Calcium-activated myosin adenosine triphosphatase (ATPase) activity has been measured in sections of rat ventricles that were rapidly frozen to preserve the structure and regulatory state of myosin occurring in vivo. These results were related to myosin isozyme composition measured in ventricles by native gel electrophoresis and by quantitative immunocytochemistry. Both total ATPase activity and percent α-heavy chain rapidly rise during the first month following birth. However, ATPase activity remains constant at a high level from 1 to 12 months following birth, even though percent α-heavy chain declines during this period. The ATPase activity of V1 myosin was specifically determined using sections in which V3 myosin had been completely inhibited by exposure to alkaline pH in the absence of adenosine 5'-triphosphate (ATP). Relative V1 specific activity, taken as the ratio of V1 ATPase activity to percent α-heavy chain, doubles in the first 2.0 months after birth and then remains approximately constant at this higher level until at least 4 months after birth. The specific activity of V1 can be further increased by the addition of adenosine-3',5'-cyclic monophosphate (cAMP). This effect of cAMP is age dependent, increasing threefold between 1 and 2 months following birth and then declining as V1 is replaced by V3. (Circulation Research 1987;61:914-924)

The contractile performance of the heart is regulated by a variety of neural and hormonal inputs. Of these, the effects of β-adrenergic stimulation are among the most profound: β-agonists can increase the peak twitch tension of cardiac muscle several times as well as shorten the duration of phasic contractions by increasing the rates of both development and relaxation of tension.1,2 In mammalian hearts, the increased contractility in response to β-agonists is accompanied by an accumulation of adenosine-3',5'-cyclic monophosphate (cAMP).3-5 Several groups have sought to identify targets of cAMP-dependent protein kinase that might affect contractility. Much of this work has focused on the structures that control the movement of calcium, namely the sarcolemma and the sarcoplasmic reticulum. These studies have led to the view that cardiac contractility is controlled by regulating the amount of calcium that is available to activate the contractile proteins.1,4,6 However, experiments with hyperpermeable cardiac cells have demonstrated that β-adrenergic stimulation and cAMP can also increase the maximum force that the myofilaments are able to develop in response to optimal concentrations of activating calcium.7 These agents also increase the calcium- and actin-activated adenosine triphosphatase (ATPase) activities of myosin.8

The extent to which isometric force production is enhanced by cAMP depends on the pattern of myosin isozymes that is present.9-11 Three isozymes of myosin have been identified in the rat ventricle on the basis of their differing mobilities on native gels during electrophoresis.12 They have been termed V1, V2, and V3 in order of decreasing mobility. Evidence from peptide mapping indicates that V1 and V3 are probably homodimers of two distinct myosin heavy chains, termed α-heavy chain (α-HC) and β-heavy chain (β-HC), respectively; V2 is believed to be a heterodimer of the two heavy chains.13 The light chain compositions of V1 and V3 are identical.12 Differences exist in the enzymatic function of the isozymes. Calcium- and actin-activated ATPase activities of V1 have been reported to be 2-8 times those of V3,12,14,15 while V2 has an intermediate enzymatic activity.14

The ventricular content of myosin isozymes changes during normal development. At birth, rat ventricular myosin is primarily V3, but by 3 weeks it is totally V1; V2 and V3 slowly reappear over the next several months.12,14 The distribution of myosin isozymes is also influenced by hormonal and hemodynamic factors. Hypothyroidism12,16-18 and mechanical overload19,20 both lead to a predominance of the V3 isozyme. As the concentration of V3 increases following thyroidectomy or during normal aging, the stimulatory effect of cAMP on maximum calcium-activated force production is reduced. This observation has led to the hypothesis that the β-adrenergic system selectively modifies V1 myosin.10,11

The aim of the present work was to examine the relation between myosin isozyme content and myosin ATPase activity in the rat heart during the first year and, in particular, the first 3 months of life, when the relative composition of the 3 myosin isozymes markedly changes. The effect of cAMP on myosin ATPase activity during this period was also measured. Hearts were rapidly frozen after dissection to preserve the structure and enzymatic function of the contractile proteins. Myosin ATPase activity was increased by cAMP during the first 2.0 months after birth and then remained approximately constant at this higher level until at least 4 months after birth. The specific activity of V1 can be further increased by the addition of adenosine-3',5'-cyclic monophosphate (cAMP). This effect of cAMP is age dependent, increasing threefold between 1 and 2 months following birth and then declining as V1 is replaced by V3. (Circulation Research 1987;61:914-924)
proteins and other cellular constituents as they exist in the intact hearts.

