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Robison, Buena Chambers, III, Ph.D.

Rice University, 1988
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PARVALBUMINS OF THE SCIAENIDAE: PURIFICATION, CHARACTERIZATION, AND POTENTIAL USE IN THE IMMUNO-IDENTIFICATION OF RED DRUM (SCIAENOPS OCELLATA)

by

BUENA CHAMBERS ROBISON III

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

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ABSTRACT

Parvalbumins of the Sciaenidae: Purification, Characterization, and Potential Use in the Immunoidentification of Red Drum (*Sciaenops ocellata*)

by

Buena Chambers Robison III

Parvalbumins are low molecular weight, water soluble sarcoplasmic proteins that are found in abundance in the fast twitch muscles of fish and amphibia. They are further characterized by a high affinity for calcium, acidic nature, polymorphism, unusual amino acid composition, unique ultraviolet absorption spectrum and high antigenicity. Parvalbumins are believed to serve in the rapid removal of calcium ions from troponin C during the relaxation phase of fast twitch muscle.

Parvalbumins are species-specific for each species of fish in terms of these physico-chemical characteristics. In view of this specificity and of the protein's considerable antigenicity, it was proposed to employ the parvalbumin from red drum (*Sciaenops ocellata*) as the basis for an immunological field test to identify the red drum species from muscle samples. The ability to identify red drum from only a sample of muscle tissue would allow game law enforcement personnel to screen contraband fish fillets on site. A field test as described would greatly strengthen enforcement efforts of parks and wildlife personnel.

The major component parvalbumins were isolated from red drum and spotted seatrout (*Cynoscion nebulosus*) by gel filtration and ion-
exchange chromatography, and antisera were produced in two rabbits against the red drum parvalbumin. This antisera was then examined
for cross-reactivity against the parvalbumin from spotted seatrout
and against the myogens of the following species of fish: sand seatrout (Cynoscion arenarius), black drum (Pogonias cromis), southern
kingfish (Menticirrhus americanus), freshwater drum (Aplodinotus
grunniens), vermilion snapper (Romboplites aurorubens), sheepshead
(Archosargus probatocephalus) and southern flounder (Paralichthys
lethostigma).

The antisera were examined by double diffusion in gels, by inter-
facial ring testing, and by enzyme-linked immunosorbent assay (ELISA).
In double diffusion in gels, the anti-red drum parvalbumin antisera-
retraced specifically with its homologous antigen when the antisera-
used was the five-week post primary inoculation antisera. As the
antisera matured, following secondary and tertiary inoculations with
antigen, the specificity lessened and the antisera reacted in varying
degrees with the heterologous antigens. With ELISA, reactivity of
the antisera was demonstrated against all antigens tested, regardless
of the stage of the antisera.
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INTRODUCTION

The purpose of the research described in this dissertation is to examine the feasibility of utilizing the major parvalbumin component of red drum (Sciaenops ocellata) as the basis for an immunochemical means of identifying that species of fish from samples of its white muscle tissue.

The red drum is a teleost of the family Sciaenidae, and has been highly valued as a game fish in Gulf coastal waters for many years. The breeding stock fishery inhabits open Gulf waters, and after spawning near passes and inlets along the shoreline, the young spend the first several years of their life within the estuary (Miles, 1950; Simmons and Breuer, 1962).

This species has been harvested commercially since the 1700s (Galtsoff, 1954) and landings reached a peak of approximately 5 million pounds in 1976 but they declined to about half that amount by 1980 (Swingle et al., 1984).

Although a population decline in a species is a complex process that can result from overharvesting, extremes of weather, fluctuations in food availability as well as other factors, and although the assessment of a decline is often hampered by imprecise and incomplete data collection, the number of red drum were deemed seriously depressed enough to warrant legislative protection. The Red Drum Conservation Act of 1977 and subsequent legislation in 1981 prohibited, respectively, the sale of native red drum and their capture by nets, seines, or trotlines (Matlock, 1980; 1984).

As state protection of the red drum increased, illegal
procurement and sale of the fish did likewise. Texas-caught fish are often sold with the claim that they were caught in Louisiana. Another significant problem that has become increasingly common is the tendency of illegal fishermen to process red drum into fillets on site, thereby making it impossible for game law enforcement officials to identify the fish (Chester Burdett, Law Enforcement Division, Texas Parks and Wildlife Department, pers. comm.).

In view of the fact that the red drum fishery contributes by direct and indirect means approximately 125 million dollars annually to the Texas economy (Matlock, 1980), legislative and scientific means to protect the fishery become obviously important.

It was therefore proposed that a simple, accurate, and reliable field test specific for red drum and based only on a sample of white muscle tissue would be of considerable value in red drum fishery management and game fish law enforcement.

The widespread use in medicine and forensics of immunological means for the identification of species, tissue types, and microbial agents prompted the decision to employ an antiserum hypothetically specific for the white muscle of red drum.

The sarcoplasmic protein known as parvalbumin was selected as the protein with which to produce the antiserum. Parvalbumin was selected because it displays several advantageous features: abundance in white muscle sarcoplasm, stability, high antigenicity, and species-specificity in such parameters as molecular weight, isoelectric point, and amino acid composition (Pechere, 1968; Pechere et al., 1973; Wnuk et al., 1982).
Therefore, in order to examine the feasibility of developing a red drum-specific field test in the future, that is, to determine the degree of specificity that an anti-red drum parvalbumin antiserum could have for red drum white muscle, parvalbumin was isolated and purified from red drum and an antiserum produced against it. This antiserum was then tested for immunological cross-reactivity versus the muscle extracts of eight other fish species as well as of red drum.

In addition, parvalbumin was purified from spotted seatrout, and both species of parvalbumin were characterized for molecular weight, isoelectric point, amino acid composition, ultraviolet absorption spectra, and calcium binding.

It was determined that red drum and spotted seatrout each have two isoparvalbumin components; in each case the major component, or more abundant of the two, was isolated.

Cross-reactivity between both purified parvalbumins was also evaluated. All immunologic analysis was carried out by the interfacial ring test, double diffusion in gels, and by enzyme-linked immunosorbant assay.

**REVIEW AND GENERAL DESCRIPTION OF PARVALBUMINS**

A little more than half a century ago Deuticke (1934) was the first investigator to observe, through ultracentrifugational analysis, that substantial amounts of low molecular weight proteins (10-12,000 daltons) were present in low-ionic-strength extracts of frog muscle.
Twenty-one years later, Henrotte (1955) reported that similar protein was present in the white muscle of the carp.

Other early investigations into the nature of fish myogens (the water-soluble sarcoplasmic proteins) revealed species-specific electrophoretic patterns of low molecular weight proteins (Connell, 1953) and that these myogenic proteins from fish displayed greater mean electrophoretic mobility than did similar extracts from the muscle of higher vertebrates (Hamoir, 1955).

Additional ultracentrifugational studies on carp (Hamoir, 1955; 1968), and cod (Connell, 1958) showed a consistent pattern of three broad peaks, including a large slow-sedimenting peak, that represented the low molecular weight components. This low molecular weight fraction appeared to be present almost exclusively in the myogen of aquatic vertebrates.

The extensive collaboration of Hamoir and Konosu (1965), Konosu, Hamoir and Pechere (1965), and Pechere and Focant (1965) on the myogen from red and white muscle of the carp demonstrated that this low molecular weight fraction of the myogen was absent in red muscle, while abundant in white muscle; that the molecular weight of these proteins lay in the 9,000 to 13,000 range; that their amino acid composition was characterized by a high proportion of phenylalanine, the absence of methionine and tryptophan, and by one residue each of tyrosine, cysteine, proline, arginine, and histidine; and that this myogenic fraction contained no enzymes from the Embden–Meyerhof pathway.

Their unusual amino acid composition also accounts for parvalbumins' unique ultraviolet absorption spectra. Because of the
predominance of phenylalanine and the lack or scarcity of tyrosine and tryptophan, a triple-peaked spectrum with maxima at or near 250, 254, and 260 nm, with very low absorption at 280 nm, is consistently characteristic of parvalbumins (Pechere, 1968; Pechere et al., 1973).

A few years later Pechere (1968) proposed the use of the name "parvalbumin" (from "parvus," Latin for "small") to denote this group of small molecular weight, acidic, sarcoplasmic proteins from aquatic vertebrates. He furthermore proposed at that time, on the basis of their similar physico-chemical properties, that parvalbumins be considered a family of homologous proteins.

Parvalbumins have subsequently been isolated from perch (Perca fluviatilis) (Lehky et al., 1979), cod (Gadus callarias) (Bhushana Rao et al., 1969), pike (Esox lucius) (Bhushana Rao et al., 1973a, 1973b), three species of Gadidae (Closset, 1976; Closset and Gerday, 1976), two species of rays (Gerday et al., 1972), the lungfish Protopterus dolloi, (Gerday et al., 1979), hake (Merluccius merluccius) (Pechere et al., 1971b, 1971c), the coelacanth (Latimeria chalumnae) (Jauregui-Adell and Pechere, 1978), carp (Cyprinus carpio) (Pechere et al., 1970), dogfish (Heizman et al., 1974), chub (Leuciscus cephalus) (Piront, et al., 1978a, 1978b), and four species of perciforms (Sullivan et al., 1975).

It was originally believed that parvalbumins occurred only in the muscle of the lower aquatic vertebrate classes, but Lehky and Stein (1974) have shown them to be present in minute quantities in the skeletal muscle of the turtle, chicken (see also Heizman et al., 1979), rabbit, and man. Maeda et al. (1984) have isolated them from
the boa, salamander, and turtle.

Parvalbumins are not present in invertebrates such as crustacea, annelids, molluscs, and the protochordates. These groups have been shown to possess sarcoplasmic calcium-binding proteins with properties different from parvalbumins (Wnuk et al., 1979).

While the location of the greatest abundance of parvalbumins is white skeletal muscle, the protein is generally not present in cardiac or smooth muscle, and therefore it appears that it is not an indispensable component of the contractile mechanism (Wnuk et al., 1982). One significant exception, however, is the occurrence of parvalbumin in the myocardium of the shrew, an extremely rapidly contracting muscle (Wnuk, op. cit.). Nor does there appear to be a precise correlation between the occurrence of parvalbumin in a muscle tissue and that tissue's use of aerobic or anaerobic metabolism; parvalbumin is not present in the breast muscle of the chicken, a primarily glycolytic muscle (LePeuch et al., 1979), while it is present in the aerobic red muscle of the electric eel (Childers et al., 1976) and of the carp (Gosselin-Rey et al., 1978).

Another distinctive feature of the parvalbumins is their polymorphism. Of fifty fish species studied, most were shown to have two isoalbuninns each, while some had four or five (Bhushana Rao et al., 1973). Certain members of the family Cyprinidae, for example, have four isoalbuninns within a single species. The polymorphic forms of a given parvalbumin show slight differences in amino acid composition, molecular weight, and in isoelectric point.

Parvalbumins have been identified as the protein responsible for
allergic reactions to fish, a well-known condition in humans (Aas, 1966a, 1966b, 1966c, 1967; Aas and Jebsen, 1967). The antigenicity of this protein, originally called Allergen M by immunologists (Elsayed and Bennich, 1975), is based upon its primary structure, and not upon its secondary or tertiary conformation.

Tryptic cleavage of the protein results in fragments that do not produce a precipitin reaction with antiserum produced against the native protein (Elsayed et al., 1972). Enzymatic digestion of the protein likewise causes a loss of immunologic reactivity with antiserum (Aas and Elsayed, 1969). Denaturation with urea and beta-mercaptoethanol, however, causes neither a loss nor a decrease in the precipitin reaction with antiserum homologous to the native protein (Elsayed and Aas, 1971).

From these investigations, and from others cited immediately below, a consistent pattern of physico-chemical characteristics can be seen that describes the family of parvalbumins as they occur in fish and amphibia:

1. high solubility in aqueous solutions
2. low molecular weight (10 - 13,000 daltons)
3. acidic nature (pI range 5.0 - 3.9)
4. polymorphism (2 - 5 isoalbumins per species)
5. abundance in fast twitch muscle (3 - 4 g./kg. muscle [Gillis et al., 1979.])
6. absent or present in minute quantities of red muscle
7. even distribution throughout fast twitch muscle (Benzonana et al., 1977)
8. high diffusion constant
9. low specific viscosity
10. unbound in muscle cell sarcoplasm (Gillis et al., 1979)
11. high antigenicity
12. unique ultraviolet absorption spectrum

Amino Acid Composition and Sequence

As a family of homologous proteins, parvalbumins display certain consistent and characteristic features regarding their amino acid content. Hamoir (1968) was among the first to recognize the unusual amino acid composition of these sarcoplasmic proteins.

They have a high and relatively constant proportion of phenylalanine (approximately 8-10 residues per molecule), usually a single arginine at position 75 in all known sequences, and a high number of the acidic residues aspartate (12-17 residues per molecule) and glutamate (8-17 residues per molecule).

In addition, methionine, cysteine, proline, tyrosine, tryptophan and histidine are found either not at all or as only a single or very few residues. By contrast, lysine (11-16 residues per molecule) and alanine (9-23 residues per molecule) are in consistently high amounts throughout the family. Parvalbumins have also been shown to be devoid of covalently bound lipid, phosphate, or carbohydrate groups (Pechere, 1968; Pechere et al., 1973; Demaille et al., 1974a; Gosselin-Rey, 1974; Kretzinger, 1980).

