Regulation of Muscular Contraction

Distribution of Actin Control and Myosin Control in the Animal Kingdom

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ABSTRACT The control systems regulating muscle contraction in approximately 100 organisms have been categorized. Both myosin control and actin control operate simultaneously in the majority of invertebrates tested. These include insects, chelicerates, most crustaceans, annelids, priapulids, nematodes, and some sipunculids. Single myosin control is present in the muscles of molluscs, brachiopods, echinoderms, echiuroids, and nemertine worms. Single actin control was found in the fast muscles of decapods, in mysidaceae, in a single sipunculid species, and in vertebrate striated muscles. Classification is based on functional tests that include measurements of the calcium dependence of the actomyosin ATPase activity in the presence and the absence of purified rabbit actin and myosin. In addition, isolated thin filaments and myosins were also analyzed. Molluscs lack actin control since troponin is not present in sufficient quantities. Even though the functional tests indicate the complete lack of myosin control in vertebrate striated muscle, it is difficult to exclude unambiguously the in vivo existence of this regulation. Both control systems have been found in animals from phyla which evolved early. We cannot ascribe any simple correlation between ATPase activity, muscle structure, and regulatory mechanisms.

INTRODUCTION

Two distinctly different control systems regulate the activity of various muscles. In vertebrate muscles, troponin and tropomyosin are apparently the only regulatory proteins and the control is therefore actin-linked (Ebashi and Endo, 1968; Weber and Murray, 1973). In molluscan muscles, a light chain of myosin acts as a regulatory subunit and the control is therefore myosin-linked (Kendrick-Jones et al., 1970, 1972; Szent-Györgyi et al., 1973). In both types of regulation, contraction is triggered by small amounts of calcium. The resting state is maintained in both because actin and myosin are unable to interact in the absence of calcium, and this occurs by the blocking of sites.
either on actin or on myosin (Eisenberg and Kielley, 1970; Parker et al., 1970; Koretz et al., 1972; Lehman and Szent-Györgyi, 1972; Szent-Györgyi et al., 1973). Despite this overall similarity in function, the interaction between actin and myosin is prevented differently in the two regulatory systems, and the two systems contain different components (Lehman et al., 1972; Kendrick-Jones et al., 1972; Szent-Györgyi et al., 1973). These components cannot be related to each other in any simple fashion, and, since common components are not found, it is very unlikely that one regulatory system could have evolved directly from the other. A comparative study may give insights into the way the two regulatory mechanisms evolved and also explain certain functional differences between various muscles.

In our previous study we presented a preliminary survey involving about two dozen species (Lehman et al., 1972). This initial investigation showed that the myosin control was not restricted to molluscs and was found in a number of invertebrate phyla. The results also led us to suggest that myosin control evolved before actin control, and showed that in a number of muscles both regulatory systems occur simultaneously. Furthermore, we described rapid methods which aided in establishing the presence of the different regulatory systems.

In the present study these observations have been extended to about 100 different animals. We show that myosin-linked regulation is widespread; however, the data are no longer consistent with our earlier view that actin control via troponin represents a relatively recent evolutionary development. In fact, we now find that muscles of many species are doubly regulated and contain both types of control, and that muscles having a single regulatory system are restricted mainly to vertebrates, some of the crustaceans and molluscs. We also describe in detail the methodology on which this survey is based.

Preparations

The actomyosin or myofibril preparations of all the species reported showed calcium-dependent ATPase activities (Table I). In general, actomyosin preparations have a greater calcium sensitivity than the washed myofibrils, and therefore actomyosin was usually studied in greater detail. Our standard approach was to determine whether a particular muscle contained a myosin-linked regulation or only an actin control by use of the competitive actin activation assay (Lehman et al., 1972). If a myosin-linked system was found, thin filaments were prepared and assayed to determine whether, in addition, a thin filament-linked system was also present in this muscle. The presence of an actin control was also explored by a competitive myosin-activation test, particularly in cases when thin filaments were not prepared because of small tissue size.
### Table Ia

**ATPase Assays on Actomyosin and Thin-Filament Preparations: Animals Showing Actin Control**

| Species | Common name | Muscles dissected | AT** activity in CaCl₂<sub>2</sub> 0.1 mM | Calcium sensitivity with rabbit actin | Calcium sensitivity of thin filaments with rabbit myosin |
|---------|-------------|-------------------|-------------------------------------------|--------------------------------------|------------------------------------------------------|
| **Vertebrata** | | | | | |
| Oryctolagus cuniculus | Rabbit | Back | 0.5 | 0 | 85 |
| Mus musculus | Mouse | Leg | 0.3 | 0 | 75 |
| Mus caroli | Hamster | Leg | 0.15 | 0 | 70 |
| Gallus domesticus | Chicken | Pectoral | 0.3 | 0 | 92 |
| Iguana iguana | Iguana | Back | 0.34 | 0 | 76 |
| Rana catesbeiana | Bullfrog (tadpole) | Leg | 0.25 | <5 | 45 |
| Rana pipiens | Grassfrog (adult) | Leg | 0.35 | <10 | 50 |
| Necturus maculosus | Mudpuppy | Back | 0.1 | <10 | 90 |
| Carassius auratus | Goldfish | Dorsal | 0.38 | <10 | 85 |
| Anguilla anguilla | Eel | Dorsal | 0.18 | <10 | 43 |
| Rana esculenta | Snake | Dorsal | 0.2 | 0 | 95 |
| Eptatretus stoutii | Hagfish | Dorsal | 0.08 | <10 | 86 |
| **Protochordata** | | | | | |
| Branchiostoma floridae | Amphioxus, lancelet | Body | 0.23 | 0 | 80 |
| **Arthropoda** | | | | | |
| **Crustacea** | | | | | |
| Mylidae | | | | | |
| Myis mixta | Opossum shrimp | Tail | 1.5 | 0 | 85 |
| Heteromysis formosa | Opossum shrimp | Tail | 0.25 | 0 | 85 |
| **Decapoda** | | | | | |
| Crangon septemspinosus | Snapping shrimp | Tail | 3.5 | 0 | 90 |
| Palaemonetes vulgaris | Prawn | Tail | 3.2 | 0 | 90 |
| Hippolyte catarinensis | Shrimp | Tail | 1.5 | 0 | 90 |
| Hemaris americanus** | Lobster | Tail | 0.7-1.1 | 0 | 90 |
| Homarus americanus | Lobster | Cutter claw | 1.0 | 0 | 90 |
| Homarus americanus | Lobster | Fast abdominal extensor | 0.7-0.9 | 0 | 90 |
| Hemaris vulgaris | Lobster | Tail | 1.1 | 0 | 90 |
| Cambarus sp. | Crayfish | Tail | 0.34 | 0 | 90 |
| Lithodes matinga | Spider crab | Carapace | 0.7 | 0 | 90 |
| Cancer irroratus | Mud crab | Claw, carapace | 1.2 | 0 | 56 |
| Uca pugnax | Black fiddler crab | Claw | 1.0 | 0 | 96 |
| Uca paguax | Gallo-back-fiddler crab | Claw | 0.45 | 0 | 95 |
| Callicrestis sappida | Blue crab | Claw | 0.8 | 0 | 80 |
| Carcinus aestuarianus | Green crab | Claw, carapace | 2.5 | 0 | 84 |
| Pagurus pullator | Hermit crab | Claw | 2.4 | 0 | 86 |
| Emerita talpoida | Sand crab | Leg | 2.3 | 0 | 77 |

* Calcium sensitivity = \(100 - \left(\frac{\text{ATPase}_{\text{CaCl₂}}}{\text{ATPase}_{\text{0}}}\right)\) X 100 of mixtures containing equal weights of actomyosin and added rabbit actin.

† Highest sensitivity obtained, usually at weight ratios of 0.2-0.3 g thin filaments to 1 g rabbit myosin.

‡ In addition, competitive actin-binding assays were performed on washed myofilaments not exposed to high ionic strength solutions and on actomyosin extracts of unwashed muscles. No evidence for myosin control was found.

¶ Payne, M. R., unpublished data.

¶ After 24-h storage in cold.

