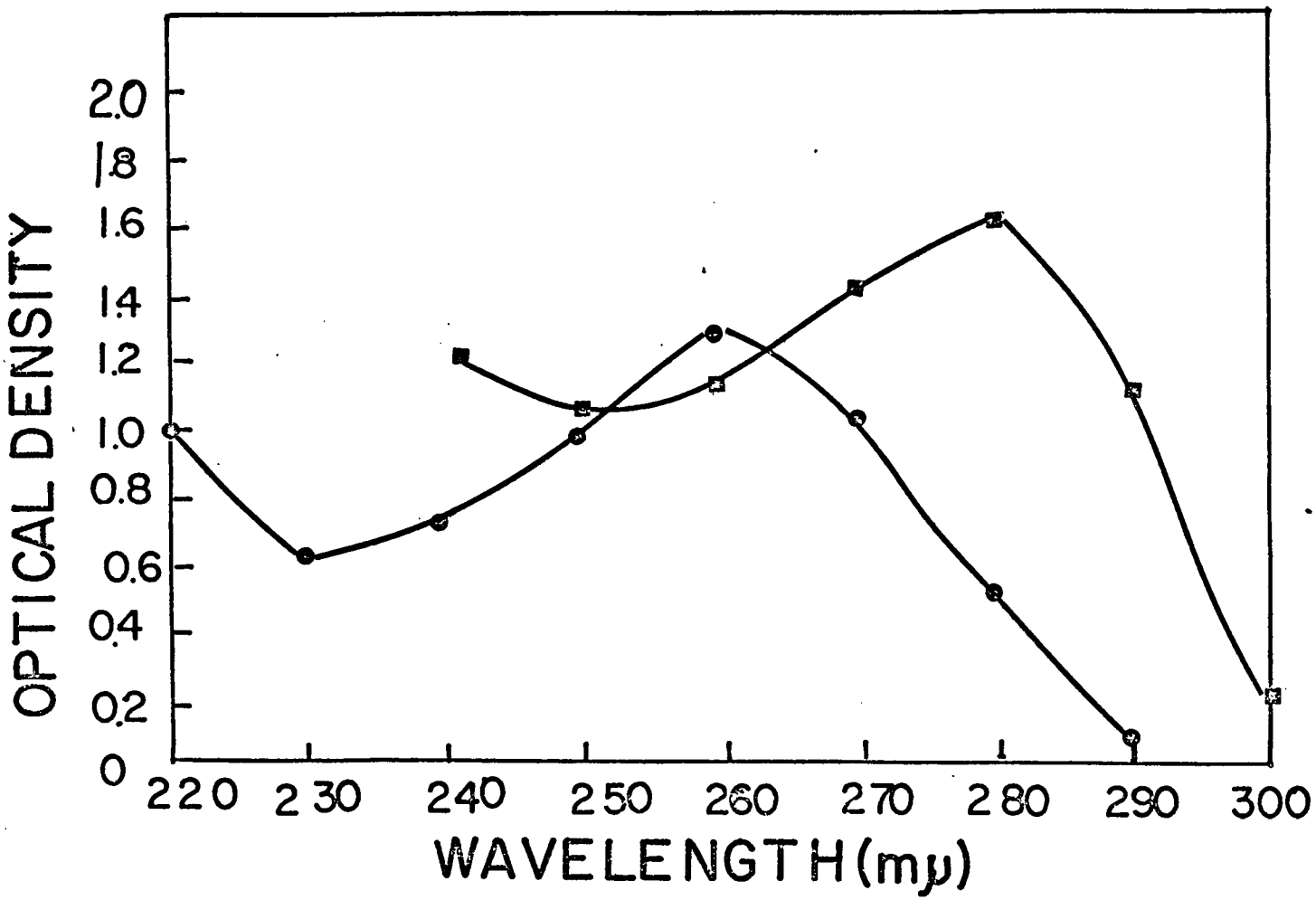

OPTICAL DENSITY
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
220
230
240
250
260
270
280
290
300
WAVELENGTH(mμ)

FIGURE 7

Spectra of yeast RNA (circles) [Davidson, 1960] and bovine serum albumin (squares) [Tanford, 1966].