Materials and Methods

Tissue Preparation for Histochemical Procedures

Animals were obtained from Charles River Breeding Laboratories, Wilmington, Mass. Male Wistar and Sprague-Dawley rats, 1-12 months old, and Wistar rats, 1-9 days old, of undetermined sex were used in this study. In addition, some male Wistar rats that had been surgically thyroidectomized 5 weeks after birth were obtained and killed 6 weeks after surgery. Rats were decapitated, and their hearts were rapidly dissected and washed free of blood in oxygenated modified Krebs buffer (in mM): NaCl 140, K$_2$HPO$_4$ 2, MgCl$_2$ 2.5, CaCl$_2$ 2.5, glucose 10, and imidazole 10, pH 7.2. The atria and great vessels were removed. In hearts from animals older than 10 days, the free walls of the left and right ventricles were dissected and rapidly frozen in isopentane cooled with liquid nitrogen. Ventricles from younger animals were left unseparated and frozen in the same manner. Tissues were stored in liquid nitrogen. Sections were cut at -15 to -20°C using an IEC cryostat and then transferred to glass coverslips. Sections were 6 μm thick unless otherwise noted.

Myosin ATPase Assay

Calcium-activated myosin ATPase activity was measured in tissue sections by a previously described microphotometric technique. Sections were exposed to preincubation solutions containing 28 mM CaCl$_2$ and 6 mM sodium barbital, with the addition of drugs and nucleotides as noted below. Exposure to alkaline pH in the absence of adenosine 5′-triphosphate irreversibly inhibits V3 without affecting V1. In some cases, this property was used to permit measurement of the activity of only V1. Following preincubation, sections were assayed in incubation solutions containing (in mM) CaCl$_2$ 18, sodium barbital 20, and ATP 5, pH 10.5, again with addition of drugs and nucleotides as noted below. All preincubations were at room temperature, and all incubations were at 37°C. Sections were exposed to preincubation solutions, denoted by P, and incubation solutions, denoted by I, according to the following experimental protocols:

I. Measurement of V1 myosin ATPase activity. a) P at pH 10.5 for 15 minutes, b) I for 10 minutes.

II. Measurement of total myosin ATPase activity. All preincubations are at pH 7.0. Control: a) P for 15 minutes, b) I for 10 minutes. cAMP Stimulated: a) P + 5 mM theophylline for 5 minutes, b) P + 5 mM theophylline + 10 μM cAMP + 5 mM ATP for 10 minutes, c) I + 5 mM theophylline + 10 μM cAMP for 10 minutes.

Microphotometry

The absorbance of sections stained for myosin ATPase activity or with antibody was quantitated using the Zonax microphotometer system (Carl Zeiss, Inc., Thornwood, N.J.) in conjunction with a Zeiss photomicroscope II. In every section, the absorbance of a 3.17-μm diameter spot in each of 30 cells was determined, and the results were averaged to give a mean absorbance for each section. A 63× dry objective with a numerical aperture of 0.9 was used for all measurements.

Analysis of Myosin Isozymes by Native Gel Electrophoresis

Samples were thawed, tissue homogenized, and protein extracted as described below for the immunoblot procedure. Cylindrical gels were run, stained for protein, and scanned at 550 nm as in Mercadier et al., except that the concentrations of acrylamide and N,N′-methylene-bis-acrylamide (BIS) were 4.5% and 0.14%, respectively, and that the gels were run for 40-45 hours. The percents of V1, V2, and V3 were calculated from the areas under the peaks; it was assumed that the peaks were symmetrical and that, in going from the top to the bottom of each gel, the first half of the V3 peak and the second half of the V1 peak were uncontaminated by other isozymes. The percent of V1 heavy chain, or α-HC%, was calculated by assuming that α-HC% = V1% + (V2%/2). In a few instances, the percent of α-HC in a single ventricle was determined from several gels, and these trials indicate that the standard deviation of this technique ranges from 5 to 10% α-HC.

