

Myosin Types and Fiber Types in Cardiac Muscle

I. Ventricular Myocardium

S. SARTORE, L. GORZA, S. PIEROBON BORMIOLI, L. DALLA LIBERA, and S. SCHIAFFINO
*Institute of General Pathology and National Research Council Unit for Muscle Biology and
Physiopathology, University of Padua, 35100 Padua, Italy*

ABSTRACT Antisera against bovine atrial myosin were raised in rabbits, purified by affinity chromatography, and absorbed with insolubilized ventricular myosin. Specific anti-bovine atrial myosin (anti-bAm) antibodies reacted selectively with atrial myosin heavy chains, as determined by enzyme immunoassay combined with SDS-gel electrophoresis. In direct and indirect immunofluorescence assay, anti-bAm was found to stain all atrial muscle fibers and a minor proportion of ventricular muscle fibers in the right ventricle of the bovine heart. In contrast, almost all muscle fibers in the left ventricle were unreactive. Purkinje fibers showed variable reactivity. In the rabbit heart, all atrial muscle fibers were stained by anti-bAm, whereas ventricular fibers showed a variable response in both the right and left ventricle, with a tendency for reactive fibers to be more numerous in the right ventricle and in subepicardial regions. Diversification of fiber types with respect to anti-bAm reactivity was found to occur during late stages of postnatal development in the rabbit heart and to be influenced by thyroid hormone. All ventricular muscle fibers became strongly reactive after thyroxine treatment, whereas they became unreactive or poorly reactive after propylthiouracil treatment. These findings are consistent with the existence of different ventricular isomyosins whose relative proportions can vary according to the thyroid state. Variations in ventricular isomyosin composition can account for the changes in myosin Ca^{2+} -activated ATPase activity previously observed in cardiac muscle from hyper- and hypothyroid animals and may be responsible for the changes in the velocity of contraction of ventricular myocardium that occur under these conditions. The differential distribution of ventricular isomyosins in the normal heart suggests that fiber types with different contractile properties may coexist in the ventricular myocardium.

Heterogeneity of cardiac muscle fibers with respect to myosin composition appears to be a general property of the vertebrate heart. Using antimyosin antibodies and immunofluorescence procedures, we have previously shown that muscle fibers from atrial, ventricular, and conduction tissue of the chicken heart contain antigenically distinct forms of myosin (27). Atrial and ventricular myosins from the chicken heart were also shown to differ in enzymatic properties and in heavy chain structure (7). Similar differences between atrial and ventricular myosins have been observed in amphibians and reptiles (our unpublished results) and in mammals (11, 18).

An even more complex picture of cardiac myosin polymorphism has recently emerged from a study of rat atrial and ventricular isomyosins based on electrophoresis of the native proteins in a nondissociating medium (14). Using an immunological approach, we have independently confirmed the

existence of myosin polymorphism in the ventricular myocardium of the rabbit heart (26). By immunofluorescence staining, we have also found that cardiac isomyosins may be variably distributed among cardiac muscle fibers: fiber type heterogeneity can thus be demonstrated within both atrial and ventricular myocardium and even within the conduction tissue. Here, we report immunohistochemical evidence for myosin polymorphism and fiber heterogeneity in the ventricular myocardium of bovine and rabbit heart. Preliminary results of this study have been presented elsewhere (28).

MATERIALS AND METHODS

Myosin was isolated from the left atrium and the left ventricle of beef heart (heart weight ~3 kg) essentially as described by Barany and Close (2) and purified by ion-exchange chromatography (24). Pepstatin 0.2 $\mu\text{g}/\text{ml}$ was added throughout the preparation (29).

Antimyosin Antibodies

Anti-beef atrial myosin (anti-bAm) antibodies were raised in rabbits. Four rabbits were injected subcutaneously at different sites with 1 mg of atrial myosin in 0.4 M KCl-0.05 M Na-phosphate buffer, pH 7.4, emulsified with an equal volume of complete Freund adjuvant. Injections were repeated after 2 and 4 wk. 7 d after the last injection the animals were bled and the serum was checked for the presence of antibodies by double immunodiffusion in agar (19). The animals were subsequently boosted with antigen in Freund adjuvant at various intervals, and the antisera from the different bleedings were stored separately at -20°C .

Specific IgG were separated from the antisera by affinity chromatography on insolubilized atrial myosin, following previously described procedures (6, 23). A typical column prepared by coupling 21 mg of atrial myosin to CNBr-activated Sepharose was found to bind 11.4 mg of antimyosin antibodies that were eluted by lowering the pH to 2.8. Anti-atrial myosin antibodies were also absorbed with insolubilized ventricular myosin to eliminate cross-reactive antibodies (see Results).