By comparison of the known sequences, the homologous nature of the parvalbumin family is evident from the numerous isologies within
their primary structures. To cite several examples from Pechere (1973), using parvalbumins from carp, hake, pike, coelacanth, and the frog, highly acidic regions (aspartate and glutamate predominating) are present at residues 50-62 and 90-101; these areas represent the highly conserved calcium-binding internal core. Among 12 calcium-coordinating positions within a given parvalbumin, nine are invariant and two are conservative throughout the known sequences.

Many other invariant positions exist among the parvalbumins. There are arginine 75, aspartate-isoleucine at 9-10, alanine-leucine at 14-15, phenylalanine at 24, 29, 30, 47, 70, and 102, glycine at 34, 93, 98, leucine at 35, 63, 67, 77 and 86, aspartate at 51, 90 and 92, serine at 55, glutamate at 59, 62, 81, 101, alanine at 72 and 88, threonine at 82, lysine at 87 and 96, and valine at 106. Among 28 residues of the internal hydrophobic core, ten are invariant and eight are conservative.

**Secondary and Tertiary Structure**

As demonstrated by Levitt and Greer (1977) and Moews and Kretsinger (1975), the secondary structure of parvalbumin consists of six alpha-helical regions, termed domains A through F, that are each separated by a loop. The loops between helices C and D and between E and F each bind a calcium ion; it is in these highly conserved regions that the acidic residues aspartate and glutamate predominate.

In carp 4.25 (NB: the number following a species names indicates the parvalbumin from that species with that isoelectric point, a common method of referring to polymorphic forms within a given species) a calcium ion is bound by the CD loop by oxygen of the side
chains of aspartate 51 and 53 and of glutamate 59 and 62, a peptide carbonyl oxygen of phenylalanine-57 and a hydroxyl oxygen of serine 55; the EF loop binds a calcium ion with oxygen from aspartate 90, 92, and 94, glutamate 101, the peptide carbonyl oxygen of lysine 96, and by one water molecule at glycine 98 (Wnuk et al., 1982).

The CD and EF domains are connected by hydrogen bonds between isoleucine 58 and isoleucine 97 that lie along an antiparallel beta-sheet.

Parvalbumins, as demonstrated from carp 4.25 and others, are globular proteins with no pits or crevasses on the surface. Within the interior of the molecule is a hydrophobic core consisting primarily of the side chains of phenylalanine, isoleucine, leucine, and valine (Wnuk, op. cit.).

This hydrophobic core gives the parvalbumin molecule a great deal of stability that results in the protein's considerable resistance to proteolysis (Cox et al., 1979) and in its tolerance of temperatures up to 80 degrees C. before thermally-induced changes are produced (Burstein et al., 1975; Cave et al., 1979).

Parvalbumins belong to the troponin C superfamily, which is a group of calcium-binding proteins that shows a high degree of homology in primary structure, especially within the ion-binding regions. This superfamily includes calmodulin, troponin C, calcineurin B, myosin light chain, oncomodulin, and vitamin D-dependent calcium-binding proteins of the avian intestine (Michiel and Wong, 1986).

These proteins are also referred to as "EF hand" proteins, after the EF domain of parvalbumin. This domain consists of the E and F
helices that are connected by a peptide loop that complexes with the metallic cation.

The term "hand" is used since the domain can be roughly illustrated by holding the index finger and thumb of the right hand at right angles to each other with the other fingers contracted. The extended fingers represent the two alpha-helices, while the contracted fingers represent the ion-binding loop. The "EF hand" configuration is found throughout this protein family (Kretsinger, 1976; 1980).

**Evolutionary Lineages**

Three separate pairs of evolutionary lineages have been proposed for parvalbumins on the basis of their amino acid composition and sequence. The features that distinguish these several lineages, however, do not necessarily apply to all parvalbumins.

Closset and Gerday (1976) proposed two lineages for parvalbumins within the genus *Gadus*. Of eight parvalbumins that they isolated from four species, they distinguished two classes: one characterized by one tyrosine, one tryptophan, three cysteine, no methionine nor histidine, and the second characterized by one methionine, one histidine, one cysteine, and no tyrosine nor tryptophan.

Pechere *et al.* (1973) likewise proposed two lineages for eight parvalbumins, designating them alpha and beta. The alpha lineage is marked by phenylalanine-valine at position 65-66, and the beta by leucine-phenylalanine at 65-66. Each of the known fourteen sequences of parvalbumins has one of these amino acid pairs at 65-66.

Sullivan *et al.* (1975) have proposed yet a third set of
evolutionary lineages, based on parvalbumins from four species of perciform fish that each have two isoalumins.

These lineages are termed "fast" and "slow." These names are based on the proteins' relative electrophoretic mobility. The more anodally migrating protein of the isoalbumin pair is called "fast," the other "slow."

The "fast" component is characterized by the presence of tyrosine, the absence of histidine and proline, 14 or more aspartic acid, fewer threonine than serine, and an alanine-glycine ratio of approximately 2.0.

The "slow" component, the larger of the two in molecular weight, is characterized by the presence of histidine and proline, the absence of tyrosine, twelve or fewer aspartic acid, more threonine than serine, and an alanine-glycine ratio of approximately 1.5.

These three sets of criteria for evolutionary lineages are not, however, universally applicable to all parvalbumins. To give one example, two proteins within Pechere's system, pike 5.0, an alpha, and carp 4.25, a beta, both have histidine at position 46, which, according to Closset and Gerday, would place them both in the same lineage.

Calcium Binding By Parvalbumins

Despite the great deal of research that has been carried out on the structure, distribution and characteristics of parvalbumin, the protein's function has yet to be precisely elucidated.

There is, however, another important characteristic of parvalbumin that must be considered an essential part of the hypotheses
concerning the protein's function: its high affinity for calcium.

The fact that parvalbumins strongly and specifically bind calcium ions was first demonstrated in the early 1970s when it was shown that parvalbumin contains two moles of calcium per mole of protein (Pechere et al., 1971c).

Later investigators showed that parvalbumins bind Ca\textsuperscript{2+} at two high affinity sites with a K-assoc. ranging from $2.5 \times 10^{6}$ to $10^{7}$ L-M (Benzonana et al., 1972) to $1.3 \times 10^{8}$ to $2.7 \times 10^{9}$ L-M (Haiech et al., 1979; Moeschler, 1980).

The two binding sites have also been shown by some researchers to be independent and equivalent with no cooperativity (Cox et al., 1977; Haiech et al., 1979; Potter et al., 1977; Moeschler, 1980; Wnuk et al., 1982).

In addition to calcium, parvalbumins also bind magnesium with a binding constant in the range of $10^{4}$ to $10^{5}$ L-M. In the muscle cell in vivo, parvalbumin never exists in the metal-depleted state but always complexed with either Ca\textsuperscript{2+} or Mg\textsuperscript{2+}. In experiments where the levels of Ca\textsuperscript{2+} and Mg\textsuperscript{2+} were approximately equal to physiological levels, namely $10^{-8}$ and 1.0 mM respectively, parvalbumin bound two moles of Mg\textsuperscript{2+} and one mole of Ca\textsuperscript{2+} (Cox et al., 1977; Potter et al., 1977; Haiech et al., 1979; Lehky et al., 1979). As ambient Ca\textsuperscript{2+} levels are increased, its uptake by parvalbumin increases along with a concomitant release of Mg\textsuperscript{2+} (Moeschler, 1980).

Due to this competition between Ca\textsuperscript{2+} and Mg\textsuperscript{2+} for binding sites, binding constants tend to be somewhat higher when calculated in Mg\textsuperscript{2+}-free systems. For example, Benzonana et al. (1972) determined
the 10(6) - 10(7) range.

Haiech et al. (1979) and Moeschler (1980) calculated the constant in the absence of Mg2+ with results in the 10(8) to 10(9) L-M range.

Cox et al. (1979) and Haiech et al. (1979) also demonstrated that parvalbumins in the Ca-form and Mg-form are practically identical in tertiary conformation. The only structural changes that do occur appear to be restricted to the calcium-binding loops that coordinate the ions (Birdsall et al., 1979).

It has also been determined that ion-binding by parvalbumin is not significantly affected by changes in pH (Pechere et al., 1977).

**Proposed Function of Parvalbumin**

Most hypotheses that have been put forth to date concerning the function of parvalbumin within the living muscle cell are centered upon the protein's possible role in the regulation of calcium flux.

This direction of thought results from several factors regarding the activation of muscle cells and the characteristics of parvalbumins. There is, first of all, the well-established role of calcium in the excitation-contraction cycle (for reviews see Ebashi and Endo, 1968, and Weber and Murray, 1973). Heizman et al. (1982) showed that concentrations of parvalbumins in various types of muscle were directly proportional to the speed of that muscle.

Furthermore, many of the characteristics of parvalbumin discussed above strongly suggest a cytosolic regulatory role in calcium dynamics in certain types of muscle: its abundance in fast twitch, glycolytic skeletal muscle of aquatic vertebrates (see also Baron et al., 1975 and Celio et al., 1982) and absence or scarcity in smooth, cardiac,
and red skeletal muscle; its presence in muscle in adequate levels to bind all calcium present (200 nM per g. of myofibrils for the cation); its lack of attachment to any organelle or macromolecule in the cytosol; its non-participation in the actual contractile mechanism; its noninvolvement in any kind of phosphorylation process; and, of course, its high and specific affinity for calcium ions in the presence of magnesium.

Pechere et al. (1975) first proposed that parvalbumins might be involved in the regulation of the movement of calcium ions during the relaxation phase of the contractile cycle in fast twitch muscle.

They suggested that parvalbumins act as a shuttle between the myofibrillar apparatus and the sarcoplasmic reticulum; the protein binds calcium as it unloads from the myofibrils and then discharges it as the ion is taken up by the sarcoplasmic reticulum.

Other pertinent data have supported this hypothesis. Gerday and Gillis (1976) demonstrated in vitro that the sarcoplasmic reticulum is able to draw calcium ions away from calcium-loaded parvalbumin, while Fischer et al. (1976) showed that parvalbumin can successfully compete for calcium with myofibrillar ATP-ase.

Thus an increasing affinity gradient can be established in this order: myofibrils -- parvalbumin -- sarcoplasmic reticulum (Gillis, 1980). Of possible additional significance is the observation that uptake of calcium by the sarcoplasmic reticulum is not fast enough to account for the rate of relaxation of living fast-twitch muscle (Ebashi, 1976).

At this point, however, a dilemma arises: the hypothetical
"parvalbumin barrier." If parvalbumin has a greater affinity for calcium than does the myofibrillar apparatus -- specifically, troponin C, whose binding constant for Ca$^{2+}$ is an order of magnitude lower than parvalbumin -- how can calcium pass from the terminal cisternae of the sarcoplasmic reticulum to the myofibrils, when the muscle is activated, without it being intercepted by the cytosolic parvalbumin?

One possible explanation is kinetic. Despite the lower affinity for calcium, uptake by the myofibrils is nevertheless faster than uptake by parvalbumin. Calcium is thus complexed first to the myofibrils, initiating the contraction cycle. Following contraction, the higher affinity of parvalbumin would allow it to mediate the removal of the cation from the myofibril during the relaxation phase (Pechere et al., 1977).

This kinetic disparity is possibly explained by parvalbumin's magnesium binding properties. In resting muscle parvalbumin exists on the Mg$^{2+}$-bound state. The half-time for dissociation of this cation from parvalbumin can be as long as 230 msec, while troponin C becomes 90 percent saturated with calcium within 20 msec (Haiech et al., 1979; Gillis et al., 1980).

**GENERAL MATERIALS AND METHODS**

Fish used throughout this investigation were obtained through the Texas Parks and Wildlife Department and from local commercial outlets. All chemicals were of reagent grade. All steps in protein purification were carried out at 4 degrees C., unless otherwise indicated. Concentrations of protein solutions were estimated by the

An itemized chronology of the isolation protocol for the major parvalbumin component from both red drum (*Sciaenops ocellata*) and from spotted seatrout (*Cynoscion nebulosus*) is given here. Each step will be described in detail immediately following.

1. preparation of the muscle extract
2. dialysis of the muscle extract
3. application and gradient elution of extract by ion exchange chromatography
4. application of parvalbumin fraction to Sephadex and Sephacryl gel filtration columns
5. concentration of protein solution by ultrafiltration and by osmotic concentration versus sucrose
6. determination of purity by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and high pressure liquid chromatography
7. lyophilization

Antiserum was prepared in two male New Zealand white rabbits against the major component of red drum parvalbumin. This antiserum was then tested for cross-reactivity against the major parvalbumin component of spotted seatrout and against the muscle extracts from eight species of fish within and outside the family Sciaenidae. Cross-reactivity was evaluated by the interfacial ring test, immuno-double diffusion in gels, and enzyme-linked immunoabsorbant assay.

Other fish species used for evaluation of cross-reactivity with anti-red drum major component parvalbumin antiserum were: from the
Sciaenidae, Cynoscion arenarius, sand seatrout; Pogonias cromis, black drum; Menticirrhus americanus, southern kingfish; Aplodinotus grunniens, freshwater drum; from the Lutjanidae, Rhomboplites aurorubens, vermillion snapper; from the Sparidae, Archosargus probatocephalus, sheepshead; from the Bothidae, Paralichthys lethostigma, southern flounder.