OPTICAL DENSITY
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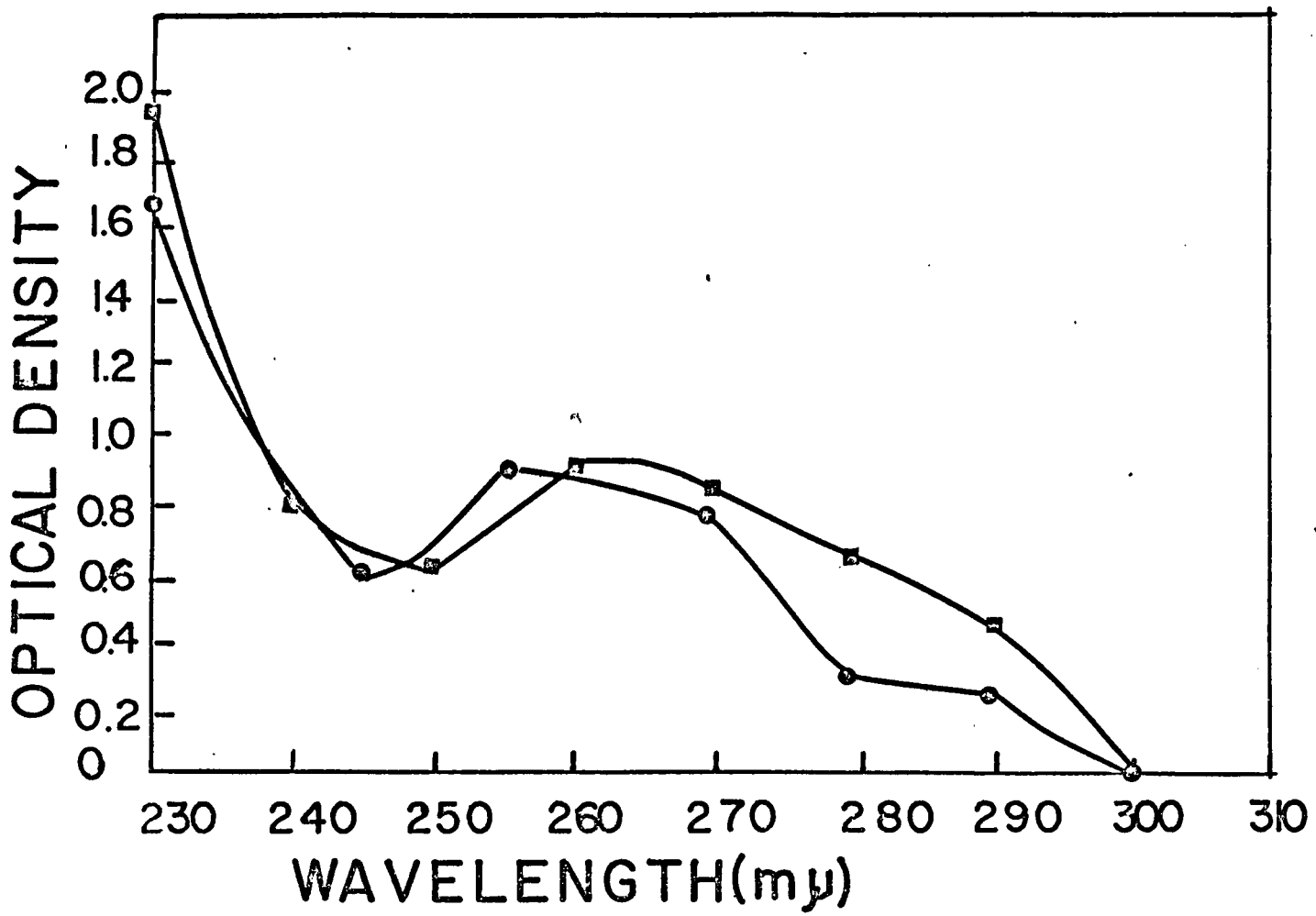
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
220
230
240
250
260
270
280
290
300
WAVELENGTH (mμ)

FIGURE 8

Spectral comparison of KCl-phosphate extracts of adult white shrimp (circles) and brown shrimp (squares). Absorbency was adjusted to zero at 300 mμ. The apparent difference in protein content is not significant and reflects only the relative efficiency of two separate extracts.