Enzyme Immunoassay

Titer and specificity of antimyosin antibodies were determined by double immunodiffusion in agarose (19) and by microplate enzyme immunoassay (3, 9). Enzyme immunoassay combined with SDS-gel electrophoresis was used to determine the localization of the antigenic determinants responsible for the specificity of the purified antimyosins. The procedure was basically similar to that described by Lutz et al. (20), except that the assay was performed in microplate wells. In brief, myosin was applied to a 5 or 10% SDS polyacrylamide gel and electrophoresis was carried out for 2 or 4 h (37). The gel was sliced and single slices were incubated in microplate wells in 0.1 M Na-carbonate buffer, pH 9.6, for 24 h at 37°C to allow the protein to elute from the gel and bind to the polystyrene surface. The gel slices were then removed and a fixed amount of anti-atrial myosin was added to each well. Bound antimyosin was revealed by goat anti-rabbit IgG conjugated with alkaline phosphatase, enzyme concentration being determined with *p*-nitrophenylphosphate as substrate. The appropriate concentrations of antimyosin antibody (usually $\sim 1\ \mu\text{g}/\text{ml}$) and enzyme conjugate (usually $\sim 1:3,000$) used in the assay were determined by titration curves, as described by Engvall and Perlmann (9). Parallel gels were stained with Coomassie Blue.

Immunofluorescence

Both direct and indirect immunofluorescence assays were performed on fresh-frozen cryostat sections of bovine and rabbit cardiac muscle. Specimens of bovine heart were obtained from four adult bulls, heart weight ranging from 2.5 to 3.5 kg; several blocks were excised within 1 h postmortem from atrial and ventricular myocardium. Specimens of rabbit heart were obtained from animals of different age: both whole heart transverse sections and through-wall blocks excised at preselected sites were examined. Specimens of cardiac muscle from hyperthyroid and hypothyroid rabbits were also processed for immunofluorescence. Hyperthyroidism was induced in seven adult rabbits by subcutaneous injections of L-thyroxine, 200 $\mu\text{g}/\text{kg}$, daily for 2 wk: this treatment has been shown to induce striking changes in myosin composition in rabbit ventricular myocardium (10). Hypothyroidism was induced in five adult rabbits by propylthiouracil, 0.02% in drinking water, for 4 wk: under these conditions complete shifts in myosin isoenzyme composition have been demonstrated in the rabbit heart (J. Hoh, personal communication). For indirect immunofluorescence, sections were first incubated with unlabeled antimyosin antibodies for 30 min at 37°C , washed with phosphate-buffered saline, then treated with fluorescein-labeled goat anti-rabbit IgG for 30 min at 37°C . Direct immunofluorescence was performed with anti-bAm antiserum absorbed with ventricular myosin. The antibodies were conjugated with fluorescein isothiocyanate as previously described (23) and applied to cryostat sections for 30 min at 37°C . Controls for direct and indirect immunofluorescence were as previously described (23). Sections were fixed in 2% paraformaldehyde, mounted in Elovanol, and examined with a Leitz fluorescence microscope.

Materials

Pepstatin, *p*-nitrophenylphosphate, L-thyroxine, and propylthiouracil were obtained from Sigma Chemical Co. (St. Louis, Mo.). CNBr-activated Sepharose was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). Goat anti-rabbit IgG conjugated with alkaline phosphatase and with fluorescein isothiocyanate were obtained from Miles Laboratories Inc. (Elkhart, Ind.).

RESULTS

Specificity of Antibodies

Two of the four rabbits immunized with beef atrial myosin produced antimyosin antibodies in high titer since early stages of immunization, as determined by immunodiffusion and immunofluorescence tests, and were therefore selected for subsequent studies. Both antisera gave a single precipitin band with beef atrial myosin and no visible reaction with ventricular myosin (Fig. 1). However, when analyzed by enzyme immunoassay these antisera were found to cross-react with ventricular myosin and by indirect immunofluorescence they showed a weak but significant reaction with ventricular fibers. This pattern of reactivity was essentially unchanged in antisera from different bleedings. To eliminate cross-reactive antibodies, anti-bAm antisera were therefore absorbed with ventricular myosin. In a typical experiment 10 ml of anti-bAm antiserum was applied to a column prepared by coupling 22 mg of beef ventricular myosin to Sepharose. A fraction of antibodies (2.4 mg) was retained by this column, which had the capacity to bind 9.6 mg of ventricular myosin. When applied to cryostat sections, these antibodies were found to stain atrial and ventricular fibers with similar intensity. The unretained fraction was subsequently applied to a column prepared by coupling 21 mg of beef atrial myosin to Sepharose: the antibodies retained by this column (9.5 mg) stained atrial but not left ventricular fibers in immunofluorescence tests (Fig. 2) and reacted with atrial but not ventricular myosin in enzyme immunoassay test.