Both red drum and spotted seatrout major parvalbumins were characterized according to the following physicochemical properties:

1. molecular weight
2. amino acid composition
3. isoelectric point
4. ultraviolet absorption spectra
5. binding constant for calcium

The characterization and immunological analysis of the proteins will be described in detail following the description of the protocol for isolation, in which specific methodologies of purification will be presented.

RESULTS AND DISCUSSION OF SPECIFIC METHODOLOGIES

ISOLATION OF THE PROTEINS

Introduction

In the literature on parvalbumins, the described protocols for purification of the protein generally proceed in one of two basic ways.

One method involves the fractionation with acetone or ammonium sulfate of a muscle extract, followed by gel filtration of the 55 -
80% fraction and then by ion-exchange chromatography of the 10 -
12,000 molecular weight peaks from gel filtration (Bhushana Rao et
al., 1969; Pechere et al., 1971a; 1971b; Gerday and Teuwis, 1972;

The second method proceeds in a reverse fashion. Dialyzed
extract is applied first to an ion-exchange column, followed by
application of the parvalbumin-containing peaks to gel filtration
chromatography (Lehky et al., 1974; Haiech, 1979; Heizmann and
Strehler, 1979; Lehky and Stein, 1979).

The purification of the major component of red drum and spotted
seatrout parvalbumins described herein follows this second
methodology.

Since parvalbumins have no enzymatic activity (Hamoir and Konosu,
1965; Konosu et al., 1965; Pechere and Focant, 1965), there is no
specific assay for them during purification of the kind employed in
classical enzymology.

Initial identification of parvalbumins during the purification
procedure, therefore, must depend instead on two of their distinctive
characteristics: their ultraviolet absorption spectra, and their low
molecular weight.

From Pechere et al. (1973):

...(parvalbumins') most remarkable physical characteristic
probably lies in their u.v. spectrum, whose maximal
absorption, in contradistinction to all other proteins,
almost always occurs at 259 nm, with the consequence that
they generally have an unusually low E280/E260 ratio...this
is due to an exceptionally high proportion of phenylalanine
in these proteins. In fact, the absorption of the aromatic
side chain is always dominant in the u.v. spectrum of
parvalbumins...In the absence of any presently known and measurable biological activity, the clearly discernible maxima at 253, 259, and 264 nm therefore often constitute a simple basis for presuming that a given acidic protein of low molecular weight is a parvalbumin.

In the protein purification to be described, ion-exchange chromatography was performed once with monitoring at 254 nm, and then performed a second time with monitoring at 280 nm. The peaks demonstrating a high absorption at 254, and lower absorption at 280 were presumptively considered the parvalbumin-containing peaks (Fig. 1).

Confirmation of the presence of parvalbumins in the peaks showing this particular absorption ratio was obtained by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (hereafter referred to as SDS-PAGE). In SDS-PAGE, parvalbumins are evident as the dominant band closest to the anodal end of the gel.

From Sullivan et al. (1975), as they describe the isolation of perciform parvalbumins:

In order to help differentiate parvalbumins from other muscle proteins, the polyacrylamide concentration was raised from the usual 7.5% to 12%...In this increased acrylamide concentration, the low molecular weight, acidic parvalbumins usually move toward the anode much more rapidly than other muscle proteins.

And from Lehky and Stein (1979), as they describe the isolation of perch parvalbumins:

Identification of parvalbumins during purification was carried out by disc gel electrophoresis in the presence of sodium dodecyl sulfate; in such a system, parvalbumins migrate as a band close to the front...upon further purification, they were identified also by their typical phenylalanine u.v. spectra...
Therefore, in the purification protocol for red drum and spotted seatrout major component parvalbumin, to be described in detail in the following sections, the parvalbumin-containing fractions that eluted during ion-exchange chromatography were monitored by both u.v. absorption ratios and by SDS-PAGE. The fractions demonstrated to be the ones containing the parvalbumins were the ones retained for further purification and ultimate characterization.

This ion-exchange - gel filtration methodology was selected for two reasons: to avoid the problem of extreme dilution of protein fractions in gel filtration and to avoid the need for the small initial volumes of sample demanded by gel filtration, and, second, to avoid the procedure of acetone or ammonium sulfate fractionation, which is time-consuming both to perform and to carry out the dialysis necessary to remove the salt or organic solvent.

For this research, two protocols were used for protein purification: an analytical one, and a preparative one.

The analytical method was carried out first to determine the point at which parvalbumins eluted during ion-exchange chromatography; this procedure involved the application of a smaller amount of extract (200 - 300 mg.) to the ion-exchange column, and elution was monitored at both 254 and 280 nm (Fig. 1) with the parvalbumin-containing fractions confirmed by SDS-PAGE.

Then this analytical procedure was scaled up three to five fold to a preparative procedure. Since one of the purposes of this research was to purify enough protein to characterize for physicochemical characteristics and for immunological cross-reactivity, it
was necessary to begin with enough extract to yield 20 to 40 mg. of purified parvalbumin.

The following sections describe in detail all the steps in the purification protocol.

Preparation of the Muscle Extract

White muscle was dissected out of intact fish, with special care to remove and discard any red muscle tissue that are present in observable quantities within the white muscle tissue.

One hundred grams of white muscle were then weighed on a Brain-weigh B 3000D (Ohaus). This tissue was then ground in 2.5 volumes (250 300 ml) of 10 mM sodium acetate buffer, pH 5.7, at high speed with a Servall Omni-Mixer for 5 minutes and low speed for 10 - 15 minutes. The container holding the sample and buffer was kept embedded in a bucket of crushed ice during the grinding process.

The resulting crude extract was then centrifuged on Sorvall RC-2 refrigerated centrifuge for 40 - 50 minutes at 15,000 r.p.m. (29,000 x g.). After centrifugation, the extract was then passed through glass wool in a Buchner funnel under suction to remove lipids and remaining gross particulate matter. After this extract was clear, and its protein content was in the range of 7 - 9 mg. per ml.

Dialysis

Once the crude extract was prepared, it was extensively dialyzed versus the operating buffer: 10 mM sodium acetate, titrated to pH 5.7 with glacial acetic acid. This buffer was selected because numerous
attempts at ion-exchange chromatography with 20 mM Tris – HCl, pH 7.6 were unsuccessful.

Whenever dialyzed myogen was applied to ion-exchange column that had been equilibrated with 20 mM Tris – HCl pH 7.6, the parvalbumin-containing peaks would not adsorb onto the column as indicated by erratic and non-reproducible elution profiles obtained when the salt gradient was passed through the column.

The higher pKa of Tris, 8.06, over that of sodium acetate (pKa = 4.76), led to its choice as first buffer, due to the low isoelectric point of parvalbumin. When the buffer was changed to 10 mM sodium acetate – acetic acid, pH 5.7, the parvalbumin adsorbed onto the column, as shown by the production of distinct, reproducible elution profiles.

Approximately 200 ml. of extract were poured into a suitable length of Spectrapor No. 1 (Spectrum Medical Industries, Inc.; molecular weight cutoff 6 – 8,000) dialysis tubing that had been soaked for ten minutes in glass distilled de-ionized water (GDW).

Dialysis was carried out in a six liter flask placed on a magnetic stirrer. Six liter complements of buffer were changed every 12 hours for 3 to 4 days. During dialysis an insoluble precipitate would form that would later be removed by centrifugation; its removal would reduce the protein concentration by 1.0 - 1.5 mg. per ml.

Dialysis was considered complete when the pH and conductivity of the myogen equalled that of the buffer. pH was measured with a Beckman Ultrace meter; conductivity, which generally fell within the range of 720 – 740 Siemens, was measured with a Radiometer CDM 83
conductivity meter. When dialysis was completed, the extract was ready to be applied to the ion-exchange column.

**Ion-exchange Chromatography**

Because of the acidic nature of parvalbumins, and thus their high negative charge at neutral pH (Hamoir, 1968), the anion-exchange medium diethylaminoethyl cellulose DEAE 52 (Whatman) was selected for the ion-exchange chromatography (Cooper, 1977; Scopes, 1982).

Before placement in the column, a batch of DEAE was prepared according to the manufacturer's instruction (Whatman Bulletin No. AIEC 201). Twelve to fifteen grams of DEAE were suspended in approximately one liter of 1.0 M sodium acetate, pH 5.7. When the medium was almost completely settled, the supernatant and the slower-settling "fines" were removed by decantation. The settled DEAE was then resuspended in a liter of 10 mM sodium acetate, pH 5.7, and the process of decanting the supernatant and "fines" was repeated 8 to 10 times. At this point the pH of the supernatant approximated that of the buffer.

The DEAE was then degassed in a 2000 ml vacuum flask with a Cole-Parmer vacuum pump, and then refrigerated to 4 degrees C. A 12-ml column with a small quantity of glass wool pressed inside at the tip was then filled with the medium to make a gel bed of the dimensions 15.8 mm. by 57 mm. with a final gel bed volume of approximately 10 ml. The column was equilibrated at 4 degrees C. and all subsequent operations were conducted at that temperature.

To bring the medium to final equilibration with the buffer, 4 to 6 liters of 10 mM sodium acetate, pH 5.7 were passed through the
column over 48 - 72 hours. When the conductivity of the outflow buffer equalled that of the original buffer, the column was considered equilibrated and ready for application of the muscle extract.

For the analytical ion-exchange chromatography, approximately 300 mg. of red drum myogen were applied on two occasions to the DEAE column with a peristaltic pump (Perista). The eluate was monitored once at 254 and again at 280 nm with a Gilson Model 111 ultraviolet detector; 2.5 ml fractions (50 drops) were collected with a Haake Buchler LC 200 fraction collector at 10 - 12 ml. per hour. The protein elution profile was recorded on an Omniscribe printer (Houston Electronics).

As mentioned earlier, the high absorption ratio of 254:280 nm suggests the presence of parvalbumins due to their high proportion of phenylalanine. This aromatic amino acid absorbs maximally between 206 and 267 nm, depending on solvating conditions, with most maxima occurring between 252 and 267 nm (Weisaufer, 1967; Cantor and Schimmel, 1980).

As the red drum myogen was being applied to the column for the analytical procedure, the non-adsorbing proteins would immediately pass through the column. When the absorbance returned to zero, a 250 ml. gradient of 10 - 250 mM sodium acetate pH 5.7 was then begun.

Under the gradient, two predominant peaks were produced that displayed a high 254:280 absorbance ratio (Fig. 1). These two peaks were then analyzed by 12.5% SDS-PAGE, which indicated that they were composed mostly of a 10 - 12,000 molecular weight protein (Figs. 11, 13 and 16). These peaks were then considered to be the ones that each
contained one of the two isoparvalbumins.

SDS-PAGE of whole muscle extract of both red drum and spotted seatrout showed that each species has two isoparvalbumins (Fig. 17). In addition, SDS-PAGE was always performed on the non-absorbed peak of ion-exchange chromatography. As shown in Fig. 15, taken from the preparative procedure, most if not all of the 10 - 12,000 molecular weight components of the myogen were retained on the column, indicating parvalbumin loss, if any, was negligible.

The conclusion was then made that when a red drum or spotted seatrout myogen that had been dialyzed versus 10 mM sodium acetate pH 5.7 was applied to a DEAE column equilibrated with the same buffer, the parvalbumins adsorbed onto the column and could be subsequently separated and eluted by the application of a 10 - 250 mM sodium acetate pH 5.7 gradient.

With this conclusion in mind, and because of the need to obtain 20 - 40 mg purified protein for characterization studies, this analytical procedure was scaled up to a preparative one.

For the preparative ion-exchange chromatography, larger amounts of myogen were applied to the same 10.0 ml column. For red drum, approximately 1300 mg protein were applied; for spotted seatrout, approximately 1000 mg were applied. Figs. 2, 3, and 7 show the elution profiles for the preparative ion-exchange chromatography. Because of the larger amounts of protein applied, the sensitivity of the ultraviolet monitor was set at 2.0 absorption units to prevent the printer from running off the page, thereby obscuring the limits of each peak. All preparative ion-exchange chromatography was monitored
only at 254 nm.

In view of the fact that very little parvalbumin, if any, passed through the column in the unabsorbed peak, as shown by SDS-PAGE (Fig. 15) and since 100 - 160 mg. of parvalbumin-containing peaks were eluted under the gradient, the capacity of DEAE for parvalbumin appears to be at least in the 10 - 15 mg. per ml range.

For the preparative procedures, 10 mM sodium acetate pH 5.7 buffer was used, and the salt gradient was also 250 ml. of 10 - 250 mM sodium acetate pH 5.7. Fractions were collected at the rate of 10 - 12 ml. per hour.

Two peaks from both red drum and spotted seatrout myogen were consistently eluted under the salt gradient, representing the two parvalbumins, and the protein content of each was estimated by the Coomassie Blue dye-binding method. The first peak to elute for each species always contained the greater amount of protein. It was this peak that was designated as containing the "major" parvalbumin component; the other was considered the "minor" component.

The minor component was retained for subsequent analysis by SDS-PAGE (Fig. 16). The major component was retained for further purification and ultimate characterization.