OPTICAL DENSITY
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
230
240
250
260
270
280
290
300
310
WAVELENGTH(mμ)

molecules of this peak have an estimated molecular weight range of a few thousand or lower as estimated from the calibration of the Sephadex G-75 column (Figure 1). The ultraviolet absorbing molecules of Peak 3 can be removed by dialyzing against KCl-potassium phosphate buffer (pH 6.85; ionic strength 0.08) or saline as shown in Figure 9. It is concluded that these molecules represent degradation products of nucleic acid derivatives present in the muscle.

Although not obviously pertinent to the aim of this thesis, a preliminary attempt was made to identify some of these small components. A tentative identification of two nucleotides, 5' IMP (inosine-5'-monophosphate) and inosine, was made (Table I). Identifications are based on the Rf values observed on paper chromatography using two solvent systems and the ultraviolet spectra of the nucleotides compared to commercial nucleotides. The identifications of these nucleotides and nucleosides can be checked by Dowex 1 chromatography. The chromatographic behavior of known nucleotides and nucleosides should be determined and compared to the behavior of the shrimp nucleotides and nucleosides under the same conditions.

A positive ninhydrin reaction was observed where nucleotides were detected on paper chromatography. Commercial 5' CMP gave positive ninhydrin reactions at concentrations of 0.01 mg/ml.

Ninhydrin positive molecules were detected by paper electrophoresis in white shrimp extract and Peak 3 from Sephadex chromatography. Although one of these ninhydrin positive substances may correspond to

FIGURE 9

Spectra of Peak 3 (circles) and dialyzed Peak 3 (squares) obtained from Sephadex G-75 chromatography of adult brown shrimp extract.