Anti-bAm antisera absorbed with ventricular myosin were analyzed by enzyme immunoassay combined with SDS-gel electrophoresis. As shown in Fig. 3, anti-bAm antibodies were found to react selectively with the heavy chains of atrial myosin and showed no significant reaction with myosin light chains or with minor contaminants present in the immunogen. Two anti-bAm preparations, obtained from two different rabbits, were used for indirect immunofluorescence with identical results. One of these preparations was also coupled to fluorescein isothiocyanate for direct immunofluorescence. The same staining pattern was obtained with this procedure; however, variations in reactivity among cardiac muscle fibers were often better revealed by the indirect immunofluorescence method.

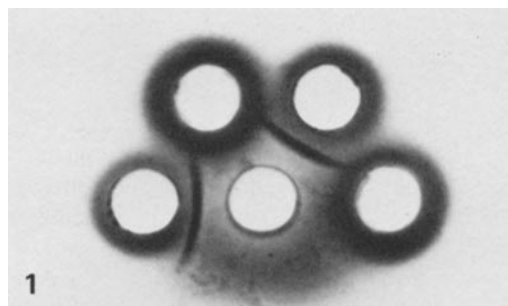


FIGURE 1 Outcherlony double immunodiffusion assay. The central well contained antiserum against beef atrial myosin. The first and third outer wells, beginning with the well on the left, contained beef atrial myosin. The second and the fourth outer wells contained ventricular myosin. Each well contained 50 μl of antigen or antiserum. Myosin concentration was $\sim 1\ \text{mg}/\text{ml}$. Immunodiffusion was carried out in 1% agarose in 0.4 M KCl, 50 mM Na-phosphate buffer, pH 7.4. The plate was incubated for 3 d at room temperature and stained in 1% amido black.

Bovine Heart

All atrial muscle cells in the bovine heart were brightly stained by anti-bAm (Fig. 4). In longitudinally oriented fibers the staining reaction was found to correspond to the A band of the myofibrils. Most ventricular fibers were completely unreactive with anti-bAm; however, specific regions in the ventricular myocardium showed variable reactivity. In particular, a significant number of fibers staining with variable intensity with anti-bAm was regularly encountered in the free wall of the right ventricle (Figs. 5 and 6). These fibers were occasionally grouped in bundles, but more frequently they were interspersed among normal unreactive fibers and could not be distinguished by size or other morphological criteria. In longitudinally oriented bundles unreactive and reactive fibers were often apparently connected to one another by intercalated disks (Fig. 7). Purkinje fibers, recognizable for the large size and rich glycogen content, also showed marked variation in their reactivity with anti-bAm: fibers brightly stained were often seen within the same bundle in close association to unstained Purkinje fibers (Fig. 6). In contrast with the right ventricle, the interventricular septum and the free wall of the left ventricle were almost homogeneously composed of negative fibers (Fig. 2). Only a proportion of Purkinje fibers and very rare muscle cells morphologically indistinguishable from normal ventricular cells were reactive with anti-bAm (Fig. 8).

Rabbit heart

Heterogeneity of cardiac muscle fibers has been observed in other mammalian species using anti-bAm. In the pig heart the response to these antibodies was essentially similar to that of the bovine heart. In the rat heart all ventricular muscle fibers were uniformly reactive with anti-bAm. An intermediate type of response was observed in the rabbit heart, which has been the object of more extensive investigations.

Rabbit ventricular fibers showed wide variation in their

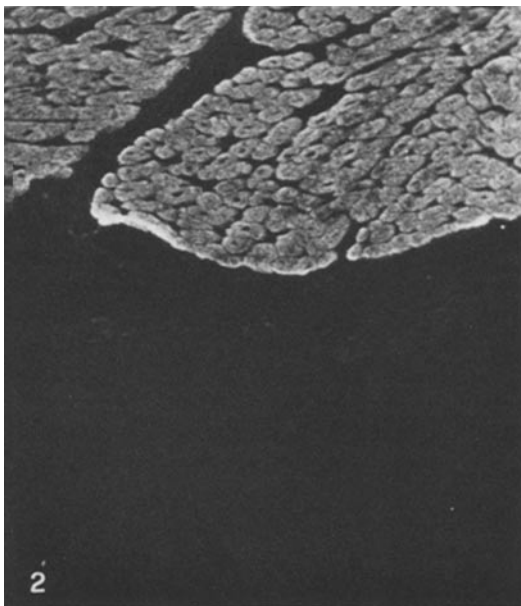


FIGURE 2 Cryostat section through a block composed of left atrial (top) and left ventricular (bottom) bovine myocardium, processed for indirect immunofluorescence with anti-bAm. Atrial fibers are reactive, whereas ventricular fibers are completely unreactive. $\times 150$.