The fractions representing the major parvalbumin components of the two components per species were then pooled and dialyzed 24 hrs. against two changes of 6.0 L of 10 mM sodium acetate, pH 5.7. The sample was then ready for the next step in the protocol, gel filtration.
Gel Filtration

Following ion-exchange chromatography of the muscle extracts of red drum and spotted seatrout, it was necessary to subject the parvalbumin-containing fractions to a three-phase gel filtration to achieve final purification (Scopes, 1977; 1982).

SDS-PAGE of the major component peaks from ion-exchange chromatography indicated several higher molecular weight contaminants as well as the predominant parvalbumin band.

For red drum major parvalbumin, the order of gel filtration chromatography was: Sephadex G-75 (1.6 x 60 cm. column), Sephacryl S-200 (1.2 x 50 cm. column), and Sephadex G-50 (0.8 x 49 cm. column) (Figs. 4, 5 and 6). For spotted seatrout, the order was: Sephadex G-75, Sephadex G-50, and Sephacryl S-200 (same column dimensions) (Figs. 8, 9 and 10). The order was slightly changed for spotted seatrout major parvalbumin to ascertain if final purification could be achieved after only two gel filtration runs.

After each individual gel filtration, SDS-PAGE was performed to test for homogeneity of the protein. Not until the third gel filtration was homogeneity achieved as indicated by a single band with SDS-PAGE (Figs. 11 and 13).

Both Sephadex gel filtration media were allowed to swell in 10 mM sodium acetate pH 5.7 at room temperature for 24 hrs. The buffer-medium slurry was degassed and refrigerated to 4 degrees C. before being poured into the columns. Once the column was poured, it was maintained at 4 degrees C. and 300 - 500 ml. buffer was pumped through it to equilibrate and settle the gel bed.
Sephacryl comes pre-swollen in a liquid suspension. It was poured into its column at 4 degrees C., and then 300 - 500 ml. sodium acetate buffer were passed through it.

Because of the need to load the column with only a volume of sample not exceeding five percent of the total gel bed volume (Reiland, 1971; Cooper, 1977) when good resolution of components is desired (as opposed to the use of gel filtration for desalting), the dialyzed protein sample was concentrated from its original volume of 25 - 35 ml. to 10 - 15 ml. by means of an Amicon ultrafiltration unit equipped with a YM5 membrane (molecular wt. cutoff 5,000). The unit was operated with compressed nitrogen at 55 psi. The ultrafiltrate was monitored for protein loss by the Coomassie Blue dye-binding method; none was observed.

To achieve a final sample volume of 3 - 5 ml. the sample was then subjected to water removal by osmosis against sucrose (Scopes, 1982). This was carried out by placing the protein sample from ultrafiltration in a dialysis bag which was then packed in reagent grade sucrose.

When the final volume was reached, the sample was then dialyzed against the operating buffer.

This reduction in volume by ultrafiltration and osmosis versus sucrose was performed on the sample before each gel filtration procedure. The sample was then applied to the gel filtration column and the operating buffer was pumped through the column at 12.0 ml. per hour. The parvalbumin always eluted at the point corresponding to a molecular weight of 10 - 12,000. The parvalbumin peak was the only one observed during elution.
Table 1 shows the balance sheet of the yield of major parvalbumin components from red drum and spotted seatrout.

**Determination of Purity: Electrophoresis**

Purity of the protein isolates was determined by sodium dodecyl sulfate-polyacrylamide tube gel electrophoresis (SDS-PAGE) and high pressure liquid chromatography (HPLC). Polyacrylamide was selected over starch gel because of the former's ability to produce smaller pore sizes needed to resolve a small protein such as parvalbumin. A concentration of 12.5% polyacrylamide was used for all electrophoretic analyses. At this concentration parvalbumin appears as a major solitary band close to the anodal end of the gel (Sullivan et al., 1973).

Stock solutions, gels, and buffers were prepared according to Hames (1981):

**Stacking gel buffer stock:** 0.5 M Tris (hydroxymethyl) aminomethane - HCl, pH 6.8. 6.0 g. Tris was dissolved in 40 ml. water, titrated to pH 6.8 with approximately 48 ml. 1.0 M HCl, and brought to 100 ml. final volume with water. (NB: "Water" always refers to glass-distilled, de-ionized water).

**Separating gel buffer stock:** 3.0 M Tris - HCl pH 8.8. 36.3 g. Tris and 48 ml. 1.0 M HCl were brought to 100 ml. final volume with water.

**Reservoir buffer stock:** 0.25 M Tris, 1.92 M glycine, 1.0% SDS, pH 8.3. 30.3 g. Tris, 144.0 g. glycine, and 10.0 g. SDS were dissolved in and brought to 1.0 L. with water.

**Acrylamide stock:** 30.0 g. acrylamide, 0.8 g. bisacrylamide, brought to a final volume of 100 ml. with water.

Separating gel solutions were prepared by mixing 12.5 ml. of acrylamide stock, 3.75 ml. separating buffer stock, 0.3 ml. 10.0% SDS,
1.5 ml. 1.5% ammonium persulfate, 11.95 ml. water, and, after degassing, 0.015 ml. (15ul) of N,N,N',N' - tetramethylethlenediamine (TEMED). This solution was immediately pipetted up to a level of 10.0 cm. in several .635 x 12.7 cm. glass tubes that had been sealed at one end with "Parafilm". A small amount of water was then pipetted onto the gel surface to flatten the meniscus and to prevent oxygen inhibition of polymerization. The solution would polymerize and harden in 15 - 20 min. at room temperature.

Once polymerization of the separating gel was complete, the overlying water was removed and the stacking gel was pipetted into the tube for a length of approximately 1.5 cm. This gel solution was prepared by mixing 2.5 ml. acrylamide stock, 5.0 ml stacking gel buffer stock, 0.2 ml 10% SDS, 1.0 ml. 1.5% ammonium persulfate, 11.3 ml. water, and, after degassing, 0.015 ml. TEMED. When the stacking gel polymerized (20 - 30 min.), the tube gels were ready for use.

Parvalbumin samples of 10 - 12 ml. at 2.5 - 3.5 mg. per ml. were dialyzed for 15 - 18 hrs. versus three changes of 6.0 L water following all gel filtration steps. An aliquot of each sample was taken and the volume of adjusted with water to produce a final concentration in the aliquot of 1.0 mg. per ml.

One-half ml. of each parvalbumin sample, representing 500 ug., was combined with 0.5 ml. 20% SDS (100 mg.) and 40 ul. beta-mercaptoethanol. This preparation was then allowed to stand 15 - 18 hrs. at room temperature. It was then heated in a 100 degree C. water bath for an additional ten minutes to complete the denaturation of the protein.
One to two drops of 50% sucrose – 0.1% bromophenol blue as the tracking dye were then added to each sample and thoroughly mixed.

Twenty, 40, and 80 µl. aliquots of each parvalbumin, representing 10, 20, and 40 µg. protein, were then applied to the gels. Reservoir buffer (25 mM tris, 192 mM glycine), was made by adding 100 ml. reservoir buffer stock to 900 ml. water, and was added to the upper and lower buffer chambers. The electrophoresis was carried out for approximately 1.5 hrs. at 25 ma and 75 v. on a Hoffer tube gel electrophoresis apparatus and power supply (current density = 3.8 ma/sq. cm.). Temperature of the lower buffer chambers was maintained at 20 degrees C. with a Forma Scientific CH/P Model 2006 bath and circulator.

When the tracking dye reached the bottom of each tube, the current was turned off, the gels removed from each tube and placed in 100 ml. 0.1% Coomasie Blue R –250 – 17% acetic acid – 41.5% methanol for fixation and staining. Gels were kept in the staining solution 15 – 18 hours and then placed in 100 ml. of 5.0% methanol – 70% acetic acid for 36 – 48 hrs. for destaining. Gels were stored in 7.5% acetic acid.

Each parvalbumin from red drum and spotted seatrout appeared as a single band near the anodal end of the gel. No other bands were evident (Figs. 11 and 13).

Electrophoresis of whole myogen was also carried out for both species of fish (Fig. 17) and on the minor parvalbumin peaks for both (Fig. 16).
Determination of Purity: High Pressure Liquid Chromatography

As an additional measure of homogeneity of the protein, the isolates were subjected to HPLC with an LDC Model III unit. Red drum major parvalbumin (750 and 850 ug.) and spotted seatrout major parvalbumin (250 and 300 ug.) were dissolved in 10 mM sodium acetate, pH 5.7, and passed through a 6.4 mm. x 25 cm. GF - 250 gel filtration column that had been equilibrated with the same buffer. The eluted protein was monitored simultaneously at 254 and 280 nm. The high 254:280 absorbance ratio characteristic of parvalbumins (Pechere, 1968), due to the protein's high content of phenylalanine, is readily evident (Figs. 12 and 14).

Purified protein was then lyophilized to a dry white powder and stored in a dessicator at 4 degrees C.

CHARACTERIZATION OF THE PROTEINS

Estimation of Molecular Weight

Molecular weights of red drum major parvalbumin and spotted seatrout major parvalbumin were estimated by means of SDS-PAGE (Weber et al., 1969; Kingsbury et al., 1970).

A standard curve plotting relative mobility versus molecular weight was calculated using six known standard proteins on 12.5% polyacrylamide (Fig. 18). The separating gel used was prepared as described above. A stacking gel was not employed due to the homogeneity of the various standard proteins used; stacking gels were used whenever a complex mixture, such as a myogen or chromatography fraction, were
being analyzed. The reservoir buffer was the same as described above for the analytical electrophoresis. Gels were poured to a length of 10.0 cm. in each tube, for both the standards and the parvalbumins.

The relative mobility (R.M.) was calculated for the following standard proteins: lysozyme, mol. wt. 14,300; beta-lactoglobulin, mol. wt. 18,400; trypsin, mol. wt. 24,000; pepsin, mol. wt. 34,700; egg albumin, mol. wt. 45,000; bovine serum albumin, mol. wt. 66,000.

These proteins were prepared for electrophoresis in the manner as described above for the parvalbumins. Ten ul. containing 25 ug. of each standard was added to each of six tubes, and electrophoresis was carried out for approximately 1.5 hrs. at 25 ma. and 75 v. (current density = 3.8 ma./sq. cm.).

When each gel was removed from the glass tube after the run, two measurements were immediately made: the total length of the tube, and the distance the tracking dye had migrated.

Staining - destaining was carried out as described above on page 32. Following this, two additional measurements were made: the total length of the gel, and the distance the protein band had migrated.

To calculate the relative mobility of each protein while at the same time compensating for the increases in gel length that occur during staining - destaining, the following formula was used (Weber et al., 1969):

\[
R.M. = \frac{G-b}{G-a} \times \frac{p}{D}
\]
where \( G-b \) = gel length before staining - destaining;
\( G-a \) = gel length after staining - destaining;
\( P \) = distance migrated by the protein band;
\( D \) = distance migrated by the dye band.

Three electrophoretic runs were performed on each set of standards, the average R.M. was calculated, and the standard curve produced from that data versus the standards' known molecular weight.

The following lists the standard proteins used and their average relative mobility on 12.5% polyacrylamide gels:

Lysozyme: 0.84
B-lactoglobulin: 0.77
Trypsin: 0.63
Pepsin: 0.38
Egg albumin: 0.37
Bovine serum albumin: 0.27.

It will be observed that pepsin displayed aberrant behavior with this electrophoretic system, and its R.M. value was not used to calculate the standard curve. It was also attempted to calculate a curve using 15% acrylamide, but a linear relationship could not be established.

The R.M. for red drum and spotted seatrout major parvalbumins was calculated four times for each protein, and from the average value the molecular weight of each was estimated from the standard curve.

For red drum parvalbumin, the following values were obtained:
0.94, 0.94, 0.92, 0.93. Avg. = 0.93.

For spotted seatrout parvalbumin, the following values were obtained: 0.89, 0.89, 0.89, 0.90. Avg. = 0.89.

These values correspond to the following molecular weight estimations: red drum major parvalbumin, 11,300; spotted seatrout major parvalbumin, 12,500.

**Determination of Isoelectric Point**

As stated earlier, a characteristic feature of parvalbumins is an isoelectric point in the range of 3.9 - 5.0. The isoelectric points for red drum and spotted seatrout major parvalbumins were estimated according to a modification of the method of Righetti (1983).

Ten mm. gels were poured into acid-cleaned glass tubes that had been sealed off at one end with "Parafilm". The gel solution was made by mixing 2.5 ml. of 30% monomer solution, 0.5 ml. glycerol, 0.5 ml. pH 2.5 - 5.0 ampholytes (Pharmacia), 50 ul. 10% ammonium persulfate, and after degassing, 23 ul. TEMED.

The tubes were overlaid with water and allowed to stand 30 min. for polymerization; they were then installed in a Hoffer tube gel electrophoretic apparatus. The anolyte used was 10 mM phosphoric acid; the catholyte used was degassed 20 mM sodium hydroxide. The gels were run overnight at 200 v. and 15 degrees C.

Fifty ug. each of red drum and spotted seatrout major parvalbumins were mixed with an equal volume (approximately 25 ul.) of a 20% glycerol solution. Fifty ul. of a 5% glycerol - ampholytes (pH
2.5 - 5.0) were placed on top of each gel. The 50 ul. protein - glycerol sample was then added. An additional gel was left blank from which to determine the pH gradient.