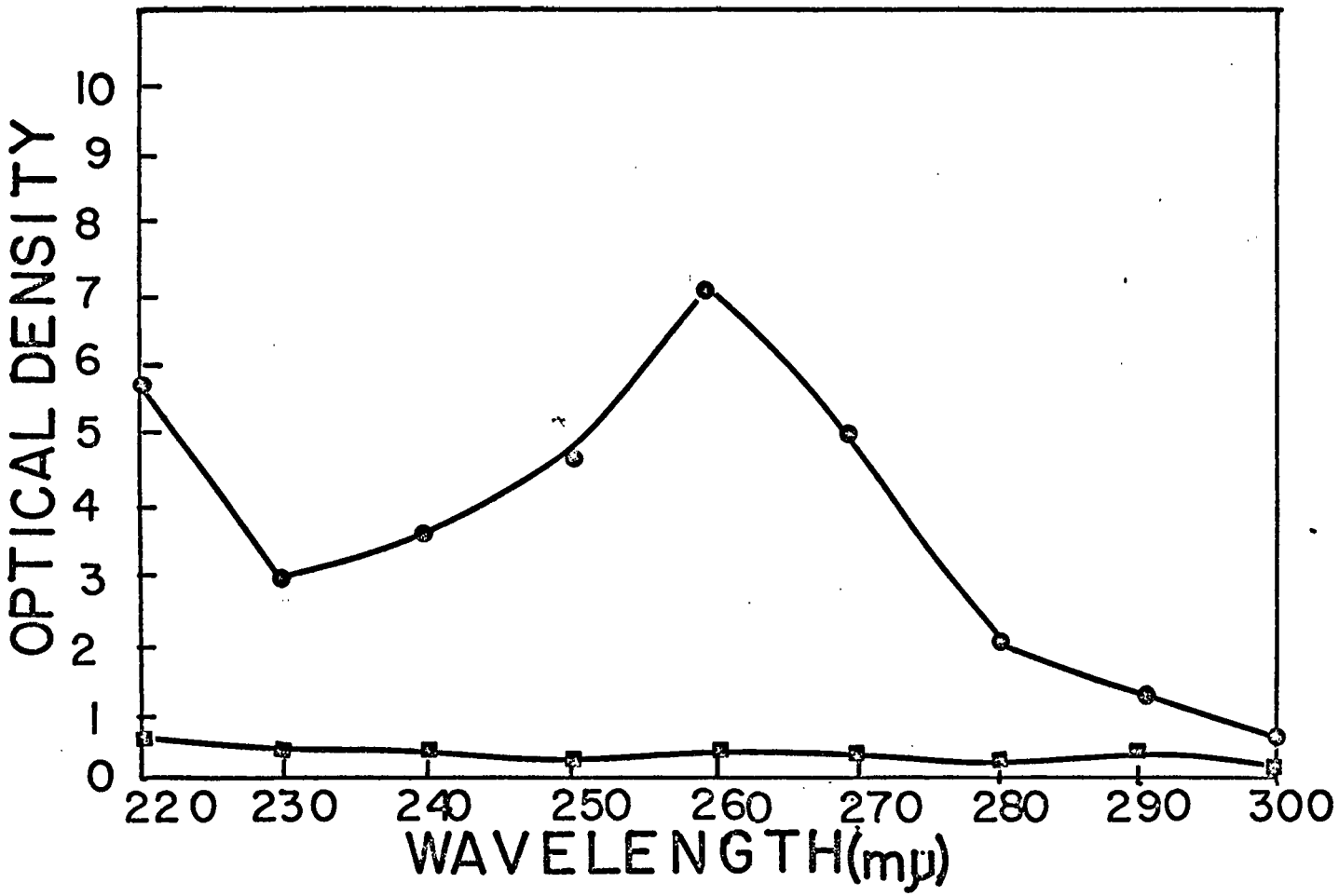

OPTICAL DENSITY
0
1
2
3
4
5
6
7
8
9
10
220
230
240
250
260
270
280
290
300
WAVELENGTH(mμ)

TABLE 1

TENTATIVE IDENTIFICATION OF CRUDE SHRIMP MUSCLE NUCLEOTIDES AND NUCLEOSIDES

Identity[1]	Spectral Comparison (1 M NH_4OH)			
	Max. (mμ)	250/260	280/290	290/260
5' IMP	251 - 252	1.12	0.06	0.00
5' IMP[2]	252	1.12	0.20	0.01
5' CMP	271 - 272	0.95	1.05	0.32
5' CMP[2]	273	0.84	0.95	0.31
Inosine	252	1.05	0.09	0.00
Inosine[2]	253	1.05	0.18	0.00

[1] 5' IMP inosine-5'-monophosphate

5' CMP cytidine-5'-monophosphate

[2] Schwartz Bioresearch Inc., Catalog 1967, pp. 116, 119

5' CMP, others probably correspond to amino acids. A yellowish-purple band, indicative of proline or hydroxyproline was observed.