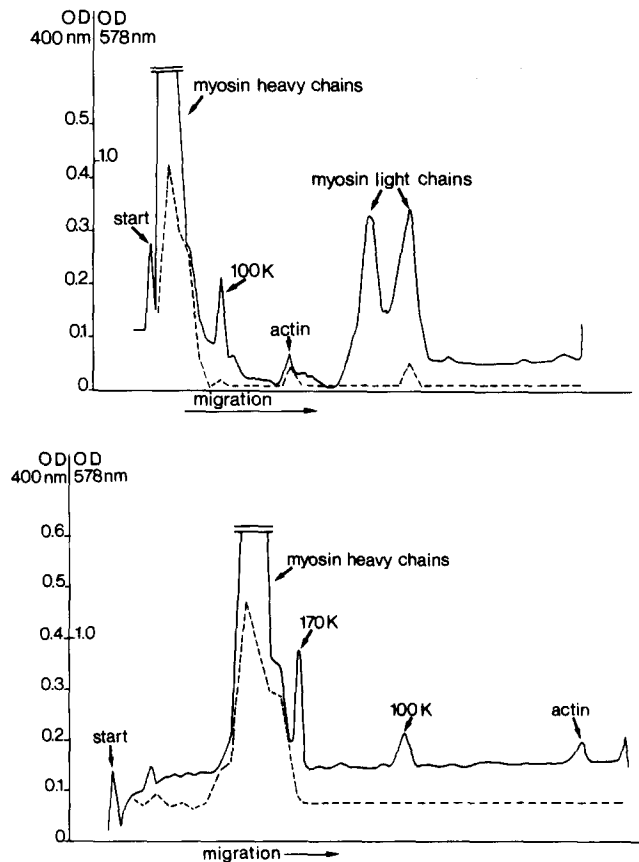
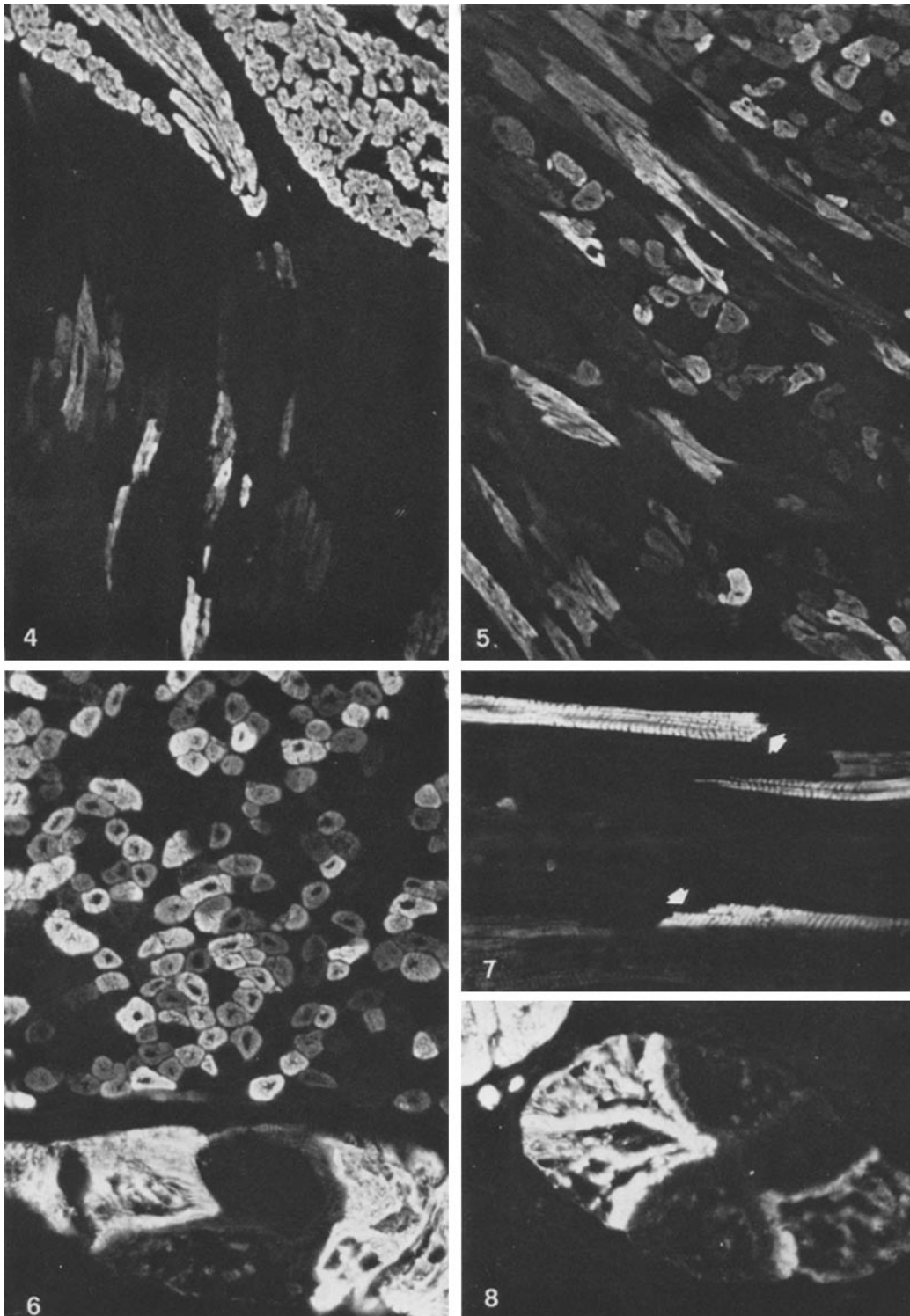