After development, the gels containing the protein were removed and stained overnight in 0.125% Coomasie Blue R-250 - 40% ethanol - 10% acetic acid and destained overnight in 40% ethanol - 10% acetic acid.

To determine the pH gradient, 2.0 mm. slices from the blank gel were sliced off and placed overnight in Eppendorf tubes with 1.0 ml. water. These samples were allowed to stand overnight, and the pH of each determined. A gradient of pH versus distance of migration along the gel could thus be established (Fig. 19).

Red drum major parvalbumin was represented by a single band; for spotted seatrout major parvalbumin by a very narrow secondary band less than a mm. from the main band on the cathodal side was observed. The migratory distance of the main bands was measured, and the isoelectric point for each was estimated from the gradient.

For red drum major parvalbumin the following values were obtained:

migratory distance: 44 mm.

isoelectric point: 4.35

For spotted seatrout major parvalbumin the following values were obtained:

migratory distance: 48 mm.

isoelectric point: 4.15
Amino Acid Analysis

Each purified major parvalbumin component from red drum and spotted seatrout was analyzed for amino acid content.

One mg. (1.00 mg.) of red drum major parvalbumin and 1.20 mg. of spotted seatrout major parvalbumin were weighed on a Sartorius analytical balance. Each sample was hydrolyzed in approximately 4 ml. of constant boiling point HCl (Sigma) for 24 hrs. at 109 degrees C.

Hydrolyzed samples were sent to the Department of Zoology at the University of Texas at Austin for analysis. The amino acid profiles have been calculated as follows:

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Red Drum</th>
<th>Spotted Seatrout</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASP</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>ASN</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>THR</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>SER</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>GLU</td>
<td>8</td>
<td>11</td>
</tr>
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<td>ALA</td>
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<td>19</td>
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<tr>
<td>VAL</td>
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<td>4</td>
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<td>1</td>
</tr>
<tr>
<td>ILE</td>
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<td>5</td>
</tr>
<tr>
<td>LEU</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Amino Acid</td>
<td>Red Drum</td>
<td>Spotted Seatrout</td>
</tr>
<tr>
<td>-----------</td>
<td>----------</td>
<td>-----------------</td>
</tr>
<tr>
<td>PHE</td>
<td>10</td>
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<td>LYS</td>
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<td>HIS</td>
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<td>1</td>
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<td>ARG</td>
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<td>1</td>
</tr>
<tr>
<td>CYS</td>
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<td>2</td>
</tr>
<tr>
<td><strong>Total:</strong></td>
<td>108</td>
<td>110</td>
</tr>
</tbody>
</table>

The following calculations were made to arrive at this amino acid composition; they were performed with the assistance of Prof. Kathy Matthews, Department of Biochemistry, Rice University.

Red Drum Major Parvalbumin

<table>
<thead>
<tr>
<th>AA</th>
<th>nmoles</th>
<th>calculated</th>
<th>no. res. mol. wt.</th>
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<tr>
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<tr>
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<tr>
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<td>Total:</td>
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**Spotted Seatrout Major Parvalbumin**

<table>
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<td>------------------</td>
<td>---------</td>
</tr>
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<tr>
<td>Total:</td>
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<td></td>
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<td>11,464</td>
</tr>
</tbody>
</table>

See also Tables 2 and 3 for amino acid composition of 11 other parvalbumins as compared to red drum and spotted seatrout major parvalbumins.

**Determination of Calcium Binding Constant**

The binding constant of the purified parvalbumins for calcium ions was determined by an adaptation of the micropartition method of ultrafiltration (Sophianopolous, 1978).

The first procedure in determining the proteins' affinity for calcium is to produce a calcium-free system; because of parvalbumin's high affinity for the ion, this presents a special difficulty.

A modification of method of Blum et al. (1977) was used to achieve a calcium-free protein for both species of parvalbumin.

Red drum major parvalbumin was incubated for 42 hours in 0.1 M EGTA and 0.1 M EDTA in 20 mM Tris – HCl, pH 7.6. The Tris buffer was used here instead of the original buffer of 10 mM sodium acetate, pH 5.7 because at the lower pH the affinity constant of EGTA for calcium ions is less than 10(4) L-M, whereas at the higher pH it approaches 10(8) L-M (Harafuji et al., 1980).

Specifically, 5.415 mg. of red drum major parvalbumin contained
in 5.6 ml. of Tris buffer were mixed with 223 mg. of EDTA and 228 mg. of EGTA. After 42 hours of incubation with chelator, dialysis versus 20 mM Tris - HCl, pH 7.6 was initiated and carried out for 19 changes of 1.0 L volumes of buffer over a period of 5 days. For the first three buffer changes 0.5 M NaCl was present.

For spotted seatrout major parvalbumin, 13.25 mg. of protein were dissolved in 10.0 ml. of 20 mM Tris - HCl, pH 7.6, and to that solution were added 190 mg. of EGTA and 186 mg. of EDTA, to produce a final concentration of 0.05 M for both chelators.

Dialysis for both proteins versus the same Tris buffer was carried out over 7 days for 26 changes of 1.0 L volumes. The first three buffer changes also contained 0.5 M NaCl.

Following dialysis, red drum major parvalbumin was present in a concentration of 0.196 mg. per ml., which represented 17.2 nmoles protein per ml. Spotted seatrout major parvalbumin was present at a concentration of 0.385 mg. per ml., which represented 32.1 nmoles protein per ml.

A calcium - 40 - chloride stock solution was made by drying a quantity of calcium carbonate for 48 hrs. at 95 degrees C. over anhydrous calcium chloride (Harafuji et al., 1980). Of this dried sample, 10.9047 gm. were dissolved in approximately 20 ml. of 12.0 N HCl. Glass distilled, deionized water was then added to produce a final volume of 108.95 ml. This yielded a 1.0 M solution of calcium chloride according to the chemical reaction:

\[ \text{CaCO}_3 + 2 \text{HCl} \rightarrow \text{CO}_2 + \text{H}_2\text{O} + \text{CaCl}_2 \]
Of this stock solution, 0.5 ml. was added to 999.5 ml. 20 mM Tris-HCl, pH 7.6 to produce a 0.5 mM solution, pH 7.6, of CaCl2. This solution was then used directly for addition to the known aliquots of calcium-free protein; it contained 1 nmole Ca-40 per 2 ul.

Calcium - 45 (Amersham) had a specific activity of 10 - 40 Ci per mg. Ca2+. Distilled water was added to the original sample to produce a final volume of 4.0 ml. at 20.75 ug. Ca2+ per ml., which equalled 0.524 nmole per ul.

To prevent contamination with extraneous calcium, all glassware used in calcium-binding experiments was kept in a 6.0 N HCl bath until needed. It was then extensively rinsed with glass-distilled water and oven dried at 39 degrees C.

To a known amount of protein was added a known amount of carrier calcium (Ca-40) and a known amount of radioactive calcium (Ca-45). A series of samples was produced for each protein using a constant amount of protein, a constant amount of radioactive calcium, and a range of increasing values of carrier calcium - 40.

For red drum major parvalbumin, 98 ug. representing 8.6 mmoles in 500 ul. buffer were used for each sample; for spotted seatrout major parvalbumin, 96 ug. representing 8.02 mmoles in 250 ul. buffer were used for each sample.

When the CA-40 and Ca-45 were added to the protein, 20 mM Tris-HCl, pH 7.6 was added to bring the sample to a final volume of 1.0 ml. Four twenty-five ul. aliquots were then each added to 5.0 ml. samples of Biofluor (DuPont), and the scintillation activity measured in a Beckman LS 7500 microprocessor controlled liquid
scintillation counter.

Each Biofluor sample was counted twice, and the eight values were averaged and corrected by a factor of 40 to give the counts-per-minute (cpm) per ml. This first cpm determination therefore represented the total calcium (Ca-t) of the sample.

The sample was then placed in a micropartition ultrafiltration unit (Amicon) equipped with a YM 5 membrane (mol. wt. cutoff 5,000). The unit was then centrifuged for 10 - 12 minutes at 4000 rpm (1900 x g.). The ultrafiltrate was then measured as above for its scintillation activity; the cpm value of the ultrafiltrate obtained after centrifugation thus represented the unbound, or free, calcium, since the protein and the calcium with which it was complexed were retained on the other side of the membrane.

The fraction produced by the ratio of the ultrafiltrate cpm to the original sample cpm represented the fraction of the total calcium that equaled the unbound calcium. This fraction multiplied by the total known calcium of the sample yields the amount of free calcium; the amount of free calcium subtracted from the total calcium yields the amount of bound calcium.

From these data a Scatchard plot was produced (Scatchard, 1949, 1964; Barrow, 1981). The method of least squares (Dunn, 1964) was used to form the regression line (Figs. 20, 21, 22 and 23) according to the equation:

\[ Y' = Y + b(X' - X) \]

where \( b = \frac{S(X' - X)(Y' - Y)}{S(X' - X)^2} \)
The use of the least squares method for determining a regression line when calcium binding data are scattered is acceptable when a reasonably accurate estimate of calcium binding affinity and number of binding sites are desired (Dr. John Dedman, Department of Physiology and Cell Biology, Univ. of Texas Health Science Center; pers. comm.).

The coordinates of the Scatchard plot are molecules calcium ion per molecule protein versus that ratio over the concentration of free calcium. The intercepts of the Scatchard line give the number of binding sites per molecule of protein and the affinity constant, according to the equation for the Scatchard line:

\[ v = n - (1/K)(v/[L]) \]

where \( v = \) molecules ligand per molecule protein;

\( n = \) number of binding sites per molecule protein;

\( K = \) equilibrium constant;

\( [L] = \) concentration of free ligand.

The following data on calcium binding were obtained: red drum major parvalbumin binds 1.9 ions calcium per molecule with a \( Ka = 0.56 \times 10^{5} \) L-M; spotted seatrout major parvalbumin binds 2.05 ions calcium per molecule with a \( Ka = 1.9 \times 10^{5} \) L-M.

The Scatchard plots and the calcium binding data for each parvalbumin are shown in Figs. 20, 21, 22 and 23.

**Ultraviolet Absorption Spectra**

Because of their high proportion of phenylalanine and low amounts of the other aromatic amino acids, parvalbumins display a distinct
absorption spectra of three peaks with maxima at or near 250, 254, and 260 nm. and very low absorption at 280 nm. (Pechere, 1973). Both red drum and spotted seatrout major parvalbumins displayed this characteristic spectra when analyzed at neutral pH on an IBM automated spectrophotometer (Figs. 24 and 25). This spectral analysis was performed with the assistance of Prof. J. W. Campbell, Department of Biology, Rice University.

**IMMUNOCHEMICAL ANALYSIS**

**Introduction**

Parvalbumins are highly antigenic, small molecular weight proteins of fish muscle that are the primary allergens for fish allergy in humans. On the basis of their antigenicity, along with their polymorphism and species-specificity in terms of molecular weight, amino acid composition and isoelectric point, as well as their abundance in fish muscle and relative ease of purification, it was proposed that parvalbumins could possibly serve as the basis for an immunological means of identification of fish species, specifically red drum.

For this purpose, an antiserum was produced against red drum major parvalbumin, and it was tested for cross-reactivity against spotted seatrout major parvalbumin and against the myogens of both sciaenid and non-sciaenid fish.

Cross-reactivity was evaluated by three methods: the interfacial ring test (IFRT), double diffusion in 2% Noble agar (method of
Ouchterlony), and by the enzyme-linked immunosorbent assay (ELISA) using a peroxidase-conjugated goat anti-rabbit immunoglobulin G. In some case frozen muscle tissue was used for preparation of extracts for immunochemical analysis, but according to Cowpie (1968) and Kennan and Shakelee (1985) neither length of freezing time nor other factors such as size, sex, age of fish, or extraction techniques affect the ability to identify fish by other biochemical means such as electrophoresis and isoelectric focusing.

Production of Antiserum

Two male white New Zealand rabbits were used in the production of antiserum to red drum major parvalbumin. Prior to immunization with the purified protein, pre-immune serum was withdrawn from each rabbit.

Protein in the amount of 250 ug. was injected into each rabbit beneath the hind foot pads. The protein was first mixed with complete Freund's adjuvant to enhance immunologic response (Herbert, 1978). The total 250 ug. of protein was contained in a final volume of 0.5 ml. of the adjuvant emulsion. Half the total dose was injected beneath each foot pad; no granuloma or abscess formation was ever observed in either rabbit.

Blood was drawn from each rabbit after this primary immunization at 10 day, 24 day, 5 week and 7 week intervals.

Three hundred micrograms of red drum major parvalbumin in aqueous solution were injected at multiple intradermal sites over the shaved abdomen nine and a half weeks after the primary immunization. Blood
was then drawn at 2, 4, 5, 6, 7, 7½, 8, and 8½ week intervals after this secondary immunization.

A tertiary booster of 500 ug. of protein was administered at multiple intradermal sites 10½ weeks after the secondary immunization. Blood was drawn at 5, 7, 9, 23, 30, 38, and 45 day intervals.

All serum samples were obtained with 20 ml. Vacutainer tubes (Becton Dickinson) from the central ear artery.

Each ear was shaved and then swabbed first with alcohol and then with xylene to dilate the vessel. The artery was punctured with a 22 ga. sterile needle, and whole blood collected in the tube. The ear was then thoroughly washed with warm water a soap and then smeared with an antibiotic-steroid veterinary ointment (Panolog, Squibb).