** In addition, competitive actin-binding assay was performed on actomyosin extracted directly from unwashed muscle. No evidence for myosin control was found.
| Species                  | Common name | Muscles dissected | ATPase activity in CaCl₂ 0.1 mM | Calcium sensitivity of thin filaments with rabbit actin* | Calcium sensitivity with rabbit myosin† |
|--------------------------|-------------|-------------------|---------------------------------|--------------------------------------------------------|---------------------------------------|
| **Arthropoda**           |             |                   |                                 |                                                        |                                       |
| **Insecta**              |             |                   |                                 |                                                        |                                       |
| Gryllus domesticus       | Cricket     | Leg               | 0.50 90                         |                                                        |                                       |
| Schizocerca gregaria     | Locust      | Leg               | 0.16 81                         |                                                        |                                       |
| Schizocerca gregaria     | Locust      | Flight             | 0.07 67 80                      |                                                        |                                       |
| Rhaphidia cornigera      | Silkmoth    | Flight             | 0.13 51                         |                                                        |                                       |
| Lethocerus erodeusanus   | Giant waterbug | Leg              | 0.35 90 78                      |                                                        |                                       |
| Lethocerus erodeusanus   | Giant waterbug | Flight             | 0.35 70 65                      |                                                        |                                       |
| Lethocerus sp. (Florida) | Waterbug   | Leg               | 0.30 88 78                      |                                                        |                                       |
| Blaberus discoidalis     | Cockroach   | Leg               | 0.16 87                         |                                                        |                                       |
| Gromphadorhina portentosa| Cockroach   | Leg               | 0.27 88                         |                                                        |                                       |
| Eublaberus passalis      | Cockroach   | Leg               | 0.23 89                         |                                                        |                                       |
| Dytiscus fuscus          | Cockroach   | Leg               | 0.19 87                         |                                                        |                                       |
| Neophila cinerea         | Cockroach   | Leg               | 0.28 64                         |                                                        |                                       |
| Leucophaea nuda          | Cockroach   | Leg               | 0.35 84 76                      |                                                        |                                       |
| Dinastes hercules        | Hercules beetle, scarab | Flight             | 0.25 90                         |                                                        |                                       |
| **Chilopoda**            |             |                   |                                 |                                                        |                                       |
| Eurypterus sp.           | Tarantula   | Leg, thoracic     | 0.10 84 88                      |                                                        |                                       |
| Limulus polyphemus       | Horseshoe crab | Leg, tail, carapace, flexor | 0.08 97 >85                      |                                                        |                                       |
| **Crustacea**            |             |                   |                                 |                                                        |                                       |
| **Girripedia**           |             |                   |                                 |                                                        |                                       |
| Mitrella polyomorpha     | Goose barnacle | Stalk             | 0.04 >80 89                     |                                                        |                                       |
| Balanus alvarness        | Ivory barnacle | Depressor       | 0.03 >80 72                     |                                                        |                                       |
| Balanus orbilis          | Giant acorn barnacle | Depressor   | 0.09 83 72                     |                                                        |                                       |
| Balanus tiburonbubunion  | Acorn barnacle | Depressor   | 0.11 77                         |                                                        |                                       |
| **Amphipoda**            |             |                   |                                 |                                                        |                                       |
| Orchestia sp. (Florida)  | Beachhopper, sand flea | Body and legs | 0.22 61 51                     |                                                        |                                       |
| Orchestia grilus (Woods Hole) | Beachhopper, sand flea | Body and legs | 0.23 60                         |                                                        |                                       |
| Orchestia trichiana (California) | Beachhopper, sand flea | Body and legs | 0.24 72                         |                                                        |                                       |
| Tubulorchis longiornis   | Beachhopper (large) | Body and legs | 0.26 62 87                     |                                                        |                                       |
| Gammarus locusta         | Sandhopper   | Body and legs     | 0.25 81 61                     |                                                        |                                       |
| Jassa falcata            |              |                   | 0.45 52                         |                                                        |                                       |
| Caprella australis       | Skeleton shrimp | Body and legs | 0.32 84                         |                                                        |                                       |
| **Isopoda**              |             |                   |                                 |                                                        |                                       |
| Cirrothula hermanni      | Pill bug     | Body and legs     | 0.10 79                         |                                                        |                                       |
| Eoitia baliosa           |              |                   | 0.45 93                         |                                                        |                                       |
| Eotia abechnae           |              |                   | 0.22 90                         |                                                        |                                       |
| Orinae asellus           | Sow bug      | Body and legs     | 0.14 78                         |                                                        |                                       |
| Ligia occidentalis       | Rock runner  | Body and legs     | 0.20 86 77                     |                                                        |                                       |
| Ligia ocellata           | Sea roach    | Body and legs     | 0.15 68 62                     |                                                        |                                       |
| **Stomatopoda**          |             |                   |                                 |                                                        |                                       |
| Squilla empusa           | Mantis shrimp | Tail              | 0.25 84 68                      |                                                        |                                       |
| **Decapoda**             |             |                   |                                 |                                                        |                                       |
| Homarus americanus       | Lobster      | Crusher claw      | 0.25 70 85                      |                                                        |                                       |
| Homarus americanus       | Lobster      | Slow abdominal extensor | 0.15-0.25 85                 |                                                        |                                       |
### TABLE 1—Continued

| Species            | Common name       | Muscles dissected          | ATPase activity in 0.1 mM CaCl Grow | Calcium sensitivity of thin filaments with rabbit myosin* |
|--------------------|-------------------|-----------------------------|------------------------------------|--------------------------------------------------------|
|                    |                   |                             | µmol/min/mg                        | %                                                     |
| **Annelida**       |                   |                             |                                    |                                                       |
| *Lumbricus terrestris* | Earth worm | Body wall                        | 0.02                              | 74                                                   | 60                                                    |
| *Nereis virens*    | Glum worm         | Body wall                        | 0.12                              | 93                                                   | 80                                                    |
| *Glycera sp.*      | Blood worm        | Body wall                        | 0.14                              | 97                                                   | 60                                                    |
| *Eudistyla polymorpha* | Featherduster worm | Body wall                   | 0.55                              | 85                                                   | 67                                                    |
| **Sipunculida**    |                   |                             |                                    |                                                       |
| *Golfgia gouldi*   | Acorn worm        | Proboscis retractor            | 0.45                              | 71                                                   | 45                                                    |
| **Priapulida**     |                   |                             |                                    |                                                       |
| *Priapulus condens*| Body wall         |                             | 0.32                              | >95                                                  | 70                                                    |
| **Nematoda**       |                   |                             |                                    |                                                       |
| *Ascaris lumbricoides* | Eel worm         | Longitudinal                       | 0.01                              | 55                                                   | 67                                                    |

*Calcium sensitivity (100 — (ATPase{EGTA}))/ATPase{EGTA} × 100) of mixtures containing equal weights of actomyosin and added rabbit actin.

Highest sensitivity obtained, usually at weight ratios of 0.2-0.3 g thin filaments to 1 g rabbit myosin.

Myofibrils solubilized in 0.6 M NaCl and 1 mM ATP.

It was difficult to obtain suitable experimental material from many animals. The problems included the small size of the animals, difficulty of isolating the muscles free from surrounding tissues, contamination with proteolytic enzymes, extraction of ATPases other than actomyosin, and resistance to homogenization. Of these problems, proteolysis was the most troublesome, and special care was taken to avoid or reduce the exposure of the muscle to intestinal contents. In some cases, proteolytic degradation was reduced by 10⁻⁴ M phenylmethylsulfonyl fluoride. Despite these precautions we were unable to obtain calcium-sensitive actomyosins from the sponge, *Porifera sp.*, the jellyfish, *Mnemiopsis leidyi*, the sea anemones, *Metridium senile* and *Haloclava producta*, the acanthocephalid, *Moniliformis dubius*, the turbellarian, *Bdelloura candida*, the planarian, *Phagocata gracilis*, a number of echinoderms, such as the starfish, *Asterias forbesii*, sea urchins, *Arbacia punctulata*, and *Strongylocentrotus droebachiensis*, the acorn worm, *Saccoglossus kowalevskyi*, and the tunicate, *Ciona intestinalis*.

**WASHED MUSCLES** Muscles were cut with scissors into 3- to 5-mm pieces and homogenized in a Sorvall Omnimixer (Dupont Instruments, Sorvall Operations, Newtown, Conn.) for 5–50 s in a solution containing 40 mM NaCl, 5 mM phosphate buffer (pH 7.0), 1 mM MgCl₂, and centrifuged and resuspended several times with the same solution.

Whenever possible, the muscles were dissected from surrounding tissues (cf. Table I). Dissection, however, was cumbersome in other instances. The
## Table IC