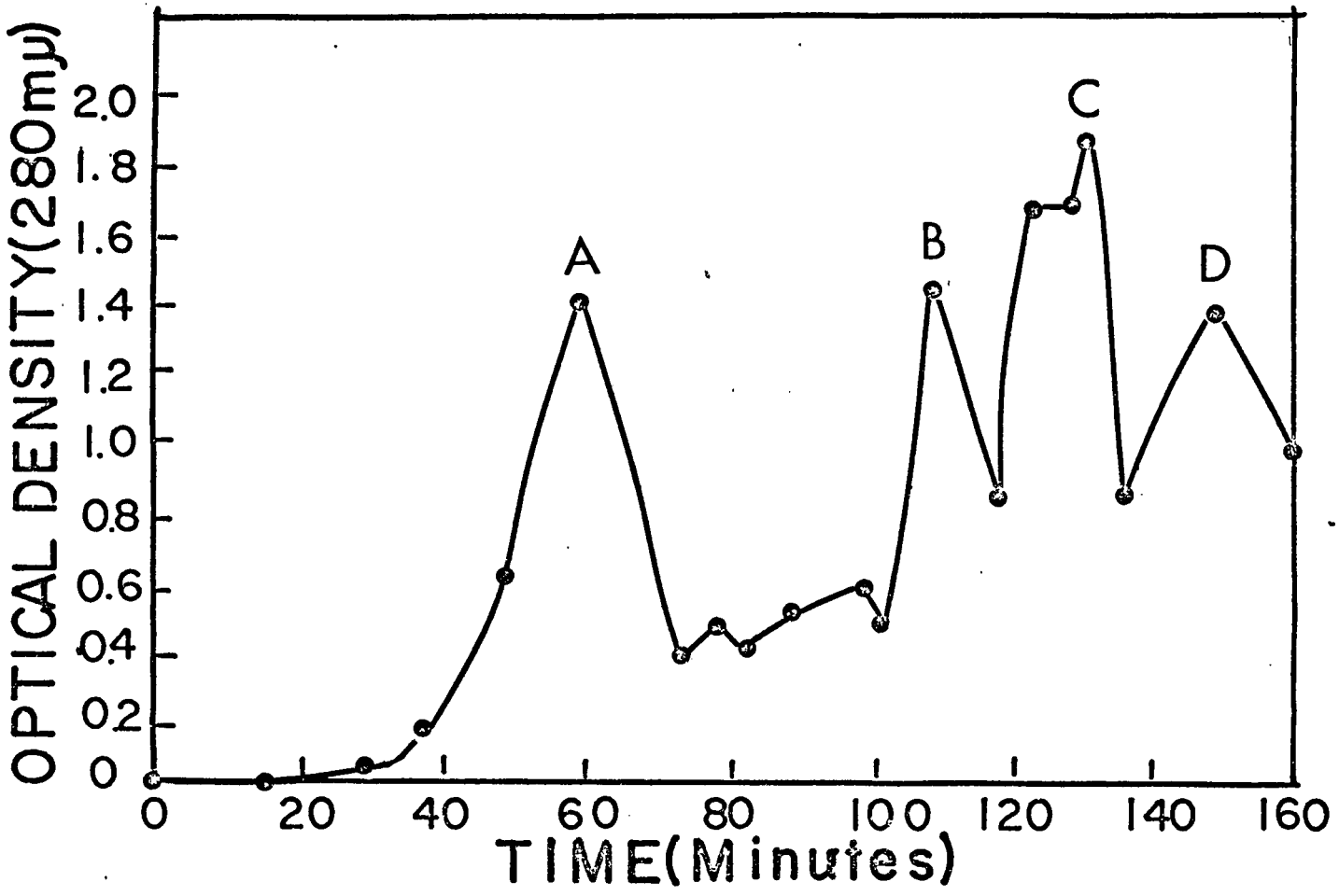
It is recommended that these small molecules be removed by dialysis or Sephadex chromatography of extracts before subsequent analysis or fractionation is carried out.

Ion Exchange Chromatography

Although electrophoresis of shrimp myogens readily allows one to identify species, the components need to be fractionated and characterized if good genetic markers are to be identified. Therefore, initial fractionation procedures were undertaken. Traditional salt fractionation was tried, but did not appear promising. Ion exchange chromatography was then tried and initial results show good promise as a means to fractionate the electrophoretic components. A typical result of DEAE cellulose chromatography with a linear salt gradient of adult brown shrimp extract is given in Figure 10. Fractions were dialyzed against KCl-potassium phosphate buffer to remove the salt before analysis by acrylamide gel electrophoresis. Comparison of the electrophoretic patterns of the major fractions of brown shrimp extract to the patterns of the whole extract showed generally that components nearest to the large pore/small pore interface were eluted first, the middle components were eluted next, followed by components near the bottom of the small pore gel. Other fractions contained components that were not in any particular order with respect to their mobility in disc electrophoresis.

FIGURE 10

DEAE cellulose chromatography of adult brown shrimp extract (0.05 M Tris-HCl and 2 M NaCl, 0.05 M Tris-HCl).


OPTICAL DENSITY(280mμ)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
A
B
C
D
0
20
40
60
80
100
120
140
160
TIME(Minutes)

It should be noted that the concentration of protein in the fraction was dilute, and analysis of fractions by disc electrophoresis may not have shown all the electrophoretic components present in the fraction.

IV.

DISCUSSION

DISCUSSION

One of the most important prerequisites to this work was to find an extraction method that yielded as many myogens as possible to compare. The solutions used for extractions of other crustacea, Manwell, et al., (1967), were tried during the initial phases of this problem, but they were found to be unsuccessful for our purposes. We found that procedures used for extraction of mammalian myogens gave more consistent results for our studies of shrimp. The solution finally selected for myogen extraction was a potassium chloride-potassium phosphate buffer (pH 6.8)