The whole blood was allowed to clot overnight at 4 degrees C. Once the clot was fully retracted, the serum was withdrawn with a Pasteur pipette and centrifuged for 5 minutes at 10,000 rpm (12,000 x g.) to remove residual erythrocytes.

The clarified serum was then stored at -16 C., labelled with the rabbit number and date of collection. Serum samples were always tested individually and never pooled.

**Interfacial Ring Test**

Interfacial ring tests (IFRT) were performed in 4 mm. (i.d.) by 60 mm. glass tubes using approximately 0.05 ml each of antiseraum and antigen and were monitored for up to 33 minutes at ambient temperature (23 - 25 degrees C.). The results were measured according to the intensity of the visible precipitin line formed at the interface
between the antiserum (the lower layer) and the antigen (the upper layer). Tests were conducted against both purified parvalbumins; results are shown in Tables 4 and 5.

At this stage of development, the IFRT appears to offer the greatest feasibility for employment as a screening test that can be used on-site in the field. Such a field test for the tentative identification of red drum or spotted seatrout would be simple in design and operation.

For example, a proposed test kit might consist of a set of glass tubes like the ones described above, a perforated rack with a solid dark background to hold them for observation, a set of standardized anti-parvalbumin antisera, a chest of ice and a utensil, such as a garlic press or mortar and pestle, for producing an aqueous extract from the muscle tissue of the fish.

The muscle fillet could be processed with the utensil and 0.05 ml of the extract could then be added to a set of tubes to which 0.05 ml of antiserum had been added. The development of a precipitin ring at the interface is then carefully timed.

It is important that the test be carried out at 0 - 4 degrees C., since the development of a precipitin reaction by both native and cross-reacting species of fish is greatly accelerated at 25 degrees C. or above (data not shown). This significant decrease in time of precipitin ring formation at this higher temperature obliterates the diagnostic capability of the interfacial ring test.

At 0 - 4 degrees C., however, the difference in reaction time is great enough to allow tentative identification of the fish species.
With the five day post tertiary anti-red drum antiserum, for example, ring formation occurs within ten minutes when tested against the homologous parvalbumin, but precipitin ring formation takes a minimum of 18 minutes when the same antiserum is tested against the parvalbumin from spotted seatrout (Table 5).

Double Diffusion in Gels

Complete sets of antisera numbers 1 and 2 were titred against both parvalbumins in serial dilution by means of Ouchterlony double diffusion in 2% Noble agar gels (Ouchterlony, 1958, 1962).

Gels were prepared by heating phosphate buffered saline pH 7.1 to 70 - 80 C. and dissolving agar in it at 2.0 g. per 100 ml. Liquid gel was then pipetted onto 1 x 3 in. glass microscope slides fitted into Gelman trays and allowed to solidify. Wells were punched out by hand: 7 mm. diameter for the central antiserum well, 3 mm. diameter for the peripheral antigen wells.

When the wells were loaded, the slide trays were placed in covered containers and allowed to sit 12 - 15 hours at room temperature before observation.

Room temperature was preferred to 4 degrees C. because the lower temperature delayed and obscured the complete antigen-antibody reactions as measured by the formation of a visible line of precipitation.

Both purified protein antigens were used in the following serial dilutions: 40, 50, 66.6, 100, and 200 ug. per ml. in 20 mM tris - HCl pH 7.6. Twenty-five ul. of antisera were used with 10 ul. antigen (Tables 6, 7, 8 and 9).
Reactions were graded according to the intensity of the precipitin reaction. No precipitin band was considered a negative reaction. A barely visible band was designated f+, faint positive. As bands became denser, they were graded +, 2+, 3+, or 4+; 3+ indicated the sharpest, most distinct band of reaction, while 4+ indicated antigen excess where the band broadened toward the antiserum well.

Three samples of each antisera were also evaluated for cross-reactivity in double diffusion gels against muscle extracts of the following sciaenid fish: red drum, spotted seatrout, black drum, freshwater drum, southern kingfish, and sand trout, and the non-sciaenid fish, vermilion snapper, southern flounder, and sheepshead. The antiserum samples used here were 5 week post-primary, 1 week post-secondary, and 1 week post-tertiary (Table 10).

Fresh muscle extracts for this set of experiments were prepared by grinding 10.0 g. of white muscle in 25 ml. 20 mM Tris - HCl pH 7.6 for 5 minutes. The crude extract was centrifuged for 45 minutes at 15,000 rpm (27,000 x g.). The protein content of each was estimated by the Bradford dye binding method, and then all samples were normalized to 5.0 mg. per ml. by the addition of Tris buffer.

Extracts were used in the following dilutions: 5.0 mg. per ml., 1:10 (0.5 mg. per ml.), 1:100 (0.05 mg. per ml.), 1:1000 (5 ug. per ml.), and 1:10,000 (0.5 ug. per ml.).

From the results produced, it can be seen in Table 10 that all non-sciaenid fish gave negative reactions with the anti-red drum major parvalbumin serum, while the sciaenid fish showed varying degrees of reactivity.
Photographs of gel diffusion slides are shown in Figs. 26, 27, 28 and 29.

**Enzyme-Linked Immunosorbant Assay (ELISA)**

ELISA was carried out on serial dilutions of the purified major component parvalbumins of red drum and spotted seatrout and of the myogens of nine species of fish (Figs. 30 and 31 and Table 11).

This method of immunological analysis was here performed by antigen blotting on nitrocellulose paper using a goat anti-rabbit IgG peroxidase-conjugated antibody as the second antibody (Clausen, 1981; Tijssen, 1985).

Specifically for this ELISA analysis a set of fresh muscle extracts was first prepared.

Ten (10.0) g. of white muscle were dissected from red drum, black drum, spotted seatrout, freshwater drum, vermillion snapper, sheepshead, sand trout, southern flounder, and southern kingfish. Each muscle sample was ground at moderate speed for 5 min. in 25.0 ml. 20 mM Tris-HCl pH 7.6 and then centrifuged for 45 min. at 15,000 rpm. (27,000 x g).

Each sample was then dialyzed versus 10 changes of 1.0 L volumes of 20 mM Tris-HCl pH 7.6. After a second centrifugation of 30 min. at 15,000 rpm., the samples were estimated for protein content by the Coomassie Blue G250 dye binding method. All samples were then diluted to 2.0 mg. per ml. by addition of the proper amount of Tris buffer; dilutions of 1:10, 1:100, and 1:1000 were made for each myogen.

ELISA was then performed as follows: a 15 x 9.2 cm. sheet of
nitrocellulose paper (Transblot, Biorad) was cut into two pieces and each was divided into 20 squares with a blunt pencil. Each horizontal row was numbered 1 through 10. The paper sections, each 7.5 x 4.6 cm., were moistened with Tris-buffered saline pH 7.5 (20 mM Tris, 500 mM NaCl) and allowed to dry on a large piece of Whatman filter paper.

Ten (10.0) μl. aliquots of each dilution of each myogen were then applied to the paper and allowed to dry. Each section of paper was then incubated in a blocking solution of 150 ml. of 3.0% gelatin (EIA grade, Biorad) in 0.05% Tween - Tris-buffered saline (TTBS) for 0.5 hr.

After this, each section was then incubated for 2 hrs. with 50 ml. of the first antibody solution, which was a 1:100 dilution of the 7 day post tertiary antiserum no. 1 with 1.0% gelatin - TTBS (the antibody buffer).

All incubations and washings were done with gentle agitation on the shaker table. Two 10-minute washings with 100 ml. of TTBS pH 7.5 followed.

The next incubation was with the second antibody solution which consisted of a 1:3000 (40 μl./120 ml.) dilution of the peroxidase-conjugated anti-rabbit IgG (Sigma) with antibody buffer. This two hour incubation was then followed with 2 ten-minute washings with 100 ml. TTBS.

Finally, the paper sections were immersed in the color development solution which was made by the addition of a mixture of 60 mg. of 4-chloro-1-napthol (HRP Color Development Reagent, Biorad) and 20.0 ml. ice cold methanol to a mixture of 60 ul. stabilized 30% hydrogen
peroxide (Mallinckrodt) and 100 ml. TTBS.

Color development would take 10 - 15 min. All myogens showed some degree of cross-reactivity with the anti-red drum major parvalbumin antiserum (Fig. 30).

To examine the possibility that the positive reactions with non-sciaenid myogens were due to non-specific binding of conjugated antibody to components of the antigen blot, 0.5% casein (Sigma reagent) was added to the blocking solution and 0.25% casein added to the antibody buffer (Fig. 31).

Non-specific reactions are common in enzyme immunoassays (Tijssen, 1985) and casein, since it is a mixture of various proteins of different sizes, amino acid sequences and surface charges, has been shown to be superior in many instances over to bovine serum albumin or gelatin as a blocking agent to prevent or minimize non-specific binding (Kenna, et al., 1985; Taborsky, 1974).

From the results shown in Fig. 31, the non-sciaenid positive reactions are still present.

ELISA was then performed using a 5 week post primary antiserum, to consider the possibility that the broad reactivity was due to the increased specificity of the older antiserum. Here a 1:50 dilution of the first antibody and a 1:2000 dilution of the second antibody were used. Results are shown in Table 11.

Additionally, the myogens of vermillion snapper and southern flounder, both non-sciaenids, were analyzed by concurrent ELISAs using 7 day post tertiary antiserum for one test, and pre-immune serum for
the other. The antiserum ELISA yielded positive for both myogens, while the pre-immune ELISA was negative.

ELISAs were also performed with 7 day post tertiary antiserum on both purified major parvalbumins. Both reacted positive with 2 ul. aliquots of 1.0 mg. per ml. and dilutions of 1:10 and 1:100, and negative with dilutions of 1:1000 and 1:10000. When pre-immune serum was used as the first antibody, all samples tested negative, indicating that positive reactions were not due to conjugated antibody reacting with endogenous IgG in the pre-immune serum.

CONCLUSIONS

The physico-chemical characteristics determined in this research for the major parvalbumins components of red drum and spotted seatrout are in close agreement with the published data on other parvalbumins.

The following data on molecular weight and isoelectric point are taken from Pechere et al. (1973) who elaborated it from multiple sources in the literature. The data is presented with the data on red drum and spotted seatrout major parvalbumins for comparison:

**Molecular weight:** thornback ray 4.45: 11,467
carp 4.25: 13,100
carp 4.1: 14,800
tilapia 4.4: 11,450
tilapia 4.1: 11,280
hake 4.36: 11,470
cod 4.4: 14,700
red drum major parvalbumin: 11,305
spotted seatrout major parvalbumin: 11,464

Isoelectric point: thornback ray: 4.45
carp: 4.25, 4.47
cod: 4.4, 4.1
tilapia: 4.4, 4.1
frog: 4.5, 4.88
pike: 5.0, 4.2
spotted ray: 4.65
red drum major parvalbumin: 4.35
spotted seatrout major parvalbumin: 4.15

Table 2, taken from Demaillé et al. (1974a), shows the amino acid composition of 15 parvalbumins. Table 3 shows the amino acid composition of red drum and spotted seatrout major parvalbumins. The high proportion of phenylalanine, glycine, alanine, leucine, and lysine and the absence of tyrosine and tryptophan, features common to the parvalbumins will be observed in the red drum and spotted seatrout proteins.

Both parvalbumins displayed the characteristic spectra with maxima at or near 250, 254, and 260 nm, with very low absorption at 280 (Fig. 24 and 25). These same spectra have been observed for all other parvalbumins that have been examined (Pechere et al., 1973).

Concerning calcium binding constants, the data obtained for red drum major parvalbumin (Ka = .56 x 10(5) L-M) and spotted seatrout
(K_a = 1.9 \times 10^{(5)} \text{ L-M}) were somewhat at the lower end of the known range of calcium binding affinity constants for parvalbumins. Most reported values lie in the range of 10^{(6)}-10^{(7)} \text{ L-M} (Benzonana \textit{et al.}, 1972) to 10^{(8)}-10^{(9)} (Haiech \textit{et al.}, 1979; Moeschler, 1980).

Several possible explanations exist for the lower values obtained for red drum and spotted seatrout major parvalbumins. There is, first of all, the problem of obtaining a completely calcium-free system, something that can be difficult with a protein with such a high affinity for calcium. Both proteins evaluated here were both incubated directly with a dual chelator system of EGTA and EDTA and then exhaustively dialyzed continuously for seven days to remove the chelators.

There is also the problem of extraneous calcium being introduced into the system and lowering the apparent affinity constant. When protein is used at the nanomolar level in calcium binding studies, even trace amounts in deionized water can depress the binding constant (Dr. John Dedman, pers. comm.).

The number of ligand ions per molecule of protein, which indicates the number of binding sites per molecule, was, however, in excellent agreement with the established number of two calcium binding sites per parvalbumin molecule. From the Scatchard plots shown in Figs. 20 and 22, red drum major parvalbumin has 1.9 binding sites per molecule, while spotted seatrout major parvalbumin has 2.05.