### ATPase Assays on Actomyosin and Thin-Filament Preparations: Animals Showing Myosin Control

| Species       | Common name   | Muscles dissected          | ATPase activity in 0.1 mM CaCl₂ | Calcium sensitivity with rabbit actin* | Calcium sensitivity of thin filaments with rabbit myosin† |
|---------------|---------------|----------------------------|---------------------------------|---------------------------------------|----------------------------------------------------------|
| **Echinodermata** |               |                            |                                 |                                       |                                                          |
| *Echinoderma*  |               |                            |                                 |                                       |                                                          |
| *Thyone briarens* | Sea cucumber | Lantern retractor          | 0.025                           | 90                                    | 0                                                       |
| *Cucumaria frondosa* | Sea cucumber | Lantern retractor          | 0.028                           | 59                                    | 0                                                       |
| **Mollusca**   |               |                            |                                 |                                       |                                                          |
| *Amphineura*   |               |                            |                                 |                                       |                                                          |
| *Cryptochiton stelleri* | Sea bread | Mantle                    | 0.04                            | 70                                    | 0                                                       |
| **Gastropoda** |               |                            |                                 |                                       |                                                          |
| *Acanthoconchus* |                |                            |                                 |                                       |                                                          |
| *Conus trunculatus* | Plate liptpet | Foot                      | 0.15                            | 76                                    | 0                                                       |
| *Polinices duplicatus* | Shark eye | Foot                      | 0.15                            | 95                                    | 0                                                       |
| *Lunatia hexa*   |                |                            |                                 |                                       |                                                          |
| *Thais lapillus*   |                |                            |                                 |                                       |                                                          |
| *Busycon canaliculatum* | Whelk | Foot                      | 0.07                            | 90                                    | 0                                                       |
| **Pectenidae**  |               |                            |                                 |                                       |                                                          |
| *Solemya velum*  |                |                            |                                 |                                       |                                                          |
| *Iroldia limatula* | Adductor | Foot                      | 0.12                            | 95                                    | 0                                                       |
| *Aequipecten irradians* |            |                            |                                 |                                       |                                                          |
| *Placopecten magellanicus* |            |                            |                                 |                                       |                                                          |
| *Pecten maximus* |                |                            |                                 |                                       |                                                          |
| **Brachiopoda** |               |                            |                                 |                                       |                                                          |
| *Lepidostoma*    |                |                            |                                 |                                       |                                                          |
| *Lamellibranchia* |                |                            |                                 |                                       |                                                          |
| *Pectinaria*     |                |                            |                                 |                                       |                                                          |
| *Lamellaria*     |                |                            |                                 |                                       |                                                          |
| *Spisula solidissima* |            |                            |                                 |                                       |                                                          |
| *Anadara ovalis* |                |                            |                                 |                                       |                                                          |
| *Astarte cantanea* |                |                            |                                 |                                       |                                                          |
| *Laevicardium mortoni* |            |                            |                                 |                                       |                                                          |
| **Nemertinae**  |               |                            |                                 |                                       |                                                          |
| *Cerebratulus lacteus* |            |                            |                                 |                                       |                                                          |
| **Cephalopoda** |               |                            |                                 |                                       |                                                          |
| *Loligo pealei* | Squid          | Ventral pharynx retractor  | 0.35                            | 97                                    | 0                                                       |
| **Echiuroidea** |               |                            |                                 |                                       |                                                          |
| *Urechis caupo* | Inns-keeper’s worm or sailor’s penis | Body | 0.09 | 76 |
| **Brachiopoda** |               |                            |                                 |                                       |                                                          |
| *Goniodoma pyramidalis* |            |                            |                                 |                                       |                                                          |
| **Nemertina**   |               |                            |                                 |                                       |                                                          |
| *Nemertes alternans* |            |                            |                                 |                                       |                                                          |
| *Sabella longissima* |            |                            |                                 |                                       |                                                          |
entire legs of the insects, tarantula and sandcrabs, were homogenized, and the cuticle or exoskeleton was removed by filtration through a single layer of gauze. The thoracic and abdominal regions of amphipods and isopods were isolated by removing the head and anal regions with the attached gonads and other internal organs under a dissecting microscope. Care was taken to avoid spilling of the intestinal contents into the thoracic and abdominal cavities. The preparation was blended and the exoskeleton was removed by filtration through a single layer of gauze. The body wall muscles of the annelids, *Urechis*, *Priapulus*, and of the nemertine worms were obtained by cutting the body open and removing the internal organs. The body wall was rinsed and homogenized. Dissection was restricted to the anterior portion of the body, i.e. the region not containing the guts, in the nemertine worms and in *Eudistylia*. Special care was exercised not to disrupt the yellow soft tissues of *Balanus nubilis* during dissection since exposure of the muscles to their content led to a loss of myosin control. The sea cucumbers were anaesthetized in seawater containing 0.1% chloretone before the dissection of the lantern muscles.

**Actomyosin** Washed myofibrils were extracted with 0.6 M NaCl, 5 mM phosphate buffer (pH 7.0), and 1–2 mM ATP (pH 7.0). The insoluble material was removed by a short centrifugation of 10 min × 30,000 g, and the supernatant was tested for ATPase activity. In many cases, the actomyosin was also precipitated by reducing the ionic strength to 0.05 by dilution or by dialysis. The actomyosin precipitate was then washed with 40 mM NaCl and 10 mM phosphate buffer (pH 7.0). The actomyosin preparations frequently contained significant amounts of paramyosin. Paramyosin contamination was reduced in some instances by extracting actomyosin at pH 6.0 with 0.4 M NaCl (Szent-Györgyi et al., 1971). These preparations were tested immediately, with the exception of actomyosin from *Amphioxus* which required overnight storage to show calcium sensitivity.

**Thin Filaments** Preparation essentially followed previous procedures (Szent-Györgyi et al., 1971; Kendrick-Jones et al., 1970; Lehman and Szent-Györgyi, 1972). 0.1 mM EDTA and 5 mM ATP (pH 6.0) was added to washed muscle preparations suspended in 40 mM NaCl, 1–5 mM MgCl₂, 5 mM phosphate buffer pH 6.0. The washed muscle was rehomogenized for a few seconds in a Sorvall Omnimixer. The suspension was centrifuged at 40,000–80,000 g for 30 min. Thin filaments were collected from the supernatant by a 2- to 3-h centrifugation at 80,000–100,000 g. The pellet was rinsed with 40 mM NaCl, 1 mM MgCl₂, 5 mM phosphate buffer (pH 7.0) and resuspended with the aid of a Teflon-coated hand homogenizer. The thin-filament preparations were clarified by centrifugation at 30,000 g for 10 min. No attempt was made to further purify the thin filaments since we wished to retain all of the components of the thin filaments even if some additional impurities were not removed. The myosin or paramyosin impurities in the thin-filament preparations, how-
ever, were negligible. The preparations had no ATPase activity, and contained little or no material with chain weight greater than 80,000 daltons (Fig. 5). Calcium-sensitive Amphioxus thin filaments were prepared from actomyosin, precipitated at low ionic strength, since the preparations from muscle were not calcium sensitive.

**MYOSIN** A rapid procedure was used for myosin preparation since in many cases myosin from invertebrate muscles proved to be highly labile and lost ATPase activity quickly. To a reprecipitated actomyosin solution, 10 mM Mg-ATP (pH 7.0) was added, and then it was immediately centrifuged at 165,000-250,000 g for 3-4 h (cf. Weber, 1956). The upper half of the supernatant solution was dialyzed against 20 vol of 5 mM phosphate pH 6.5 for 3-5 h. The myosin was then diluted with an equal volume of 5 mM phosphate solution, collected by centrifugation and resuspended and washed once in 40 mM NaCl, 5 mM pH 7.0 phosphate buffer and tested immediately for ATPase activity. This procedure removed most of the actin and the ATPase activity of the myosin preparations was activated 4- to 10-fold on addition of rabbit actin (Table III; Figs. 3 and 4). No attempt was made to remove the paramyosin impurity. Whereas this procedure yielded active myosin preparations from a number of invertebrate muscles, in many cases activity diminished significantly after a 1-day storage. Unfortunately, we have been unable to obtain active myosin from a number of insects, gastropods, and polychaete muscles, even though the actomyosin preparations from the same muscles were active and calcium sensitive for several days.

Purified calcium-sensitive myosin was prepared from Limulus muscle with a slight modification. Washed myofibrils were resuspended in a solution consisting of 0.6 M NaCl, 5 mM phosphate buffer (pH 7.0), 1 mM MgATP (pH 7.0) and sedimented for 4 h at 200,000 g. The top half of the supernatant was collected and dialyzed overnight against 40 mM NaCl, 1 mM MgCl₂, 5 mM phosphate buffer (pH 7.0), and then diluted twofold with this solution. The precipitate was collected by centrifugation, dissolved in 0.6 M NaCl, 1 mM MgCl₂, 5 mM phosphate buffer (pH 7.0) and recentrifuged at 100,000 g for 3 h. The top half of the supernatant contained myosin and some paramyosin.

**SCALLOP CALCIUM-BINDING PROTEIN** A calcium-binding protein was obtained from scallop striated muscle. The initial low ionic strength extract of muscles containing soluble proteins was lyophilized and redissolved in 1/10 vol of water. This solution was centrifuged for 2-3 h at 200,000 g. The supernatant was dialyzed against 40 mM NaCl, 5 mM phosphate buffer (pH 7.0), then brought to pH 4.3 by a dropwise addition of 0.5 M HCl and the precipitated protein was removed by centrifugation. The supernatant was neutralized,
lyophilized, and then redissolved in a small volume of water and chromato-
graphed on a Sephadex G-100 column (2.6 X 80 cm). A calcium-binding pro-
tein comprised the last peak and showed only trace impurities on SDS acryla-
mide gel electrophoresis (Fig. 6).

SOURCES OF MATERIAL Animals were obtained from the following
sources: Marine Biological Laboratory, Woods Hole, Mass.; Gulf Specimen
Corporation, Panacea, Fla.; Pacific Biomarine, Venice, Calif.; Peninsula Bio-
logicals, Sand City, Calif.; Sheepsfoot Supply, West Southport, Me.; Millport
Marine Station, Scotland; Southwestern Supply Co., Tucson, Ariz.; Con-
necticut Valley Biological Supply Co., Inc., Southampton, Mass., and various
bait and pet shops. Cockroaches were given by Dr. L. Roth of the Natick Army
Laboratories, Natick, Mass. *Ascaris lumbricoides* was a gift of Dr. D. Fairbairn,
Zoology Department, University of Massachusetts, Amherst, Mass. *Lethocerus
cordofanus* was a gift of Mr. Richard Tregear, Department of Zoology, Oxford
University.

METHODS

The Mg-activated actomyosin ATPase was measured in a pH stat at pH 7.5, 25°
as previously described (Szent-Györgyi et al., 1971). The assay solution consisted of
0.7 mM ATP, 1 mM MgCl₂, and 20-40 mM NaCl. Calcium sensitivity was measured
by comparing the ATPase rates in the presence of 0.1 mM EGTA before and after the
addition of 0.2 mM CaCl₂.