As soon as good extraction procedures were found, it was easy to show that electrophoresis is a quick and ready means to distinguish biochemically between species of juvenile and adult commercial shrimp from the Gulf of Mexico. In figures 2 and 3, it can be seen that the electrophoretic pattern of white shrimp differs from the others primarily in the presence of deeply staining bands that migrate almost as fast as the marker dye. Furthermore, the relative numbers and mobilities of the slower components differ between the brown and white shrimp. Results

of a few extracts of pink shrimp during the early phases of this work indicate that fewer electrophoretic components are observed. It is not known whether this reflects fewer myogens in pink shrimp or extraction methods optimal for brown shrimp may not be for pink shrimp. However, the pattern of the pink shrimp does resemble the brown more than the white in the lack of the fast moving, intense bands seen in the white shrimp patterns. The difference in patterns between the brown and pink shrimp support the viewpoints of taxonomists who believe that the pink and brown represent two distinct species. Mr. Lindner, of the Bureau of Commercial Fisheries, recommended that further examination of the pink shrimp be made in which individuals from the western and eastern portion of the Gulf should be compared, primarily because they believe for biological reasons that geographical subpopulations of pink shrimp exist.

Cellulose acetate electrophoresis, although providing different patterns for white and brown shrimp, did not give good resolution of bands nor is it as sensitive as acrylamide gel electrophoresis.

The aim of this work was to demonstrate the feasibility of identifying

species of commercial shrimp readily and easily by biochemical means. This has been shown in the case of juvenile and adult shrimp. The need exists, nowever, to identify the larval stages of shrimp, especially in samples that probably contain a mixture of species. To accomplish this aim, specific biochemical markers must be found which are sufficiently well characterized that their properties can be utilized for larval identification. The second stage of this research, therefore, was to examine various means of fractionating shrimp myogens to determine the method which shows the greatest promise of providing relative pure specific myogens.

The first fractionation procedure investigated was Sephadex column chromatography. Three fractions were obtained with Sephadex G-75. Although not providing good fractionation, molecular weights of myogens could be estimated. From observing the elution volumes of known proteins on the Sephadex, a calibration curve for molecular weights can be drawn following the method of Andrews (1964). From Figure 1, it can be seen that our results compare qualitatively to his results, but the elution volumes are systematically lower. This difference can be attributed

to differences in the size of the columns and relative packing of the gels. The three peaks observed for both brown and white shrimp extracts had estimated molecular weight ranges of 69,000 or greater, around 30,000, and 3,000 or less.

The absorption spectra of these peaks indicated that the first two peaks were predominately protein, while the third peak contained nucleic acid components (nucleotides and nucleosides) and ninhydrin positive material. The nucleotides and nucleosides were observed in the extract two and a half hours after the initial extraction was made (this is the time needed to clarify the mixture). It is not known whether they represent products of enzymatic action on extracted nucleic acids or products occurring free in the muscle.

Tentative identification of some nucleotides and nucleosides was made by determining their behavior on paper chromatography and their absorption spectra. 5' IMP, 5' CMP and inosine were found. These identification are tentative as the preparations are crude and the identifications are based primarily on the spectra of the separated bands.

Obviously it is desirable to remove these nucleotides and nucleosides in the extract before the myogens are further analyzed. It is recommended that these molecules can be removed most easily by dialysis of the extract.

Preliminary fractionation of these myogens using DEAE cellulose ion exchange chromatography indicates that this method shows a greater promise than Sephadex chromatography in the fractionation of these myogens. The chromatographic run showed four major components and several minor components for the brown shrimp extract. Preliminary studies have shown that these fractions have electrophoretic heterogeneity.

One of the next stages of this project will be to use immunological methods to show finer distinctions among the shrimp myogens. This approach as well as the identifications of some of the enzymes in the shrimp extracts is being studied by another student, Miss Donna Sanders, in this laboratory.

The ultimate aim of this research is to show the proteins from the larval and postlarval stages of shrimp can be used to identify the species. Electrophoresis will probably be possible in pure samples of one species, but for samples collected in the field where a mixture of species are present immunochemical and enzymatic methods will probably be necessary.

Using these techniques, it should be possible to define specific reactions of each species. With an understanding of the specificities of these reactions, the amount of reaction obtained from pure samples and mixed samples of known ratios could be calibrated. When field samples are collected and this method is used, the ratios or the relative abundance of each of the species could be determined.

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