From the immunological data, it is apparent that immunoidentification of sciaenid fish will be able to be carried out only with certain constraints. These constraints are directly related to the sensitivity of the tests employed in this research.
ELISA is sensitive at the picogram level (Tijssen, 1985), while the less sensitive double diffusion in gels relies upon multivalent antigens that are capable of forming a lattice-like aggregate that creates a visible precipitin band (Cowles, 1961). Nor is double diffusion in gels responsive to slight changes in protein structure in the manner of, for example, microcomplement fixation (Arnhheim, 1967).

The interfacial ring test (Garvey et al., 1977; Otterness and Karush, 1982), according to the data in Tables 4 and 5, could provide at least a reasonably accurate screening test for the on-site identification of red drum from samples of white muscle tissue, as discussed on page 49.

Based on comparative studies between the two purified parvalbumins, there is a strong difference in the time course of the formation of a precipitin reaction between the anti-red drum major parvalbumin antiserum and the antigens. Although distinct cross-reactivity is observed between the two species of parvalbumins as the test progresses, the red drum parvalbumin is by far the faster reacting of the two.

The data given here indicate that there is at least a threshold below which the IFRT and double diffusion in gels demonstrate specificity of the anti-red drum major parvalbumin antiserum for the homologous antigen. This specificity is evident from the above-mentioned time course of the IFRT with purified parvalbumins in which red drum major parvalbumin reacts more rapidly than spotted seatrout major parvalbumin, and in gel diffusion, in which the red drum parvalbumin and the red drum myogen which reacts the most intensely with the five week
post primary antiserum.

There is also the level of antigen concentration, as shown in Tables 6 through 10, at which only red drum parvalbumin or red drum myogen is reactive with the homologous antiserum. Photographs of double diffusion gels illustrating the sole reactivity of red drum parvalbumin and myogen are shown in Figs. 26 through 29.

The concept of affinity maturation (Landsteiner, 1936; Carpenter, 1956; Crumpton, 1974), in which the specificity of antiserum broadens with time to include not only the original homologous antigen but also increasing numbers of heterologous antigens, become an important consideration here. This increased specificity is evident from the comparison of gel diffusion analysis of the 5 week post primary versus the 7 day post tertiary antisera against the serial dilution of myogens.

For example, red drum myogen tested positive down to 1:100 against 5 week post primary antiserum, whereas other sciaenids such as sand trout and spotted seatrout were completely negative. Against the 7 day post tertiary taken from the same rabbit 17 weeks and two booster injections later, sand trout and spotted seatrout are positive and the remaining sciaenids that were positive against the 5 week antiserum react more strongly with the more mature antiserum.

This broadened specificity is also evident in ELISA analysis using these same two antisera. No myogen tested positive below 1:10 with the 5 week antiserum whereas four sciaenid myogens tested positive at the 1:100 level with the older serum.

These data suggest that it would be advantageous to the future
development of an immunodiagnostic tool for red drum identification to employ the antiserum at which reactivity with the homologous antigen becomes first apparent.

The wide reactivity of all antisera tested against all myogens by ELISA deserves comment.

As mentioned above, non-specific positive reactions are not at all uncommon problems in ELISA analysis (Tijssen, 1985).

These reactions may result from the non-specific binding of conjugated antibody to non-antigenic components of the antigen blot of the nitrocellulose, or they could result from the secondary antibody binding to endogenous IgG within the primary antiserum. Another possible cause is insufficient washing between incubation steps.

Reaction of conjugated antibody with endogenous IgG can most likely be ruled out since all ELISAs done with pre-immune serum were negative. Furthermore, the rabbits used for antiserum production in this study had never before been inoculated with any other antigen.

The fact that ELISAs were positive not only for sciaenids but also for taxonomically distant species such as southern flounder (order Pleuronectiformes), vermillion snapper (family Lutjanidae), and sheepshead (family Sparidae) (Nelson, 1984), raises a number of questions.

First, we must consider the nature of antigenic determinants.

There are two main factors in the formation of an antigenic determinant, or epitope, on the surface of a globular protein: the primary sequence of amino acid residues, and the tertiary structure, or the three-dimensional conformation of the molecule (Landsteiner, 1936;

It is amino acid residues on the surface, moreover, that are exclusively involved in formation of the epitope (Prager, 1971) and, in a family of homologous proteins, the surface residues will vary while the interior core will remain conserved (Kretsinger and Nockolds, 1973; Reichlin, 1975).

The precise nature of an epitope is still a matter of debate. Atassi (1977), using detailed studies on the antigenic determinants of sperm whale myoglobin and hen egg-white lysozyme, asserts that epitopes are discreet, delineated regions on the surface of the molecule. On the other hand, Benjamin et al. (1984) have proposed that the entire surface of the molecule is a continuum of potential epitopes, with such factors as the host animal playing a part in which epitopes on a given molecule become antigenically active.

It is well-established that parvalbumins are a family of homologous proteins that have been conserved throughout the evolution of the lower vertebrates. Abundant sequence isologies exist among the 13 parvalbumins whose sequences have been determined, especially in the regions of positions 50 - 61 and 90 - 101, where ligand binding occurs (Pechere, 1968; Pechere et al., 1973; Gosselin-Rey, 1974; Demaille et al., 1974a, 1974b).

In addition to their considerable sequential isologies, the parvalbumins also display extremely similar tertiary structures (Pechere et al., 1973; Kretsinger, 1980). According to Coffee et al. (1974), the calcium binding octahedra of parvalbumins and their related helices are very similar in tertiary order.
Whatever the exact nature of the epitope, antibodies combine with antigens at these surface regions; if similarities exist in either sequential or conformational determinants, cross-reactivity among homologous proteins or otherwise related or similar proteins results, since antibodies recognize epitopes, and not molecules (Glynn and Stewart, 1977; Kimball, 1983; Clark, 1986).

Because of the number of different possible epitopes on a given antigen, an antiserum will usually be made up of several subpopulations of antibodies, each specific toward one or more epitopes. Antisera will be polyspecific either in that different antibodies will recognize different epitopes or antibodies subpopulations will vary in their affinity for the same epitope (Berzovsky et al., 1981).

This polyspecificity, as mentioned above, increases with the time from the point of inoculation. Thus, as an antiserum matures in the animal, its cross-reactivity increases (Carpenter, 1956).

From the data produced in this research, this increase in affinity for heterologous antigens is demonstrated, and therefore suggests that an early antiserum will be necessary if immunoidentification of fish based on parvalbumins is to be developed.

This considerable cross-reactivity of parvalbumins is not unusual when considered in the light of their probable evolution.

When the 34 out of 100 amino acid differences between hake 4.36 and frog 4.50 components (both beta lineages) are considered with a branching time of 400 million years ago (MYA) for the divergence of fish and ancestral higher vertebrates, a unit evolutionary period of 9.6 million years is obtained, according to Pechere et al. (1973).
When this rate is extrapolated back (assuming the rate is constant) to the amino acid difference between hake 4.36 and pike 5.0 (an alpha lineage), a gene duplication event can be placed at approximately 500 MYA, which is comparable to the alpha-beta duplication in globins (Pechere et al., 1973) and to the agnathan – gnathostome radiation (Demaille et al., 1974a).

Lack of cross-reactivity among parvalbumins is frequently observed among the different evolutionary lineages. For example, an antiserum against the hake major component parvalbumin, a beta protein, reacts with carp 4.37, 4.25, and 3.95, and with frog 4.50, all beta proteins but this same antiserum does not react with two alpha lineage proteins, coelacanth 5.0 and frog 4.88 (Pechere et al., 1973).

Poor cross-reactivity has been observed among the parvalbumins of carp and of chub, and complete lack of cross-reactivity has been observed between parvalbumin II and III of pike (Gosselin-Rey, 1974). Nor do parvalbumins cross-react with either troponin-C or troponin-I (Demaille et al., 1974b).

Additional complex immunochemical relationships have been observed among the parvalbumins of the Gadidae and of the Cyprinidae. For example, among the Gadidae parvalbumins, the proteins behave immunochemically according to their amino acid composition which reflects their evolutionary lineage. Those parvalbumins showing reactivity against anti-haddock II antiserum are all characterized by the absence of histidine and methionine and by the presence of one residue of tryptophan and tyrosine and 3 cysteine.
The other group of Gadidae parvalbumins, by contrast, react against anti-haddock III antiserum and are all characterized by one histidine and methionine, one cysteine, and no tryptophan and tyrosine (Piront and Gosselin-Rey, 1974; 1975).

Of additional significance is the fact that the immunogenicity and cross-reactivity of parvalbumins are heavily dependent on the proteins' primary structure, as shown by the demonstration that denaturation of a parvalbumin with urea or bet-mercaptoethanol does not result in the loss or decrease of cross-reactivity with the homologous antiserum (Elsayed and Aas, 1971). The amino acid compositions of the red drum and spotted seatrout parvalbumins shows a high degree of similarity in their composition, and suggests considerable sequential similarity.

Parvalbumins are thus probably fairly old proteins and their age, homology and origin by gene duplication are reflected in their immunochemical similarities as demonstrated by IFRT, ELISA, and double diffusion in gels.
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Fig. 1. Analytical ion-exchange chromatography of red drum myogen. Three hundred mg. (300) of myogen dialyzed against 10 mM sodium acetate-acetic acid pH 5.7 were applied to a 12.0 ml. DEAE column equilibrated with the same buffer. Solid line indicates elution profile monitored at 254 nm; dotted line indicates elution profile monitored at 280 nm. Salt gradient was 250 ml. of a 10 – 250 mM sodium-acetate pH 5.7 gradient. Fractions were 2.5 ml. collected at 12 ml. per hr. SDS-PAGE of each of the two large peaks indicated the predominance of a 10 – 12,000 molecular weight protein.
Fig. 1
Fig. 2. Preparative ion-exchange chromatography of 185 ml. (1283.4 mg.) of dialyzed myogen of red drum. Column dimensions: 1.6 x 6.5 mm. 12 ml. syringe. Exchange medium: DEAE-52 equilibrated with 10 mM sodium acetate pH 5.7. Absorbance read at 254 nm. Fractions were 2.5 ml. collected at 12 ml. per hr. Dotted line indicates 250 ml. gradient of 10-250 mM sodium acetate pH 5.7. Peak I was contained in fractions 53-59 and contained 129 mg., of which most was the major parvalbumin component.
Fig. 3. Preparative ion-exchange chromatography of red drum myogen. Peak II (right) eluted at fractions 67-71 and contained 28.6 mg. of protein representing the minor parvalbumin component.
Fig. 4. Sephadex G-75 gel filtration chromatography of Peak I from DEAE 52 column of Fig. 2. Column dimensions: 1.6 x 90 cm. Buffer: 10 mM sodium acetate-acetic acid pH 5.7. Absorbance read at 254 nm. Fractions: 5.1 ml. collected at 12 ml. per hr. Peak eluted at fractions 25-31 and represents 69.3 mg. protein.
Fig. 5. Sephacryl S-200 chromatography of peak from Sephadex G-75 column of Fig. 4. Column dimensions: 1.2 x 50 cm. Buffer: 10 mM sodium-acetate pH 5.7. Fractions: 2.55 ml. collected at 12 ml. per hr. Peak eluted at fractions 9-14 and represented 34.6 mg. protein.
Fig. 6. Sephadex G-50 chromatography of peak from Sephacryl S-200 column of Fig. 5. Column dimensions: 0.8 x 49 cm. Buffer: 10 mM sodium acetate pH 5.7. Fractions: 2.55 ml. eluted at 12 ml. per hr. Peak eluted at fractions 12-14 and contained 21.0 mg. protein which represented the final purified major parvalbumin component of red drum as subsequently determined by SDS-PAGE and HPLC.
Fig. 7. Preparative ion-exchange chromatography of approximately 1000 mg. of dialyzed myogen of spotted seatrout. Column dimensions: 1.6 x 6.5 cm., 12.0 ml. syringe. Exchange medium: DEAE 52 equilibrated with 10 mM sodium acetate pH 5.7. Dotted line represents 250 ml. of 10-250 mM sodium acetate pH 5.7 gradient. Absorbance read at 254 nm. Fractions: 5.1 ml. collected at 12 ml. per hr. Peak I, containing the major parvalbumin component, represented 67.5 mg. protein; peak II, containing the minor component, represented 43.7 mg. protein. Peak I eluted at fractions 43-47, peak II at 54-59.
Fig. 8. Sephadex G-75 gel filtration chromatography of peak I of DEAE 52 column of Fig. 7. Column dimensions: 1.6 x 60 cm. Buffer: 10 mM sodium acetate pH 5.7. Absorbance read at 254 nm. Fractions 11.5 ml. collected at 25 ml. per hr. Peak eluted at fractions 4 and 5.
Fig. 9. Sephadex G-50 gel filtration chromatography of peak from Sephadex G-75 column of Fig. 8. Column dimensions: 1.2 x 50 cm. Buffer: 10 mM sodium acetate pH 5.7. Absorbance read at 254 nm. Fractions: 11.5 ml. collected at 30 ml. per hr. Peak contained 53.2 mg. protein.
Fig. 10. Sephacryl S-200 gel filtration chromatography of peak from Sephadex G-50 column of Fig. 9. Column dimensions: 1.2 x 50 cm. Buffer: 10 mM sodium acetate pH 5.7. Absorbance read at 254 nm. Peak eluted at fractions 20-25 and contained 24.0 mg. protein that represented the final purified major parvalbumin component of spotted seatrout as subsequently demonstrated by SDS-PAGE and HPLC.
Fig. 11. 12.5% polyacrylamide gels from electrophoresis of purified major component of red drum parvalbumin. Left to right, 10, 20 and 40 µg. of protein. Stain: 0.1% Coomassie Blue R-250.
Fig. 12. HPLC of red drum major parvalbumin component. Left peak = 820 ug.; right peak - 750 ug. Absorbance read at 254 (upper line) and 280 nm.
Fig. 13. 12.5% polyacrylamide gels from electrophoresis of purified major component of spotted seatrout parvalbumin. Left to right: 8.75, 17.5 and 35 ug. protein. Stain: 0.1% Coomassie Blue R-250.
Fig. 14. HPLC of major parvalbumin component of spotted seatrout.
Left peak = 250 ug. protein; right peak = 300 ug. Absorbance read
at 254 (upper line) and 280 nm.
Fig. 15. 12.5% polyacrylamide gels from electrophoresis of unabsorbed peak from DEAE 52 ion-exchange chromatography. Left to right: red drum, 100 and 50 ug.; spotted seatrout, 50 and 100 ug. protein. Stain: 0.1% Coomassie Blue.
Fig. 16. 12.5% polyacrylamide gels from electrophoresis of peak II (minor parvalbumin component) from DEAE 52 ion-exchange chromatography. Left to right: red drum, 39 and 78 ug.; spotted seatrout, 40 and 80 ug. protein. Stain: 0.1% Coomassie Blue R-250.
Fig. 17. 12.5% polyacrylamide gels from electrophoresis of whole myogen. Left to right: red drum, 50 and 100 ug., spotted seatrout, 50 and 100 ug., protein. Stain: 0.1% Coomassie Blue R-250.
Fig. 18. Graph indicating molecular weight versus relative mobility of protein on 10.0 cm. 12.5% polyacrylamide gels. Red drum major parvalbumin component: relative mobility = .93, corresponding to an estimated molecular weight of 11,300. Spotted seatrout major parvalbumin component: relative mobility = .90, corresponding to an estimated molecular weight of 12,500.
Fig. 18
Fig. 19. Graph indicating isoelectric point versus distance of migration on polyacrylamide gels. Red drum major parvalbumin component: migratory distance = 44 mm., indicating a pI of 4.35. Spotted sea-trout major parvalbumin component: migratory distance = 48 mm., indicating a pI of 4.15.
Fig. 20. Scatchard plot of calcium ion binding by red drum major par-valbumin component. Intercept with y-axis indicates number of ligand ions bound by one molecule of protein. Intercept with x-axis indicates binding constant of protein for ligand. Regression line computed by method of least squares. Graph shows 1.9 ions of Ca++ per molecule of protein with a binding constant of \(0.56 \times 10^5\) L·M·. 
Figure 20