In addition, one gel was analyzed by digitizing the image of the gel obtained with a television camera and deconvoluting the peaks using a least-squares curve fitting routine; the shapes and areas of the peaks were calculated by assuming that all 3 peaks were normally distributed. This calculation yielded values for the percents of V1, V2, V3 and α-HC of 58, 11, 31, and 63, respectively, and indicated that the assumptions of symmetrical peaks and pure half-peaks for V1 and V3 were reasonable. The percents of V1, V2, V3, and α-HC obtained from this gel by the typical method were 58, 4, 39, and 60, respectively. Although the deconvolution indicates that the typical method of gel analysis overestimates the percent of V3 at the expense of V2, the two methods yield percents of V1 and α-HC that are in close agreement.

Antibody Production and Purification

Actomyosin was prepared from the isolated ventricles of 4-5-week-old male rats by the method of Adelstein et al., except that homogenization took place in 6 volumes of KCl 0.6 M, dithiothreitol 10 mM, sodium pyrophosphate 10 mM, MgCl$_2$ 2 mM, and Tris 15 mM, pH 7.5 with the addition of butanol to give a final concentration of 3%. The final actomyosin precipitate was suspended in 40 mM sodium pyrophosphate, 1 mM EDTA, 0.025% β-mercaptoethanol, pH 8.5 and centrifuged for 3 hours at 55,000g. The pellet was discarded, and the myosin-rich supernatant fraction was further purified by gel filtration on a 2.5×40 cm column of Bio-gel A15m 200-400 mesh agarose beads. The column buffer was KCl 0.6 M, dithiothreitol 1 mM, sodium pyrophosphate 10 mM, MgCl$_2$,
2 mM, and imidazole 10, pH 7.0, and 5-mL fractions were collected. Protein elution was monitored by measuring absorbance at 280 nm, and representative samples were analyzed by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Fractions containing the purest myosin, usually eluting between 60 and 120 mL, were pooled. After myosin was precipitated by overnight dialysis against 2 mM MgCl₂, 60 and 120 mL, were pooled. After myosin was eluted by placing the gel in 77 mM NaCl and 77 mM glycine, pH 2.7, for 20 minutes at 4°C and collected by filtration. This procedure was repeated 2 more times with an equal volume of Freund's adjuvant. Two rabbits were each initially injected with 2 mg of V1 myosin, followed by 5 injections of 1 mg each given at 2-week intervals. The rabbits were periodically bled from an ear vein, their sera were pooled, and IgG was isolated by precipitation with 50% saturated ammonium sulfate.

Thirty milligrams of V1 prepared as above and 30 mg of V3 prepared from thyroidecimated rats by the same method were each attached to 8 ml of Bio-gel A15m 200—400 mesh agarose beads by the method of March et al. Typically, 500 mg of IgG were reacted with the immobilized V1 for 2 hours. Unbound IgG was removed by filtration of the gel slurry; bound IgG was eluted by placing the gel in 77 mM NaCl and 77 mM glycine, pH 2.7, for 20 minutes at 4°C and collected by filtration. This procedure was repeated 2 more times using the unbound IgG fraction. The IgG fractions that had been bound to the immobilized V1 were then pooled and reacted with the immobilized V1 in the same manner for a total of 4 times to remove cross-reacting antibodies. The final unbound fraction was considered to be specific, affinity-purified anti-V1 antibody.