Isolated thin filaments were mixed with rabbit myosin in ratios of 0.3-0.5:1 (wt/wt)
in 0.6 M NaCl and then diluted for the ATPase assays. When sufficient amounts of
thin filaments were available, they were tested at several different weight ratios. Myos-
in preparations were assayed alone and mixed with rabbit actin (2:1 wt/wt) in high
salt, and the specific activities and calcium sensitivities were compared.

The calcium binding of muscle protein suspensions was determined as previously
described (Kendrick-Jones et al., 1970), using a double-labeling technique. In this
technique calcium binding is measured on sedimented protein. Correction is made for
the void volume with the aid of a second label, [³H]glucose, which is not bound by
muscle proteins. The proteins were washed twice with 4.0-8.0 ml [⁴⁴Ca]EGTA buffer,
containing labeled glucose, to ensure that the free calcium concentration was not
significantly altered by the binding on the protein. Calcium binding of the scallop
calcium-binding protein was measured by equilibrium dialysis. The protein was
equilibrated twice for 24 h against 50–100 vol of 40 mM NaCl, 1 mM MgCl₂, 10 mM
imidazole-HCl pH 7.0, containing 25 µM [⁴⁴Ca]EGTA buffers. The dissociation con-
stant of the calcium-EGTA was taken as 1.9 X 10⁻⁷ M at pH 7.0 (Chaberek and
Martell, 1959).

Protein concentrations were measured by the method of Lowry et al. (1951) stand-
ardized by Kjeldahl nitrogen determinations of bovine serum albumin. Sodium
dodecyl sulphate (SDS) polyacrylamide gel electrophoresis was performed using
Coomassic Blue as a stain according to Weber and Osborn (1969).
RESULTS

Distribution of Regulatory Systems

The control systems regulating the contraction of the muscles of approximately 100 species have been categorized (Table II; Fig. 1). The single actin-linked regulation operates in the muscles of all the chordates tested (13 species), including the cephalochordate, *Amphioxus*, which represents an early example of chordate evolution. Among invertebrates, the single actin control is found in most decapod muscles (14 species) and mysidacea (2 species). In addition, *Dendrostomum pyroides*, one of the three sipunculids tested, showed only an actin-linked regulation, although *Dendrostomum* myosin binds calcium (Table III).

The single myosin-linked regulation operates in all of the molluscs tested (23 species), in the two echinoderms, in the two nemertine worms, and in the single examples of echiuroids and brachiopods studied. Both the myosin-linked and the actin-linked regulations function together in the rest of the animals examined. Double regulation was demonstrated in all of the insects tested (12 species), in the two chelicerates (*Limulus* and *Eurypelma*), in the cirripeds (4 species), isopods (6 species), amphipods (7 species), and stomatopods (1 species). The slow crusher claw muscles and the slow superficial abdominal extensor muscles of the lobster also have both controls operating. Double regulation was found in all the annelids tested (four species), and in *Golfingia gouldi*, one of the three sipunculid worms examined. Myosin control is present also in *Phascolosoma agassizi*. The only nematode studied, *Ascaris*, has both regulations functioning.

Experimental Basis for Classification of Control Systems

COMPETITIVE ACTIN-ACTIVATION ASSAY. This assay probes for the myosin control in actomyosin and in washed myofibrils by measuring the effect of excess pure rabbit actin on the ATPase activity in the absence of calcium (Lehman et al., 1972). If a myosin control operates, the myosin is unable to combine with pure actin, and the ATPase activity remains low until calcium is introduced (Fig. 2 d and f). In contrast, pure actin activates those preparations that have an actin control, even in the absence of calcium. This activation results from the ability of myosin to combine with pure actin in these systems, a combination which is not influenced by the troponin-tropomyosin-containing thin filaments present in the preparation (Fig. 2 e).

Full activation of the ATPase by excess pure actin in the absence of calcium, i.e. the loss of calcium sensitivity, demonstrates that a particular muscle contains solely an actin-linked system and that myosin control is not functioning. The interpretation is straightforward and the identification is unambiguous. The lack of activation by pure actin demonstrates that the system contains a
myosin-linked regulatory system. This result, however, does not exclude the additional presence of an actin-linked regulation, and the competitive actin activation assay needs to be complemented by tests probing for actin control (cf. Fig. 2 a and c).

### Table II

**Regulation in Different Animals**

| Species | Ca** binding on TF SDS gels | Troponin sensitivity test (presence of My control) | Competitive Ac activation test (presence of Ac control) | Type of regulation |
|---------|----------------------------|-----------------------------------------------|-------------------------------------------------------|-------------------|
| **Vertebrata** | | | | |
| Oryzalis curvdus | My | TF | My + Ac | Ac |
| Mus musculus* | My | TF | My + Ac | Ac |
| Mecercistus auratus* | My | TF | My + Ac | Ac |
| Gallus domesticus* | My | TF | My + Ac | Ac |
| Iguna ignana | My | TF | My + Ac | Ac |
| Rana catesbeiana (tadpole) | My | TF | My + Ac | Ac |
| Rana pipiens | My | TF | My + Ac | Ac |
| Necturus maculosus | My | TF | My + Ac | Ac |
| Carassius auratus | My | TF | My + Ac | Ac |
| Anguilla anguila | My | TF | My + Ac | Ac |
| Rana esculenta | My | TF | My + Ac | Ac |
| Eptatretus rusti | My | TF | My + Ac | Ac |
| **Protochordata** | | | | |
| Branchiostoma floridae | My | TF | My + Ac | Ac |
| **Echinodera** | | | | |
| **Arthropoda** | | | | |
| **Insecta** | | | | |
| Gryllus domesticus | My | TF | My + Ac | Ac |
| Orthocerus gregaria | My | TF | My + Ac | Ac |
| **Leg** | | | | |
| flight | My | TF | My + Ac | Ac |
| Hylophora comoria (flight) | My | TF | My + Ac | Ac |
| Laphroa corticatus (flight) | My | TF | My + Ac | Ac |
| leg | My | TF | My + Ac | Ac |
| Laphroa sp. (Florida) (leg) | My | TF | My + Ac | Ac |
| Heterocerus dichotoma (leg) | My | TF | My + Ac | Ac |
| Gromphadorhina portentosa (leg) | My | TF | My + Ac | Ac |
| Eulebher pusia (leg) | My | TF | My + Ac | Ac |
| Syprio aegypia (leg) | My | TF | My + Ac | Ac |
| Nympheo cinerea | My | TF | My + Ac | Ac |
| Leucophaeus mediocris | My | TF | My + Ac | Ac |
| Dynastes hercules | My | TF | My + Ac | Ac |
| **Chelicerata** | | | | |
| Limulus polychrous | My | TF | My + Ac | Ac |
| *Eurydbs* sp. | My | TF | My + Ac | Ac |
| **Cephalocra** | | | | |
| **Chirripedia** | | | | |
| Mitella polymorpha | My | TF | My + Ac | Ac |
| Balanus eburneus | My | TF | My + Ac | Ac |
| Balanus nubilis | My | TF | My + Ac | Ac |
| Balanus tintinabulum | My | TF | My + Ac | Ac |
| Species                      | Ca** binding | Troponin test (presence of Ac control) | Ca** sensitivity | Competitive Ac activation test (presence of Ac control) | Type of regulation |
|------------------------------|--------------|----------------------------------------|-----------------|--------------------------------------------------------|-------------------|
| Amphipoda                   |              |                                        |                 |                                                        |                   |
| *Orchei* sp. (Florida)      | +            | +                                      | My              | My + Ac                                                |                   |
| *Orchei* grilibis (Wood Hole) | +            | +                                      | My + Ac         |                                                        |                   |
| *Orchei* traskiana (California) | +            | +                                      | My + Ac         |                                                        |                   |
| *Tularechei* longicornis    | +            | +                                      | My + Ac         |                                                        |                   |
| *Gammarus* locusta          | +            | +                                      | My + Ac         |                                                        |                   |
| *Jassa* falata              | +            | +                                      | My + Ac         |                                                        |                   |
| Caprella acutifrons         | +            | +                                      | My + Ac         |                                                        |                   |
| Isopoda                     |              |                                        |                 |                                                        |                   |
| *Cirrana* harfordi          | +            | +                                      | My + Ac         |                                                        |                   |
| *Idotea* helena             | +            | +                                      | My + Ac         |                                                        |                   |
| *Idotea* ochotensis         | +            | +                                      | My + Ac         |                                                        |                   |
| *Oxyurus* asellus           | +            | +                                      | My + Ac         |                                                        |                   |
| *Ligia* noddensalis         | +            | +                                      | My + Ac         |                                                        |                   |
| *Ligia* sifersis            | +            | +                                      | My + Ac         |                                                        |                   |
| Stomatopoda                 |              |                                        |                 |                                                        |                   |
| Squilla empusa              | +            | +                                      | My + Ac         |                                                        |                   |
| Myxtacea                    |              |                                        |                 |                                                        |                   |
| *Mysis* mixta               |              |                                        | Ac              |                                                        |                   |
| *Heteromysis* formosa       |              |                                        | Ac              |                                                        |                   |
| Decapoda                    |              |                                        |                 |                                                        |                   |
| *Crangon* setaspinosus      |              |                                        | Ac              |                                                        |                   |
| *Palaeomontessa* vulgaris   |              |                                        | Ac              |                                                        |                   |
| *Hypholytus* mexicanus      |              |                                        | Ac              |                                                        |                   |
| *Homarus americanus* (tail) | -            | +**                                    | Ac              |                                                        |                   |
| cutter claw, fast abdominal extensor |  | | | | |
| *Homarus* vulgaris          |              |                                        | Ac              |                                                        |                   |
| *Cambarus* sp.              |              |                                        | Ac              |                                                        |                   |
| *Lithraea* aegagrostis      |              |                                        | Ac              |                                                        |                   |
| *Cancer* irroratus          |              |                                        | Ac              |                                                        |                   |
| *Uca* pagunus               |              |                                        | Ac              |                                                        |                   |
| *Uca* pagulator             |              |                                        | Ac              |                                                        |                   |
| *Callinectes* sapidus       |              |                                        | Ac              |                                                        |                   |
| *Carcimus* noens            |              |                                        | Ac              |                                                        |                   |
| *Pagurus* pallicaris        |              |                                        | Ac              |                                                        |                   |
| *Emerita* talpoida          |              |                                        | Ac              |                                                        |                   |
| Annelida                    |              |                                        |                 |                                                        |                   |
| *Lumbricis* territrix       | +            | +                                      | My + Ac         |                                                        |                   |
| *Hesiers* stern             | +            | +                                      | My + Ac         |                                                        |                   |
| *Olivea* sp.                | +            | +                                      | My + Ac         |                                                        |                   |
| *Echistella* polymorphica   | +            | +                                      | My + Ac         |                                                        |                   |
| Mollusca                    |              |                                        |                 |                                                        |                   |
| *Amphineura*                |              |                                        |                 |                                                        |                   |
| Cryptochiton littleri       | -            | -                                      | My              |                                                        |                   |
TABLE II—Continued