FIGURE 3 *Upper panel:* Specificity of anti-bAm antibodies as determined by enzyme immunoassay combined with 10% polyacrylamide gel electrophoresis. The continuous line represents the densitogram of a gel stained with Coomassie Blue after electrophoresis of bovine atrial myosin (40 μ g). The superimposed dashed line shows the enzyme immunoreaction of anti-bAm (1 μ g/ml) against slices of a parallel gel (see Materials and Methods). *Lower panel:* Same as in upper panel except that electrophoresis was performed in 5% polyacrylamide gel to obtain a better resolution of high molecular weight components. Note specific immunoreaction of anti-bAm with myosin heavy chains, whereas no reaction is shown by myosin light chains, actin, and unidentified high molecular weight components (100,000 and 170,000).

response to anti-bAm, ranging from a completely negative to a strong positive reaction (Figs. 9 and 10). At variance with bovine heart, fibers giving a positive reaction were more numerous than negative fibers in both right and left ventricular myocardium. Unreactive fibers were mostly concentrated in subendocardium (Fig. 11), whereas reactive fibers were especially frequent in subepicardium (Fig. 12) and were generally more numerous in the right than in the left ventricle. However, there was no clear-cut separation between areas showing fiber type predominance, the various fiber types being often intermingled and closely associated. Fiber type heterogeneity was also seen in small bundles of muscle fibers crossing the ventricular cavity (trabeculae carneae), which appear to correspond to the "Purkinje strands" described by Sommer and Johnson (30).

The response of rabbit ventricular myocardium to anti-bAm varied with age and with the thyroid state of the animal. All fibers reacted strongly with anti-bAm up to the fourth postnatal week, when unreactive fibers were first seen (Fig. 13). The overall degree of the reactivity of ventricular fibers was found



FIGURES 4-16 Cryostat sections of bovine and rabbit heart processed for indirect immunofluorescence with anti-bAm.

FIGURE 4 Bovine heart. Section through a block composed of right atrial (top) and right ventricular (bottom) myocardium. All atrial fibers are strongly reactive and a number of ventricular fibers are variably reactive with anti-bAm. $\times 150$.

FIGURE 5 Bovine heart; right ventricle; subepicardial area. Muscle cell heterogeneity after anti-bAm staining. $\times 150$.