**Immunoblot Procedure**

Ventricles from a young normal and a chronically thyroidecimated rat were each homogenized in 6 volumes of NaCl 0.5 M, sodium phosphate 20 mM, MgCl₂ 0.1 M, 6-aminocaproic acid 0.1 M, benzamidine hydrochloride 5 mM, and EDTA 50 mM, pH 7.0, agitated for 20 minutes at 4°C and centrifuged at 27,000g for 10 minutes. The supernatant fractions were diluted 1:1 with 6% SDS, 10% β-mercaptoethanol, 20% glycerol, and 125 mM Tris, pH 6.8. Six micrograms protein were loaded onto each lane of a slab gel composed of a top to bottom 10–20% gradient of acrylamide, 0.1% SDS, 375 mM Tris, pH 8.8, and electrophoresis took place in a reservoir buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS. Proteins were electrophoretically transferred from the gel to a sheet of nitrocellulose paper essentially as described by Towbin et al. Transfer took place in the presence of 50 mM Tris, 187 mM glycine, and 30% methanol for 1 hour using a 12-volt battery charger. Part of the nitrocellulose was stained with amido black to verify that transfer of all proteins was satisfactory.

The remaining portion of the nitrocellulose was washed with agitation in the following: a) wash buffer composed of 150 mM NaCl, 50 mM Tris, 0.05% nonidet P-40, and 0.1% bovine serum albumin (BSA), pH 8.0 (overnight); b) wash buffer + 5 mg/ml normal goat IgG (30 minutes); c) wash buffer (3 × 5 minutes); d) wash buffer + 5 mg/ml normal goat IgG and 0.5 mg/ml affinity-purified anti-V1 antibody (15 minutes); e) wash buffer (3 × 5 minutes); f) wash buffer + 2 mg/ml normal goat IgG and peroxidase labelled goat anti-rabbit IgG diluted 1 in 100 (15 minutes); g) wash buffer (2 × 5 minutes); h) 150 mM NaCl and 50 mM KH₃PO₄, pH 7.2 (2 × 5 minutes); i) 15 mM NaCl and 50 mM Tris, 0.02% H₂O₂, 0.033% 3,3′-diaminobenzidine-tetrahydrochloride, pH 7.6 (5 minutes); j) water (5 × 1 minute). The stained nitrocellulose was then blotted with filter paper and allowed to dry.

**Antibody Staining Protocol**

The antibody staining procedure consisted of immersing sections attached to coverslips in the following solutions with continuous agitation: a) 50 mM Tris, 187 mM glycine, and 30% methanol (15 minutes); b) 0.05% nonidet P-40 in wash buffer composed of 50 mM Tris and 150 mM NaCl, pH 8.0 (3 × 5 minutes); c) wash buffer (3 × 5 minutes); d) wash buffer + 20 mg/ml goat γ-globulins (30 minutes); e) wash buffer + 20 mg/ml goat γ-globulins + 0.05 mg/ml affinity-purified anti-V1 antibody (30 minutes); f) wash buffer (3 × 5 minutes); g) wash buffer + 10 mg/ml goat γ-globulins and 0.2 mg/ml goat anti-rabbit IgG (30 minutes); h) wash buffer (3 × 5 minutes); i) wash buffer + 10 mg/ml goat γ-globulins and rabbit peroxidase anti-peroxidase complex diluted 1 in 50 (30 minutes); j) wash buffer (3 × 5 minutes); k) 0.05 M Tris, pH 7.6 (2 × 5 minutes); l) 0.05 M Tris, pH 7.6, 0.015% H₂O₂, and 0.05% 3,3′-diaminobenzidine tetrahydrochloride (15 minutes); m) water (5 × 1 minute). The stained sections were mounted in a standard salt solution containing 25% glycerol.

This protocol is a modification of Sternberger's unlabelled antibody peroxidase anti-peroxidase technique. The concentrations of goat anti-rabbit IgG and peroxidase anti-peroxidase complex used were found to be saturating, as a threefold increase in either of the two produced no significant change in the absorbance of stained serial sections.

**Quantitative Immunocytochemistry**

Because cell-to-cell heterogeneity in the relative concentration of myosin isozymes has been shown in mammalian hearts, myosin isozyme concentration was measured in the same cells as ATPase activity. A quantitative immunocytochemical procedure that measured the relative concentration of V1 in sections serial to those used for ATPase measurements was developed.