| Species                      | Type of regulation |
|------------------------------|--------------------|
| Gastropoda                   |                    |
| Acma testudinalis            |                    |
| Polinices duplicatus         |                    |
| Lusoria heros                | +                  |
| Thais latillus               | +                  |
| Bucephara canaliculatum      | +                  |
| Pelagicola                   |                    |
| Solenpseud lumtula           | +                  |
| Actinopteryx irredens        | +                  |
| Pterosoma majoritans         | +                  |
| Pten um magnificus           | +                  |
| Mytilus edulis               | +                  |
| Polyplis densus              | +                  |
| Giacello virgulino           | +                  |
| Exotic strenchus             | +                  |
| Mya aruna                    | +                  |
| Mercenaria meruenservia      | +                  |
| Spireta solidissima          | +                  |
| Anadara ovalis               | +                  |
| Astrea santae               | +                  |
| Latistium montani            | +                  |
| Macoma tana                  | +                  |
| Aspesa siresse              | +                  |

| Cephalopoda                  |                    |
| Loligo pealeri               | +                  |
| Brachiopoda                  |                    |
| Glossole pyramidalis         | +                  |
| Echiuroidea                  | +                  |
| Sigueracida                  | +                  |
| Goniola gaudii               | +                  |
| Pholadroma agassiz           | +                  |
| Dendrotermum pyr/>.           | +                  |
| Priapulida                   |                    |
| Priapus caudatus             | +                  |
| Nematoda                     |                    |
| Ascaris lumbricoides          | +                  |
| Nematida                     |                    |
| Carbotholus tectus           | +                  |
| Linnea longissima            | +                  |

| Species                      | Ca** binding | Troponin | Ca** sensitivity | Competitive Ac activation | Competitive My activation test | Type of regulation |
|------------------------------|--------------|----------|------------------|---------------------------|-------------------------------|--------------------|
|                             | My | TF | on TF | My | SDS gels rabbit Ac | rabbit My | control |              | My | control |
| Gastropoda                   |    |    |        |    |                      |                        |          |          |              |    |          |
| Acma testudinalis            | +  | ?  | +     | +  |  -                  | +                      | +        | +        | +              | My |  -       |
| Polinices duplicatus         | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Lusoria heros                | +  | ?  | -     | +  |  -                  | -                      | +        | -        | -              | My |  -       |
| Thais latillus               | +  | ?  | -     | +  |  -                  | -                      | +        | -        | -              | My |  -       |
| Bucephara canaliculatum      | +  | ?  | -     | +  |  -                  | -                      | +        | -        | -              | My |  -       |
| Pelagicola                   |    |    |        |    |                      |                        |          |          |              |    |          |
| Solenpseud lumtula           | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Actinopteryx irredens        | +  | ?  | +     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Pterosoma majoritans         | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Pten um magnificus           | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Mytilus edulis               | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Polyplis densus              | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Giacello virgulino           | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Exotic strenchus             | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Mya aruna                    | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Mercenaria meruenservia      | +  | -  | +     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Spireta solidissima          | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Anadara ovalis               | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Astrea santae               | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Latistium montani            | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Macoma tana                  | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Aspesa siresse              | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Cephalopoda                  |    |    |        |    |                      |                        |          |          |              |    |          |
| Loligo pealeri               | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Brachiopoda                  |    |    |        |    |                      |                        |          |          |              |    |          |
| Glossole pyramidalis         | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Echiuroidea                  | +  | ?  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Sigueracida                  | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Goniola gaudii               | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Pholadroma agassiz           | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Dendrotermum pyr/>.           | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Priapulida                   |    |    |        |    |                      |                        |          |          |              |    |          |
| Priapus caudatus             | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Nematoda                     |    |    |        |    |                      |                        |          |          |              |    |          |
| Ascaris lumbricoides          | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Nematida                     |    |    |        |    |                      |                        |          |          |              |    |          |
| Carbotholus tectus           | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |
| Linnea longissima            | +  | -  | -     | +  |  -                  | +                      | +        | -        | -              | My |  -       |

* Payne, M., unpublished data.
† Ohtsuki et al. (1971) and Hitchcock et al. (1973), isolated troponin from chicken muscles.
‡ Assayed on 2-day old actomyosin preparation having a reduced ATPase activity.
¶ Presence of actin control not tested.
# Lehman et al. (1974).
** Based on the finding of troponin in scarabs by Bullard et al. (1973). Based on the finding of troponin in lobster by Regenstcin and Szent-Gyorgyi (1975).
†† Kendrick-Jones et al. (1970).
‡‡ Szent-Gyorgyi et al. (1973). Troponin-C has been prepared from the hake, Merluccius merluccius, the lizard, Saranus exanthematicus, and the python, Philo rhaco, by Demallie et al., 1974.
FIGURE 1. Distribution of actin- and myosin-linked regulation. Evolutionary tree modified from Borradaile et al., 1963. Horizontal lines: presence of myosin control. Vertical lines: presence of actin control. Horizontal and vertical lines: double systems.
Table III

**ATPase activity, calcium sensitivity, and calcium binding of myosin preparations having regulatory function**

|                   | ATPase activity in 0.1 mM Ca²⁺ | Calcium sensitivity with rabbit actin | Calcium binding at 3 × 10⁻⁷ M |
|-------------------|-------------------------------|--------------------------------------|-------------------------------|
|                   | Myosin with rabbit actin*     | %                                    | µmol/g                        |
| Locust§           | 0.004                         | 0.26                                 | 58                            |
| Tarantula         | 0.001                         | 0.10                                 | 75                            |
| Limulus           | 0.007                         | 0.07                                 | 64                            |
| Eudistyla         | 0.05                          | 0.32                                 | 90                            |
| Urechis           | 0.04                          | 0.2                                  | 76                            |
| Golffinga         | 0.07                          | 0.42                                 | 62                            |
| Dendrostomum¶     | 0.03                          | 0.22                                 | 5                             |
| Lunatia           | 0.01                          | 0.07                                 | 82                            |
| Mya               | 0.03                          | 0.18                                 | 90                            |
| Mercenaria        | 0.05                          | 0.19                                 | 80                            |
| Mytilus           | 0.02                          | 0.1                                  | 89                            |
| Scallop           | 0.02                          | 0.25                                 | 80                            |
| Squid             | 0.05                          | 0.37                                 | 92                            |
| Glottidia         | 0.02                          | 0.34                                 | 77                            |
| Priapulus         | 0.04                          | 0.18                                 | 95                            |
| Squilla empusa    | 0.03                          | 0.22                                 | 87                            |

* 0.3-0.5 g actin to 1 g of myosin.
† (1 - (ATPaseKAT)/(ATPaseCa²⁺)) × 100.
§ Lehman et al., 1974.
§ Tropomyosin also present in the ATPase tests with actin.
¶ Regulation is not linked to myosin. Specific activities between various preparations may vary by about 50%.
** Kendrick-Jones et al., 1970.
†† Szent-Györgyi et al., 1973.

The competitive actin activation assay is an important one and has been employed with virtually all the muscles that we have examined (Table I). It has the advantage of being simple and requiring small amounts of material. Apart from its simplicity, this test is particularly significant since it is performed on contractile systems which have undergone minimal amounts of biochemical manipulations. Thus, the contractile proteins are most likely to be unaltered and present in their in situ molar ratios.