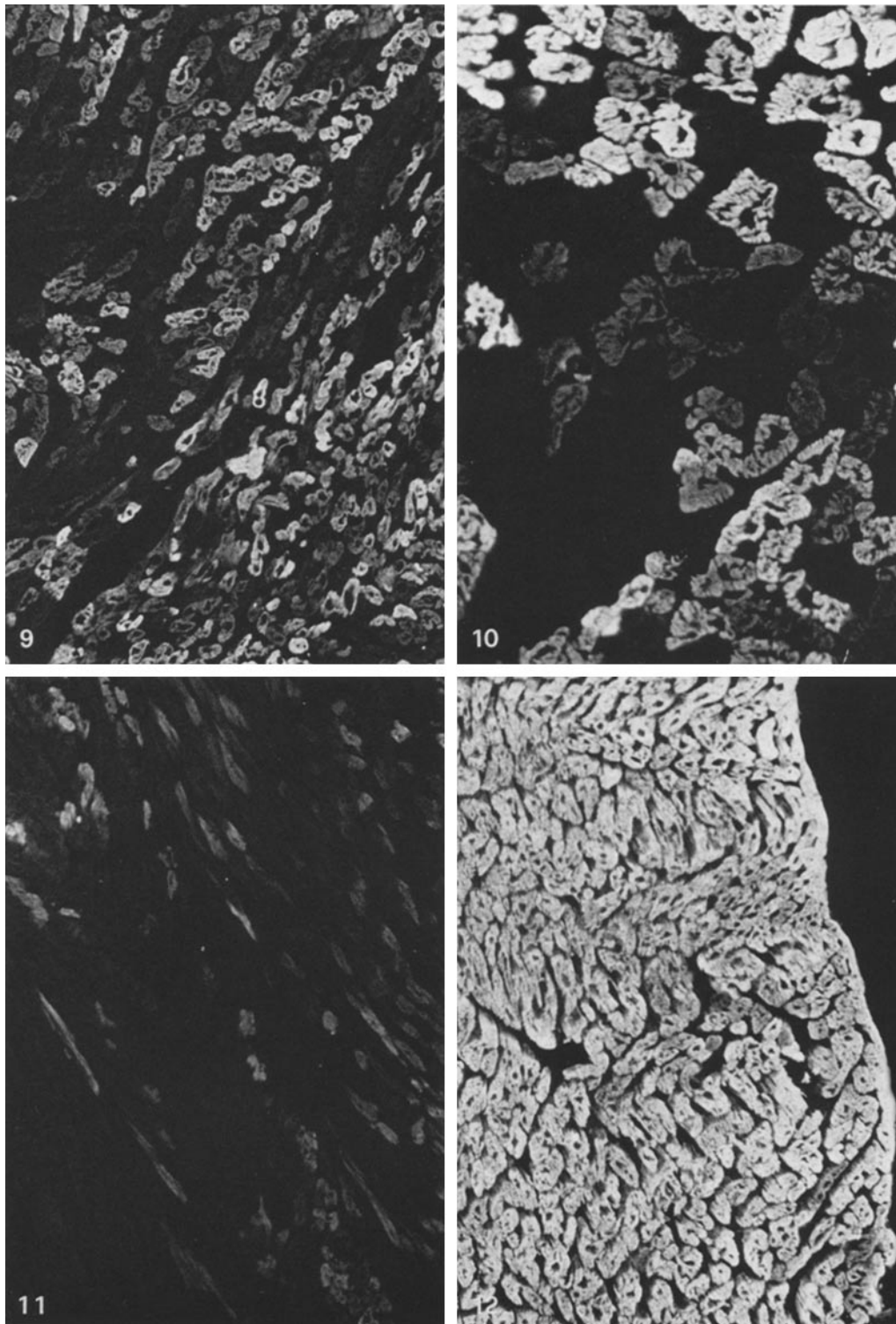
FIGURE 6 Bovine heart; right ventricle; subendocardial area. Muscle cell heterogeneity after anti-bAm staining in both the working myocardium (top) and within a bundle of large Purkinje fibers (bottom). $\times 300$.

FIGURE 7 Bovine heart; right ventricle. Section through longitudinally oriented muscle fibers. Intercalated disks (arrows) connect labeled and unlabeled fibers. Transverse striations in labeled fibers correspond to the A bands. $\times 300$.

FIGURE 8 Bovine heart; left ventricle; subendocardial area. Muscle cell heterogeneity within a bundle of Purkinje fibers. $\times 300$.

to decrease during subsequent stages and the proportion of completely unreactive fibers became progressively greater. In adult rabbits the relative proportion of the different fiber types showed marked variation from animal to animal. Striking changes in the fiber type profile of the ventricular myocardium

were observed in hyper- and hypothyroid states. After 2 wk of L-thyroxine treatment, unreactive fibers completely disappeared and all ventricular fibers were strongly reactive with anti-bAm (Fig. 14); the intensity of the staining reaction was almost identical in atrial and ventricular fibers. In contrast,



FIGURES 9 and 10 Rabbit heart, right ventricle. Muscle cell heterogeneity after anti-bAm staining. Note presence of fibers with intermediate degrees of reaction and close association of different fiber types. Fig. 9: $\times 150$; Fig. 10: $\times 400$.

FIGURES 11 and 12 Rabbit heart; left ventricle; subendocardial (Fig. 11) and subepicardial (Fig. 12) regions from the same through-wall block. Note the striking difference in reactivity to anti-bAm between the two regions. $\times 150$.

propylthiouracil treatment induced a striking depression of the reactivity of ventricular muscle cells: under these conditions most muscle fibers in subendocardial areas were completely unreactive (Fig. 15), whereas in subepicardial regions weakly

stained fibers were found to persist (Fig. 16).