A polyclonal antibody against V1 was raised in rabbits and affinity purified. The specificity of the antibody for V1 was evaluated using the isolated antigen and, more relevant to the purpose of this study,
by examining its binding to the different isozymes of myosin in tissue sections. The purified antibody binds to the V1 heavy chain in immunoblots with a specificity of over 4 to 1. It does not react with the myosin light chains or any other cardiac proteins (Figure 1). The most important test for antibody specificity was the nature of its binding to myosin in sections of frozen ventricles. In micrographs of sections from a 2-month-old rat containing only V1 myosin, staining is clearly localized to myofibrils, while there is only a diffuse, nonspecific staining in sections from thyroidectomized animals devoid of V1 (Figure 2).

In sections up to 6 μm in thickness, the microphotometric technique is clearly sensitive to changes in the amount of antigen present (Figure 3); penetration of antibody is not a significant problem in the 6-μm-thick sections typically used in this study. The small deviation from linearity in the relation for sections less than 6 μm in thickness is probably a consequence of a small impairment of penetration in the sections. Even in the thicker sections, antibody penetration would not limit the quantitation of myosin isozymes if they were homogeneously distributed within individual cells, as is indicated by immunofluorescent studies.

To convert the microphotometric measurements of antibody binding to V1 heavy chain concentration, V1 was measured by immunocytology and native gel electrophoresis on the same tissues. Ventricles with different concentrations of V1 were obtained from normal animals ranging in age from 1 to 12 months and from animals that had been thyroidectomized. Three to seven nonserial sections from randomly selected portions of each ventricle were stained with antibody, and microphotometric measurements were averaged to yield a mean absorbance for each ventricle. The unsectioned portion of each ventricle was used for the measurement of myosin isozyme composition by native gel electrophoresis. Since the percent of cytoplasmic volume occupied by myofibrils is independent of age and thyroid function, concentration of myosin is the same in all tissues, and differences in the intensity of antibody staining should indicate changes in the percent of V1. This issue is not as important in the detection of changes in specific activity of myosin by measurement of ATPase activity and antibody concentration in serial sections. The microphotometric measurement of antibody binding exhibits a nonlinear relation to the electrophoretic measurement of α-HC% (Figure 4). The data were fit by a second-order equation of the form:

\[
\text{Absorbance} \ (6.29 \times 10^{-6}) (\alpha\text{-HC%})^2 + 0.128
\]

The correlation coefficient is 0.90, and the addition of a linear term to the equation does not improve the correlation. The average of the deviations of the points from the calibration curve corresponds to a mean error of 7.98% in the estimation of α-HC%. (A curve using the absorbance measurements from individual sections instead of the average of values obtained from several sections for each ventricle is almost identical.) This relation was used to calculate α-HC% from the absorbance measurements obtained from individual sections. Because the vast majority of data obtained in this study was from tissue containing greater than 50% α-HC, the relative insensitivity of the immunocytochemical assay at lower concentrations was not a limiting factor. The nonlinearity of the curve relating the absorbance of sections stained with antibody to the α-HC% as determined by native gel electrophoresis is consistent with the positive cooperativity expected in the binding of bivalent antibodies to an immobilized matrix of antigens. Identical antigens of myosin should exist every 14.3 nm, the periodicity observed with x-ray diffraction, and be well within the 20 nm spanned by a single bivalent antibody. This type of cooperativity arises because the probability of a single antibody being able to span the distance between two antigenic sites, and therefore binding to the section twice as strongly, is a nonlinear function of antigen concentration. Therefore, the observed curve most likely represents the bottom portion of a sigmoidal relation. Positive cooperativity has also been observed in an in vitro enzyme-linked immunoabsorbant assay for measuring the percent of V1 in a sample of purified myosin.