Although the competitive actin activation assay is a simple one, certain precautions must be followed. Actin is mixed with actomyosin in 0.6 M NaCl in various ratios (0.3-1.5 mg actin to 1 mg actomyosin) to ensure that actin is in excess and its combination with myosin is not sterically hindered. The myofibrils are also regularly solubilized with 0.6 M NaCl and 1 mM ATP immediately before the addition of pure rabbit actin. The effect of pure tropomyosin should also be checked since some myosins are only fully activated by an
Figure 2. Assay for regulatory systems with the aid of rabbit myosin and actin. (a) 0.19 mg Glottidea thin filaments with 0.8 mg rabbit myosin. (b) 0.25 mg Amphioxus thin filaments with 0.8 mg rabbit myosin. (c) 0.4 mg Tarantula thin filaments with 0.5 mg rabbit myosin. (d) 1.9 mg Glottidea actomyosin with 0.75 mg rabbit actin. (e) 1.2 mg Amphioxus actomyosin with 1.7 mg rabbit actin. (f) 2.3 mg Tarantula actomyosin with 1.2 mg rabbit actin. 30 mM NaCl, 1 mM MgCl₂, 0.7 mM ATP with 0.1 mM EGTA without calcium (lower curves), with 0.2 mM CaCl₂ (upper curves). These tests show myosin control in Glottidea; actin control in Amphioxus, and a double control in Tarantula.

Actin-tropomyosin complex (Lehman and Szent-Györgyi, 1972). Actomyosins are also frequently reprecipitated to guarantee the removal of a possible excess of troponin-tropomyosin that may be present in the initial actomyosin extract. The concentration of actomyosin in the ATPase activity assays should exceed 30 µg/ml to ensure that most of the protein stays precipitated. The competitive actin activation assay can be readily applied to actomyosin preparations having a wide range of ATPase activities (Table I). The test does not require purification of actomyosin as long as the impurities do not have ATPase activities. In fact, the specific ATPase activities given in Tables I and III are only approximate values since the myosin and actomyosin preparations were contaminated with paramyosin to various degrees.
With proper precautions the actin activation test gave consistent results and could be applied to all muscles. This assay turned out to be the most reliable of the tests we have employed.

**COMPETITIVE MYOSIN ACTIVATION ASSAY** This test probes for the presence of an actin control in actomyosin or in myofibrils. The ATPase rates of an actomyosin are compared with and without calcium in the presence and in the absence of rabbit myosin (0.5–1.5 mg rabbit myosin mixed with 1 mg actomyosin in 0.6 M NaCl). The differences in the ATPase activities give the rates for the complex formed from the rabbit myosin and from the actin originally present in the actomyosin. The calcium dependence of the incremental ATPase activity shows the presence of an actin control in the actomyosin; lack of calcium dependence indicates the absence of actin control. In practice, however, this assay cannot be employed with muscles having a high ATPase activity; because of the large background ATPase level it is difficult to evaluate the calcium sensitivity of the added rabbit myosin. Hence this assay was restricted to muscles whose specific ATPase activities are less than that of rabbit muscle. This assay was particularly useful with muscles from which thin filaments were not prepared (Table II), or where gel patterns of thin filaments indicated considerable losses of tropomyosin.

**MYOSIN** The myosin-linked regulation can be directly demonstrated using purified myosins. Preparations with greatly reduced actin content have been obtained from a number of different organisms (Figs. 3 and 4; Table III). The magnesium-activated ATPase activity of these preparations increased in the presence of calcium 4- to 10-fold with added actin, further indicating that the actin content of the myosin preparation was low. A number of these myosin preparations required calcium for full ATPase activity and also bound calcium (Table III). The presence of the myosin control was established with the aid of partially purified myosin preparations in a number of different molluscan muscles, including scallops, clams, snail, and the squid, in the brachiopod, *Glottidia pyramidata*, in the polychaete worm, *Eudistylia polymorpha*, in tarantulas, *Limulus*, locusts, mantis shrimps, *Priapulus*, *Golfingia*, and *Urechis*.

We note, however, that the lack of calcium-regulated ATPase activity in certain myosin preparations may not necessarily exclude the existence of a myosin-linked regulatory system. Regulation in molluscs may be lost as a result of experimental manipulations (cf. Szent-Györgyi et al., 1973). The calcium response is lost from precipitated myosin preparations of locusts (Lehman et al., 1974) and frequently from dilute samples of *Limulus* myosin.

**THIN FILAMENTS** The calcium sensitivity of the ATPase activation conferred by thin filaments directly demonstrates the presence of an actin control. Thin filaments were prepared from most of the muscles in which the competi-
Figure 3. SDS gel electrophoretic pattern of actomyosin preparations and of myosins depleted of actin. 7.5% gels Coomassie Brilliant Blue staining. 23-50 μg protein applied. Myosin was obtained from actomyosin by high speed centrifugation (ca. 200,000 g) for 3-5 h in the presence of 10 mM Mg-ATP. Myosin was precipitated at low ionic strength and washed. Loligo, Mya, Mercenaria, and Urechis extracted at pH 6.0 with 0.4 M NaCl, 3 mM ATP; other muscles at pH 7.0 with 0.6 M NaCl, 3 mM ATP. Actomyosin and myosin preparations in each pair: (a) Goffingia, (b) Loligo, (c) Mya, (d) Mercenaria, (e) Urechis, (f) Squilla, (g) Tarantula, (h) Eudistyla, (i) Dendrotoomum, (k) Glottidia. My: myosin; Pa: paramyosin; Ac: Actin. With the exception of Dendrotoomum, all these myosin preparations showed calcium sensitivity with excess pure actin. Myosin preparations contain reduced amounts of actin and variable amounts of paramyosin.

The actin activation assay demonstrated the presence of myosin control in order to determine whether actin control was also present. Thin filament preparations from all species activated the ATPase activity of rabbit myosin in the presence of calcium; however, not all of them formed a calcium-sensitive actomyosin complex (Table I). The thin filaments were combined in various proportions with rabbit myosin since calcium sensitivity depends on the
relative proportions of thin filaments to myosin. Frequently, calcium sensitivity was greatest at a thin filament to myosin weight ratio of about 1:2–3.

For most thin filaments there is a good correlation between thin-filament composition and the functional tests, (Fig. 5), i.e., thin filaments containing regulated actin also have low molecular weight components that correspond in size to the subunits of invertebrate troponin (Regenstein, 1972; Regenstein and Szent-Györgyi, 1975; Bullard et al., 1973). Frequently, these thin filaments contain three major components, in addition to actin and tropomyosin, on SDS gels. Of these a component having a chain weight of about 25,000–32,000 daltons, probably corresponding to rabbit troponin-I, is seen clearly on all thin-filament preparations showing control. The band with a chain weight of about 15,000–20,000 daltons, probably corresponding to rabbit troponin-C, is less prominent and frequently stains poorly. With the exception of annelids, a third component with a larger chain weight than actin is also present on invertebrate thin filaments. These thin filaments bind calcium (Lehman et al., 1972).

The thin filaments obtained from muscles that have only myosin-linked regulation, are, on the whole, relatively free of low molecular weight components and consist mostly of actin and tropomyosin (Fig. 5). Minor bands, however, can be seen on the thin filaments of *Busycon, Lunatia, Loligo, Glottidia,* and *Urechis,* and can even be detected on *Aequipecten* (Fig. 6), *Anadara,* and
Figure 5. SDS acrylamide gel electrophoretic pattern of thin filaments. 10% gels stained with Coomassie Brilliant Blue. 8–16 μg proteins except Cryptochiton which was less than 5 μg. Note lack of myosin or paramyosin in significant quantities and the presence of actin and tropomyosin in all preparations.
Minor components of scallop myofibrils. 10% SDS acrylamide gel electrophoresis stained with Coomassie Brilliant Blue. (a) Isolated soluble calcium-binding component present in unwashed muscle. (b) Whole muscle. (c) Washed muscle. (d) Thin filaments. Preparations of muscles and thin filaments are overloaded (100 μg myofibrils and 30 μg thin filaments) to demonstrate the presence of minor components. Note that calcium-binding protein is not found in washed muscle. Three minor components are detectable on scallop thin filaments, and migrate similarly to invertebrate troponins. Thin filaments, however, do not sensitize rabbit myosin and the molar ratios of the minor components to tropomyosin are less than 1:5.

*Ensis* preparations. Low molecular weight components are present in larger amounts on the thin filaments of the ribbon worm, *Cerebratulus*. Nevertheless, none of these thin filaments, including eight different preparations from *Cerebratulus*, show calcium regulation when mixed with rabbit myosin. Myosin competition tests on actomyosin or on muscle suspensions of these animals fail to detect the presence of the actin control. With the exception of *Busycon* and *Lunatia* the molluscan thin filaments do not bind calcium (Kendrick-Jones et al., 1970).