DISCUSSION

This study shows that ventricular muscle fibers from bovine

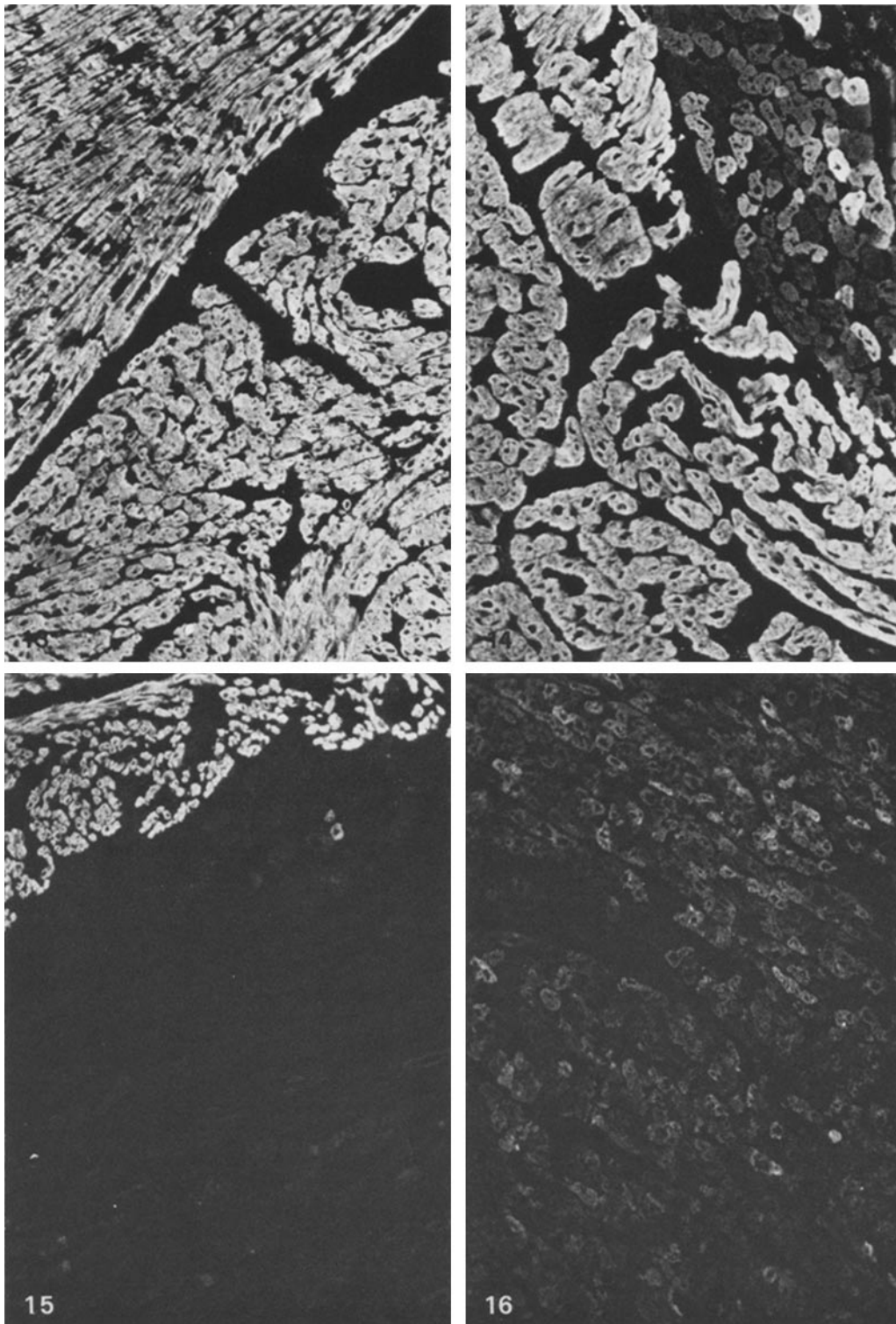


FIGURE 13 Developing rabbit heart. Strong and uniform reaction with anti-bAm in both atrial (top) and ventricular (bottom) myocardium from 3-wk-old animal. $\times 150$.

FIGURE 14 Hyperthyroid rabbit heart. Section through a block composed of left ventricular myocardium from normal (right) and hyperthyroid (left) rabbits, showing facing subendocardial regions. $\times 150$.

FIGURES 15 and 16 Hypothyroid rabbit heart. Fig. 15: section through a block composed of left atrial (top) and subendocardial ventricular (bottom) myocardium. Fig. 16: section through the subepicardial ventricular myocardium. $\times 150$.

and rabbit heart differ in their reactivity towards antibodies specific for myosin heavy chains of bovine atrial myocardium. This finding can be interpreted by assuming that (a) multiple isomyosins are present in mammalian ventricular myocardium, and (b) those ventricular isomyosins antigenically cross-reactive with atrial myosin are variably distributed among ventricular muscle cells. This appears to be true for muscle cells in both the working ventricular myocardium and the Purkinje system.