\[ \frac{[\text{mole protein}]}{\text{M/L}} \times 10^5 \]
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Fig. 21
Fig. 22. Scatchard plot of calcium ion binding by spotted seatrout major parvalbumin component. Intercept with y-axis indicates number of ligand ions bound by one molecule of protein. Intercept with x-axis indicates binding constant of protein for ligand. Regression line computed by method of least squares. Graph shows 2.05 ions Ca++ bound per molecule protein with a binding constant of $1.9 \times 10^{5}$ L-M.
Calcium Binding Data: Spotted Seatrout Major Parvalbumin

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<th>Ca-unb</th>
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Fig. 23
Legend for Figs. 21 and 23: Ca-T, total calcium of sample; CPM-pre, scintillation counts per minute per ml. of sample prior to ultrafiltration; CPM-post, scintillation counts per minute per ml. of supernatant after ultrafiltration; %, ratio of CPM-post/CPM-pre; Ca-bnd, nmoles of bound calcium; Ca-unb, nmoles of free calcium; v, Ca-bnd/nmoles protein in sample (8.6 n mole for red drum major parvalbumin, 16.05 nm for spotted seatrout major parvalbumin); [L], concentration of free ligand.
Fig. 24. U.V. absorption spectrum of 0.5 mg./ml. red drum major parvalbumin component in aqueous soln. Maxima observed at 252, 260, and 265 nm.
Fig. 24
Fig 25. U.V. absorption spectrum of 1.0 mg/ml. spotted seatrout major parvalbumin component in aqueous soln. Maxima observed at 250, 254 and 260 nm.
Fig. 26. Double diffusion gel. Large well: 25 ul. 5wk. post primary AS. Small wells: right, 10 ul. 66.6 ug./ml. red drum major parvalbumin component; left, 10 ul. 66.6 ug./ml. spotted seatrout major parvalbumin component.
Fig. 27. Double diffusion gel. Large well: 25 ul. 1 wk. post secondary AS. Small wells: left, 10 ul. 50 ug./ml. red drum major parvalbumin component; right, 10 ul. 100 ug./ml. spotted seatrout major parvalbumin component.
Fig. 28. Double diffusion gel. Large well, 25 ul. 5 wk. post primary AS. Small wells, left to right, 10 ul. of 1:10 (0.5 mg./ml.) muscle extract of: vermillion snapper, southern flounder, sheepshead, southern kingfish.
Fig. 29. Double diffusion gel. Center well: 25 ul. 5 wk. post primary AS. Small wells, left to right, 10 ul. of 1:10 (0.5 mg./ml.) muscle extract of: red drum, black drum, spotted seatrout, freshwater drum, and sand trout.
Fig. 30. Results of ELISA analysis of serial dilution of myogens of 9 species of fish. Test was carried out on nitrocellulose paper using 3% gelatin-Tween/Tris-buffered saline pH 7.5 as blocking agent. Antiserum used was 7 day post-3° AS No. 1; secondary antibody was antirabbit IgG peroxidase conjugated antibody. Legend: 1, freshwater drum; 2, sand trout; 3, spotted seatrout; 4, sheepshead; 5, red drum; 6, black drum; 7, southern flounder; 8, southern kingfish; 9, vermillion snapper.

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Note: 1:1 equals 2.0 mg. per ml. myogen in 20 mM Tris-HCl pH 7.6 3+ is maximal reaction, f+ is faintly visible reaction.
Fig. 31. Results of ELISA analysis of serial dilution of myogens of 9 species of fish. Test was carried out on nitrocellulose paper using 3% gelatin - 0.5% casein (Sigma) in Tris-buffered saline pH 7.5 as blocking agent. Antiserum used was 7 day post-3° AS No. 1; secondary antibody was anti-rabbit IgG peroxidase conjugated antibody. Legend: 1, freshwater drum; 2, sand trout; 3, spotted seatrout; 4, sheepshead; 5, red drum; 6, black drum; 7, southern flounder; 8, southern kingfish; 9, vermilion snapper.

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Note: No. 10 represents (left to right) red drum peak II 2 ul. and 5 ul. and spotted seatrout peak II 2 ul. and 5 ul. For 1 - 9, 1:1 equals 2.0 mg. per ml. in 20 mM Tris-HCl pH 7.6 3+ is maximal reaction, f+ is faintly visible reaction.
### Red Drum Major Component Parvalbumin

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### Spotted Seatrout Major Component Parvalbumin

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</tbody>
</table>

Legend on following page.
Table 2 Legend: Parvalbumins are identified according to isoelectric point. Total amino acid residues are given in parentheses.

1. Thornback ray 4.45 (110)
2. Spotted ray 4.65 (112)
3. Carp 4.47 (108)
4. Carp 4.37 (108)
5. Carp 4.25 (108)
6. Carp 3.95 (108)
7. Pike 5.0 (109)
8. Pike 4.1 (108)
9. Hake 4.36 (108)
10. Cod 4.4 (107)
11. Cod 4.1 (109)
12. Coelacanth 5.4 (111)
13. Coelacanth 5.0 (111)
14. Coelacanth 4.5 (108)
15. Frog 4.50 (108)

This table was adapted from Demaille et al. (1974a).
Table 3
Amino Acid Composition of Red Drum and Spotted Seatrout Major Component Parvalbumins

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Red Drum</th>
<th>Spotted Seatrout</th>
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<tbody>
<tr>
<td>Gly</td>
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Table 4
Interfacial Ring Test Data

Red Drum and Spotted Seatrout Major Component Parvalbumin, both at 30 ug. per ml., versus serial antisera. Legend: AS30#1 = 30 day post-inoculation serum no. 1. All antisera are post 3° inoculation.

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<td>RF</td>
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<td>RF</td>
<td>ST</td>
<td>ST</td>
<td>ST</td>
<td>ST</td>
</tr>
<tr>
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<td></td>
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<td>4</td>
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</tbody>
</table>

Reactions are graded on intensity of preception band: 0 = no reaction, 1 - 4 = weaker to stronger reaction.
Table 5
Interfacial Ring Test Data

Red Drum and Spotted Seatrout Major Component Parvalbumin. Red Drum at 30 ug./ml, seatrout at 300 ug./ml. All antisera are post 3º No. 1. Legend: AS5 = 5 day post inoculation, etc.

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<th>3</th>
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<td>RF</td>
<td>RF</td>
<td>RF</td>
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Time:

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</tbody>
</table>

Antisera are 5, 7, 9, and 23 days post 3º No. 1. Reactions graded on intensity of precipitin band. 0 = no reaction, 1 - 4 = weaker to stronger.
Table 6
Ouchterlony Titration of Antisera Against Serial Dilution of Red Drum Major Component Parvalbumin.

All antisera were from rabbit No. 1. Gels were 2% Noble agar run for 12-15 hrs. at 25° C. Volumes: anti-serum, 25 ul.; antigen, 10ul.

<table>
<thead>
<tr>
<th></th>
<th>40 ug./ml.</th>
<th>50 ug./ml.</th>
<th>66.6 ug./ml.</th>
<th>100 ug./ml.</th>
<th>200 ug./ml.</th>
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</thead>
<tbody>
<tr>
<td>Pre-immune</td>
<td>-</td>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10 day post 1°</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>24 day post 1°</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>2+</td>
<td>2+</td>
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<td>+</td>
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<tr>
<td>6 week post 2°</td>
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<td>f+</td>
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<td>f+</td>
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<td>f+</td>
<td>f+</td>
</tr>
<tr>
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<td>f+</td>
<td>f+</td>
<td>f+</td>
<td>-</td>
</tr>
<tr>
<td>7 day post 3°</td>
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</tr>
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<td>2+</td>
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<td>+</td>
<td>+</td>
<td>2+</td>
<td>2+</td>
</tr>
</tbody>
</table>

Legend: For this and all following tables, reactions are graded according to intensity of precipitin band or staining reaction: - = no reaction; f+ = faint positive; + to 4+, reactions of increasing intensity.
Table 7
Ouchterlony Titration of Antisera Against Serial Dilution of Spotted Seatout Major Component Parvalbumin.

All antisera were from rabbit No. 1. Gels run as in Table 6.
Volumes: anti-serum, 25 ul.; antigen, 10ul.

<table>
<thead>
<tr>
<th></th>
<th>40 ug./ml</th>
<th>50 ug./ml</th>
<th>66.6 ug./ml</th>
<th>100 ug./ml</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Pre-immune</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>24 day post 1°</td>
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Table 8
Ouchterlony Titration of Antisera Against Serial Dilution of Red Drum Major Component Parvalbumin.

All antisera were from rabbit No. 2. Gels conditions and volumes identical to Tables 6 and 7.

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<th>40 ug./ml</th>
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Table 9
Ouchterlony Titration of Antisera Against Serial Dilution of Spotted Seatrout Major Component Parvalbumin.

All antisera were from rabbit No. 2. Gels conditions and volumes are identical to Tables 6, 7, and 8.

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Table 10
Analysis of cross-reactivity of serial dilutions of myogens of 9 species of fish versus anti-red drum major parvalbumin by double diffusion in 2% Noble agar gels.

Volumes: antiserum, 25 ul.; antigen, 10 ul. Legend: 1, red drum; 2, black drum; 3, vermillion snapper; 4, freshwater drum; 5, sand trout; 6, southern flounder; 7, southern kingfish; 8, sheepshead; 9, spotted seatrout.

<table>
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<th>5.0 mg./ml.</th>
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<th>1:100</th>
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Table 10 (continued)

1 Week Post 3° AS No. 1

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<th>1:10⁴</th>
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5 Week Post 1° AS No. 2

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| 4.          | 2+   | 2+    | f+    | -     | -     |
| 5.          | +    | -     | -     | -     | -     |
| 6.          | -    | -     | -     | -     | -     |
| 7.          | -    | -     | -     | -     | -     |
| 8.          | -    | -     | -     | -     | -     |
| 9.          | -    | -     | -     | -     | -     |
### 1 Week Post 2° AS No. 2

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### 1 Week Post 3° AS No. 2

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| 4.          | +    | +     | -     | -     | -     |
| 5.          | f+   | f+    | -     | -     | -     |
| 6.          | -    | -     | -     | -     | -     |
| 7.          | +    | -     | -     | -     | -     |
| 8.          | -    | -     | -     | -     | -     |
| 9.          | 2+   | +     | -     | -     | -     |
Samples of antigen were serial dilution of myogens of 8 species of fish. Legend: 1, red drum; 2, black drum; 3, sand trout; 4, freshwater drum; 5, vermilion snapper; 6, southern kingfish; 7, spotted seatrout; 8, sheepshead.

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