All the muscles tested and all the thin filaments prepared contain tropomyosin. Densitometry of acrylamide gels indicates that in many thin-filament preparations the weight ratio of tropomyosin to actin is about 1:3–4, suggesting that a molar ratio of about 6 actins to 1 tropomyosin characterizes invertebrate thin filaments, and that there is little or no actin free of tropomyosin. The data suggest that actin is complexed with tropomyosin even in muscles where tropomyosin has no regulatory function. Tropomyosin, however, may in some cases be lost from thin filaments during preparation, and the tropomyosin to actin ratio in thin filaments may fall below that found in
muscle, especially in species which do not contain significant amounts of troponin. The losses are particularly great from *Cucumaria*, *Thyone*, and *Cryptochiton* thin filaments, where special precautions were necessary to retain even some of the tropomyosin. These precautions included the use of high protein concentrations during preparations, higher magnesium concentrations (5 mM), and a pH of 6.0 at every step of the preparation. The competitive myosin-activation assay of these muscles indicating the lack of actin control is particularly important.

There appears to be a one-to-one molar ratio between the 25,000- to 32,000-dalton component, corresponding to troponin-I and tropomysin in a number of thin-filament preparations having a regulatory function (cf. Lehman et al., 1972). The lower chain weight component, corresponding to troponin-C, is less intensely stained, and the staining varies considerably. The molar ratio of one troponin to one tropomyosin to 5–7 actins is particularly relevant for our understanding of the double systems because the information argues against the presence of two populations of muscles, one with only myosin-linked regulation and the other with an actin-linked regulation. The fact that some doubly regulated muscles retain fully their calcium sensitivity in the presence of excess pure actin (Table I) indicates that these muscles contain predominantly a single population of regulated myosin.

The minor components of the thin filaments are distinct from the light chains of myosin. In some cases these components can be clearly identified in actomyosin preparations. For example, *Limulus* thin filaments contain four components in addition to actin and tropomyosin, while *Limulus* myosin has three different light chains. With the exception of components migrating at about 18,000 daltons which are present in both myosin and thin filaments, these low molecular weight components move differently in SDS acrylamide gel electrophoresis and the bands seen in actomyosin preparations may be easily traced either to myosin or to thin filaments (Fig. 4). The low molecular weight components of molluscan actomyosins can be largely accounted for by the light chains of myosin (Fig. 3).

High molecular weight components occasionally present on thin filaments may represent α-actinin or other components of Z-line and dense body structures. Significantly, little or no protein remains at the origin of the gels, indicating the absence of myosin and paramyosin.

The presence of both actin- and myosin-linked regulation can increase the fidelity of calcium control. The calcium sensitivity of regulated myosin together with regulated thin filaments from *Limulus* or from locust is greater than the sensitivity of the individual components tested with rabbit actin or myosin (Table IV).

Regulated invertebrate thin filaments bind fewer calcium ions than regulated vertebrate thin filaments (Lehman et al., 1972). Lobster troponin binds approximately one calcium for each mole of troponin (Regenstein and Szent-
Table IV

COMBINED EFFECTS OF CALCIUM-SENSITIVE THIN FILAMENTS AND MYOSINS

| ATPase activity | Calcium sensitivity* |
|-----------------|----------------------|
|                 | 0.1 mM EGTA | 0.1 mM Ca²⁺ | %    |
| Limulus thin filaments + rabbit myosin | 0.12 | 0.6 | 80 |
| Limulus myosin + rabbit actin + rabbit tropomyosin | 0.022 | 0.062 | 65 |
| Limulus myosin + Limulus thin filaments | 0.003 | 0.120 | 98 |
| Locust thin filaments + rabbit myosin† | 0.1 | 0.5 | 80 |
| Locust myosin + rabbit actin + rabbit tropomyosin | 0.068 | 0.27 | 75 |
| Locust myosin + locust thin filaments† | 0.008 | 0.31 | 97 |

0.3-0.5 g thin filament or actin-tropomyosin to 1 g myosin.
* (1 - (ATPase₀ EGTA)/(ATPase₀ Ca²⁺)) × 100.
† Lehman et al., 1974.

Györgyi, 1975) in contrast to the four calciums bound by a mole of rabbit troponin (Potter, 1974). Full ATPase activation by Limulus thin filaments requires large changes in calcium concentration (Fig. 7). This broad transition in the pCa curves reflects single noncooperative calcium-binding sites on the

![Figure 7](image-url)

**Figure 7.** Calcium dependence of ATPase activities. △ scallop myofibrils, □ rabbit thin filaments with rabbit myosin, ■ rabbit troponin-tropomyosin and rabbit actin with rabbit myosin, ● Limulus thin filaments with rabbit myosin. The calcium dependence of the preparations was normalized to 100%. The actual sensitivity of scallop myofibrils amounted to 95%, the sensitivity of the rabbit and Limulus thin filaments to 85%, the sensitivity of the reconstituted rabbit relaxing system to 70-80%. The pCa values for 50% calcium sensitivity were also normalized to the Limulus thin-filament values (1.4 × 10⁻⁸ M Ca²⁺). The halfway point was reached at 0.5 × 10⁻⁷ M Ca²⁺ in scallop and 1.5 × 10⁻⁷ M in rabbit preparations at neutral pH.
thin filaments. The pCa curves for vertebrate thin filaments, or the pCa dependence of molluscan muscles are sharp, indicating that more than one calcium is involved at each regulatory site (Fig. 7). Likewise, the transition for the doubly regulated *Limulus* myofibrils is abrupt. On the other hand, the calcium dependence of the thin-filament-regulated lobster tail myofibrils is broad.

**Discussion**

**Interpretation of the Evidence**

The presence of a particular regulation in a muscle can be established unambiguously by functional tests. It is more difficult, however, to interpret the evidence indicating the absence of a regulatory system since the lack of function may on one hand represent in vivo conditions; alternatively, it could result from inactivation of the regulatory proteins due to experimental manipulations or other experimental artifacts. The fact that calcium-sensitive actomyosin preparations could not be obtained from a number of animals indicates some of the experimental difficulties. The organisms with double regulation thus may be underestimated; therefore, the evidence for single regulation has to be examined with particular caution.

In these studies we have tried to retain all components contributing to the function of myosin and thin filaments. Preparations were performed rapidly employing only a few steps to limit possible inactivation or loss of components. It was important, however, to reduce cross contamination of myosin in thin-filament preparation, and the components of the thin filaments in the myosin preparations. Other impurities may be and are present. Myosin preparations may contain considerable amounts of paramyosin; some of the minor bands in the thin-filament preparations may represent membrane fragments or other impurities.

The pattern of the distribution of the regulatory systems indicates a relative simplicity (Fig. 1). Single systems are not randomly distributed in the animal kingdom, and organisms within a major phylum or class tend to behave similarly. Single actin control is restricted to the chordates and, among the invertebrates, to the fast muscles of decapods and mysidaceae. Single myosin control is restricted to molluscs, echinoderms, and several other minor phyla (brachiopod, echiuroid, and nemertine worms). The relative simplicity of the distribution of single regulatory systems is perhaps the most significant evolutionary aspect of these comparative studies. A similar consistency is seen among doubly regulated systems. All the insects and annelids tested behave similarly. Crustaceans, cirripeds, stomatopods, amphipods, and isopodes all show double control, although pure rabbit actin partially reduced the calcium sensitivity of amphipod actomyosins.

In lobster, however, the fast tail muscles show a single actin control, and
the slow muscles are doubly regulated. The sipunculids also seem to be an exception as regulation varies among the members of this phylum. *Dendrostomum* myosin is, however, unusual by binding calcium without a demonstrable regulatory function. This may suggest a partial loss of regulatory function, or reflects an experimental artifact. Smooth muscles of chicken gizzard may also be an exception and evidence for myosin control has been reported (Bremel, 1974).

**Evolutionary Aspects**

The major evolutionary features which have emerged from this investigation are the wide occurrence of both regulatory systems and the relative simplicity of the distribution of single regulatory systems. When taken in the context of the differing properties of the components of actin- and myosin-linked regulation, these features are of particular interest. Troponin consists of three different subunits, two of these are considerably larger than the "regulatory" light chains. Troponin combines only with actin and tropomyosin but not with myosin. In contrast the "regulatory" light chain binds only to myosin. A common evolutionary origin for troponin-C, myosin light chains and parvalbumin has been proposed recently on the basis of similarities in amino acid sequences (Tufty and Kretsinger, 1975; Collins, 1974; Weeds and McLachlan, 1974); however, there is no functional overlap between troponin-C and the regulatory light chains. The two regulations act independently of each other, although their effect may be additive (Table IV).

Both the myosin-linked and the actin-linked regulations are found in phyla which appeared early in evolution, and at present there is no evidence for assuming that myosin-linked regulation evolved before actin-linked regulation, even though the myosin control requires only a single regulatory component, whereas actin control involves the interaction of a number of different regulatory components.

In our initial studies we speculated that myosin-linked regulation evolved first (Lehman et al., 1972). This hypothesis became untenable after finding both regulation systems in the nematode, *Ascaris lumbricoides*. Recently the presence of actin control was reported in the slime mold, *Physarum polycephalum* (Nachmias and Asch, 1974). It is no longer necessary to assume a convergent evolution for the thin-filament-linked regulatory systems. The different calcium-binding properties of invertebrate and vertebrate troponins may have stemmed from an ancestral mutation.

**Functional Aspects**

In vivo regulation may be altered genetically in several different ways: the synthesis of normal regulatory components may be decreased; inactive regulatory components may be produced; the binding sites on myosin or on actin for the regulatory proteins may be changed. Alternatively, mutations may
have made a regulatory system particularly sensitive to experimental manipulations and the apparent loss of regulation would not reflect the in vivo properties of the muscle.