We have reported elsewhere (26) that the differential response of rabbit ventricular fibers to anti-bAm is caused by the variable distribution of a particular form of ventricular myosin antigenically related to atrial myosin. This isomyosin (Va) has been separated as a minor component from rabbit ventricular myosin by affinity chromatography using insolubilized anti-bAm as immunoabsorbent, and shown to differ in heavy chain structure from the major ventricular component (Vb) unrecovered by the anti-bAm column. Va can also be differentiated from rabbit atrial myosin on the basis of the light chain pattern (L. Dalla Libera, unpublished results). Direct evidence for myosin polymorphism in mammalian ventricular myocardium was first reported by Hoh et al. (14) using electrophoretic analysis in pyrophosphate gels of intact myosins. The rat ventricular myocardium was found to contain three distinct isomyosins, V₁, V₂, and V₃, differing in heavy chain structure and Ca²⁺-activated ATPase activity. V₁ shows the highest Ca²⁺-ATPase and is the predominant form in young animals and hyperthyroid animals, while V₃ has the lowest ATPase activity and is the predominant form in hypothyroid rats. According to the model proposed by Hoh et al. (15) these isomyosins are composed of two distinct heavy chains, HC_α and HC_β, V₁ being the homodimer of HC_α, V₃ the homodimer of HC_β, and V₂ the hybrid heterodimer. Thyroid hormone stimulates directly the synthesis of HC_α (13). Similar findings, including changes in isomyosin composition in hyper- and hypothyroidism, have recently been obtained also for the rabbit heart, the only significant difference between the two species being the greater proportion of V₃ vs. V₁ in the normal adult rabbit compared to the rat (J. Hoh, personal communication). By comparing the changes in reactivity of rabbit ventricular fibers to anti-bAm in hyper- and hypothyroidism with the corresponding changes in isomyosin composition observed by Hoh, it appears that our Va corresponds to Hoh's V₁ or, more precisely, that anti-bAm is specific for HC_α in rabbit ventricular myocardium.

The segregation of different forms of myosin in different types of ventricular muscle fibers may have profound influence on cardiac muscle physiology. The notion that contractile properties of muscle fibers are to a large extent determined by the form of myosin they contain is well documented for skeletal muscle fibers, whose speed of shortening is directly proportional to myosin ATPase activity (1). Several lines of evidence indicate that the relation between myosin ATPase and muscle speed is valid for cardiac muscle as well: (a) The maximum velocity of shortening of the ventricular myocardium varies in different mammalian species in parallel with the Ca²⁺-ATPase activity of myosin (8, 12, 34, 38). (b) The speed of atrial muscle is greater than that of ventricular muscle (36) and myosin ATPase is correspondingly higher (7, 11, 18, 35). (c) Myocardial hypertrophy induced by mechanical overloading is accompanied by a reduction in shortening velocity, as measured in isolated papillary muscles (31) or in chemically skinned ventricular preparations (21), and by a parallel decrease in Ca²⁺- and actin-stimulated myosin ATPase activity (see references 21 and 33). These myosin changes are the result of a shift in the

relative proportion of different ventricular isomyosins (17, and L. Gorza, manuscript in preparation). (d) The intrinsic velocity of contraction of cardiac muscle is increased in hyperthyroid and decreased in hypothyroid animals (5, 16) with corresponding changes of Ca²⁺- and actin-stimulated myosin ATPase activity (see references 22 and 25). Using myosin preparations from the same rabbits processed for immunofluorescence, we have found that, in agreement with a previous report (39), Ca²⁺-ATPase of ventricular myosin from hyperthyroid rabbits is about three times that of hypothyroid animals and, like atrial myosin, is not significantly changed by exposure to alkaline pH, whereas ventricular myosins from hypothyroid rabbits is markedly inactivated under these conditions (Dalla Libera et al., manuscript in preparation). These differences in alkali lability between ventricular isomyosins remind those reported for fast-skeletal and slow-skeletal myosins (32). In the light of this evidence the demonstration of a differential distribution of ventricular isomyosins in bovine and rabbit heart raises the possibility that ventricular muscle fibers may differ in their contractile properties. On the basis of changes in anti-bAm reactivity in hyper- and hypothyroid animals and the correlated changes in contractile properties, one would predict that fibers reactive with anti-bAm are faster than unreactive fibers. However, information on the distribution of other ventricular isomyosin would be needed to characterize more precisely the various fiber types in ventricular myocardium. Newly developed methods for dissociating single cardiac muscle fibers and measuring their contractile properties (4) could permit a direct correlation of physiological and immunohistochemical properties of single fibers. Regional variation in the distribution of fiber types may be related to differences in functional demand and contractile performance between different portions of ventricular myocardium, such as right vs. left ventricle, or subepicardial vs. subendocardial layers. Because different fiber types are apparently connected within a common network the question arises as to how their function is precisely coordinated. One may also wonder whether selective activation or recruitment of different fiber types may occur under certain conditions, thus affording a means for modulating the contractile performance of cardiac muscle.

We wish to thank Mr. Massimo Fabbri for technical assistance.

This work was supported in part by grants from Muscular Dystrophy Association of America and from the Legato Dino Ferrari per la Distrofia Muscolare.

Received for publication 8 July 1980, and in revised form 6 October 1980.

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