Absence of actin control in molluscan and brachiopod muscles has a simple explanation; regulatory proteins are not present in sufficient quantity to regulate actin (cf. Lehman et al., 1972). The reasons for the lack of significant amounts of troponin in these muscles are not known. Minor bands can be detected on overloaded SDS acrylamide gels of scallop thin filaments or washed muscles (Fig. 6). These bands may correspond in chain weight to the subunits of invertebrate troponin. However, these components are present only in small quantities; the molar ratio of the 25,000-chain weight peptide to tropomyosin is less than 0.2. Although we have not been able to demonstrate any actin control in molluscan muscles, and we have not been able to isolate a functional troponin from scallops, we cannot exclude that some troponin may be synthesized in the muscle. It is also possible that a nonfunctional mutant of troponin is synthesized that may have lost its ability to combine with actin and tropomyosin. If so, some of the troponin subunits may be found in the soluble protein fraction. We have isolated a protein from the soluble fraction of scallop striated muscle which consists of a single chain of about 22,000 daltons and binds about 1 mol of calcium at $3 \times 10^{-8}$ M Ca$^{2+}$ concentrations in the presence of 1 mM MgCl$_2$ (Fig. 6). This calcium-binding protein amounts to less than 0.5% of the muscle proteins, and does not complex with other soluble proteins or with the thin filaments. We have not as yet demonstrated that it has any regulatory function. This calcium-binding protein of scallop may be related to the parvalbumins, a group of calcium-binding proteins obtained from a number of vertebrate muscles. One notes, however, that the chain weight of the scallop calcium-binding protein exceeds the size range reported for parvalbumins (11,000-15,000 daltons) (Pechere et al., 1973), and it has a relatively high tryptophan and tyrosine content with an absorption peak at 280 nm and an extinction coefficient of about 1.4 OD units (milligrams per milliliter per centimeter), in contrast to the parvalbumins that have few or no aromatic residues and show absorption maxima at around 260 nm.

The lack of myosin control in vertebrates and decapods is not due to the lack of "regulatory" light chains. Kendrick-Jones has shown that the DTNB-(5,5'-dithio-bis-(2-nitrobenzoic acid)) light chains of rabbit hybridize with a desensitized scallop myosin and the hybrid formed is regulated (1974). Similarly, "regulatory" light chains can be prepared from a number of other vertebrate myosins and also from lobster (Kendrick-Jones et al., to be published). In contrast, neither rabbit myosin nor any hybrid of the rabbit heavy chains is calcium sensitive. The lack of a myosin control is thus due either to an alteration in the heavy chain of myosin, such that it will not respond to regulatory light chains, or that the myosin control is particularly sensitive to
the relatively limited manipulations required even for the competitive actin-activation assay. At present, it is difficult to decide experimentally between these possibilities, and it will not be easy to detect alterations or mutations on a molecule the size of the myosin heavy chain. We have performed competitive actin-activation assays on unwashed mouse myofibrils not exposed to high ionic strength, and on actomyosin extracts from unwashed lobster muscles in order to avoid the possible loss of a myosin control during preparation. Nevertheless, we failed to detect the presence of a myosin-linked regulation in either case. It is not obvious how to devise a more direct biochemical approach which more faithfully approximates the in vivo conditions of a muscle, and the evidence strongly suggests that in at least some muscles myosin control is lacking. The importance of positive evidence, however, cannot be overstated.

The disappearance of the ordered cross-bridge lattice from frog sartorius upon stimulation of stretched muscle (Hazelgrove, 1972), the calcium-dependent fluorescent change of the DTNB light chain of rabbit myosin (Werber et al., 1972), and the calcium dependence of the viscosity and sedimentation properties of isolated and reconstituted thick filaments (Marimoto and Harrington, 1974) indicate that calcium may interact with vertebrate myosin. These observations, however, do not demonstrate directly that a myosin-linked control functions in vertebrate striated muscles. The ATPase activity of vertebrate myosins and actomyosins in the presence of pure actin is not stimulated by calcium ions, in fact, calcium may inhibit by about 15–20% when magnesium is low (Weber and Murray, 1973).

The evidence at present that molluscan muscles are controlled by a single myosin-linked system is firm. In these muscles the lack of actin control is due to the lack of troponin. In vertebrates and in most decapod muscles, on the other hand, "regulatory" light chains are likely to be present although they do not seem to function in vitro, and it is difficult to establish with absolute certainty that in vitro results apply to in vivo conditions.

There is no obvious relationship between ATPase activity, the structure of the muscle, and a particular regulatory system. ATPase activities range widely irrespective of the nature of the control (Table I). Furthermore, both myosin-linked and actin-linked regulation are displayed by both striated and smooth muscles. Nevertheless, the studies reported here are relevant for interpreting some of the structural studies on muscle. The movement of myosin cross bridges in insect muscles upon addition of calcium, before contact with actin filaments is established (Miller and Tregear, 1971), i.e. the calcium-activated state, may be readily explained by the demonstration of myosin control in Lethocerus flight muscles. It is also of interest that the increase in the intensity of the second layer line of actin during rigor in the byssus retractor muscle of Mytilus edulis indicates that tropomyosin may move in the absence of functioning troponin (Lowy and Vibert, 1972).
The advantages of double regulation are obvious: the accuracy and precision of the calcium control over rest and activity may be enhanced. Functional advantages of single regulation are less apparent. The evolutionary pressures for the development of single systems may be explained in the case of molluscs if one assumes that the presence of a regulation acting on the thin filaments is not compatible with the maintenance of "catch," a property important for the survival of these animals (cf. Johnson, 1962). One may argue that the evolution of a troponin with multiple calcium-binding sites allowed for a sharp transition between rest and activity in vertebrates, and hence the importance of myosin control was reduced. There is no apparent advantage, however, for losing myosin-linked regulation in the crustacean decapods since these muscles would have additional requirements for rapid Ca\(^{++}\) removal if their muscles were regulated in vivo solely by invertebrate troponin.

The calcium dependence of tension measurements in skinned fibers of the carpopodite flexor muscle of the crayfish, *Orconectes*, is similar to that of frog muscles; both preparations are brought to full activity over a narrow calcium-concentration range (Brandt et al., 1972; Orentlicher et al., 1974). These crayfish muscles, however, are slow muscles with 8- to 10-\(\mu\)m long sarcomeres and are very likely doubly regulated.

The decapods are of particular interest because one species may contain both doubly regulated and singly regulated muscles. There are additional structural and biochemical differences. The muscles with single actin control in general have several-fold higher ATPase activities than the other doubly regulated crustacean muscles (Table I). The fast tail, cutter claw, and deep abdominal extensor muscles of the lobster that show a single actin control have shorter thick filaments and sarcomere lengths (2-3.5 \(\mu\)m) compared to the slow crusher claw and slow superficial abdominal extensor muscles (6- to 9-\(\mu\)m sarcomere length) (Jahromi and Atwood, 1969, 1971). Paramyosin is found in the crusher claw muscle but not in the tail muscles of lobster (Weisel and Szent-Györgyi, to be published). However, the two light chains of the myosins of both muscles migrate identically on SDS gel electrophoresis. It thus appears that the heavy chains of the two myosin types of the lobsters differ and that the lack of the myosin control in fast muscles is the result of alterations in the heavy chains of myosin. Similarly, a functional myosin control in vertebrate smooth muscles (Bremel, 1974) and the lack of such a control in vertebrate striated muscles may be simply explained by assuming differences in the heavy chains.

In summary, we suggest that the genome of most, possibly of all, animals contains the information for both regulatory systems. This information may be expressed fully and all the components of both regulations are present in significant amounts in most animals with the exception of molluscs, brachiopods, echinoderms, and echiuroids which lack in troponin. Regulation may
also be lost as a result of changes in the myosin molecule without altering or losing regulatory components.

We thank Drs. Carolyn Cohen, Hugh E. Huxley, John Kendrick-Jones, Eva M. Szentkiralyi, Annemarie Weber, Joe M. Regenstein, John Weisel, and Michael Payne for discussions. We also thank Drs. Carolyn Cohen, Eva M. Szentkiralyi, Peter Vibert, Annemarie Weber, John Weisel, and Michael Payne for criticizing the manuscript. We acknowledge the help of Dr. John Kendrick-Jones in some of the experiments. We thank Ms. Debbie Wyalgal for experiments on the calcium-binding protein of the scallop, Ms. Ruth Hoffman for expert assistance during most of this work, and Ms. Regina Niebieski for assistance at the later stages.

We are grateful to the late Mr. Harold Williams for collecting Lethocerus sp. (Florida), and to the Supply Department at the Marine Biological Laboratory, Woods Hole, Mass., for collecting a large number of relatively rare specimens. Some of this work was performed at the Department of Zoology at Oxford University when one of us (W. L.) was on the A. R. C. staff. Some experimentation was done at the M. R. C. Laboratory of Molecular Biology at Cambridge when one of us (A. G. S. G.) was a visiting scientist. We thank Richard Tregear, Professor John Pringle, and Dr. Hugh E. Huxley for their hospitality.

This work was supported by grants from the National Science Foundation (GB-40308) (W. L.) and from the Public Health Service (AM-17062) (W. L.) and (AM-15963) (A. G. S. G.).

Received for publication 14 January 1975.

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