For the data obtained from normal adult animals by immunocytocchemistry, the mean error in the estimation...
of \( \alpha \)-HC\% compared with data from electrophoresis was 8%. The average cytoplasmic concentration of myofibrils in hearts from different animals varied by approximately 6%.\(^{3,9,21} \) Therefore, most of the error in the estimation of \( \alpha \)-HC\% in a single ventricle is probably due to variation in the cytoplasmic concentration of myofibrils.

**Data Analysis**

In order to correct for any minor day-to-day variation in procedures, values for total myosin ATPase activity were always normalized by using the average ATPase activity of sections from 1-12-month-old normal animals assayed with each experiment under control conditions. Even without this normalization, day-to-day variability of the average ATPase activity measured in sections from 1-12-month-old animals was small, having a standard error of the mean of less than 8%.

Comparisons between \( \alpha \)-HC\% as determined by microphotometry and ATPase activity were made only for data obtained from serial sections.

In calculations and figures, each ventricle was treated as a single sample from the population (i.e., as \( n = 1 \)). In some cases, more than one set of serial sections were used from the same ventricle.

All regressions were computed by the method of least squares, and a Student's \( t \) test was used to determine whether correlation coefficients and the differences between means were significantly different from zero. All errors indicated are standard errors of the mean.

**Results**

**Changes in \( \alpha \)-Heavy Chain Percent and Myosin ATPase Activity in the Maturing Heart**

The percent of each of the isoforms of myosin in the ventricles of hearts from rats between the ages of 1 day and 12 months has been measured by native gel electrophoresis and quantitative immunocytology (Figures 5 and 6). The two techniques give similar results. As reported by others, the relative amount of the \( \alpha \)-HC increased from about 30% in the immediately neonatal period to 100% by 1 month of age. From age 2 months, the relative amount of \( \alpha \)-HC begins to decline, and by 1 year, it represents only about 70% of the total myosin.

The activity of calcium-activated myosin ATPase has been measured in cryostatic sections taken from the same hearts (Figure 7). Relative changes in the amounts of myosin isozymes and total ATPase activity do not follow each other precisely. The enzymatic activity is lowest in the immediately neonatal period and then rises over the first 10 days of life as the amount of V1 increases. Myosin ATPase activity remains nearly constant from 2 to 12 months during which some of the \( \alpha \)-HC is being replaced by \( \beta \)-HC. A least squares
fit to the values for ATPase activity from 4 to 12 months gives a straight line with a correlation coefficient of 0.9 and a slope that is not significantly different from 0. A similar analysis of the values for α-HC (Figure 6) gives a slope that is significantly different from that for ATPase activity (p<0.05).

The ATPase activity of V1 can be directly measured by taking advantage of the irreversible inhibition of the ATPase activity of V3 that occurs with exposure to alkaline pH in the absence of ATP. Preincubation for 15 minutes at pH 10.5 inhibited ATPase activity by 94±2% in sections from thyroidectomized animals containing only V3 (n=3 ventricles) but had no significant effect on the ATPase activity in sections from 1-2-month-old animals containing only V1 (the change was 7±9%, n=1 ventricles). The ability to inhibit V3 in frozen sections by alkalinity has already been demonstrated.28 The inability of Yazaki and Raben22 to produce total inhibition in rabbit heart probably has two explanations: they used pH 9.0, which has been found to be inadequate for total inhibition of V3 in rat hearts (unpublished work, A. Weisberg and S. Winegrad), and there was no indication that their rabbit hearts contained only the slow isozyme of myosin. Both the concentration and the ATPase activity of V1 rapidly increase during the first month of life, but the ATPase activity of V1 continues to rise even after its concentration has reached its maximum (Figures 6 and 7). These measurements of V1 ATPase activity and α-HC% were used to calculate the relative specific activity of V1 in each ventricle. Following birth, there is a significant age-dependent increase in V1 specific activity. By 2.0 months, V1 specific activity has doubled and remains at this elevated level (or higher) at least until 12 months after birth (Figure 8).

The same changes in the specific activity of V1 during the first month were observed regardless of whether the content of V1 was measured by electrophoresis or immunocytochemistry. Heterogeneity of isozyme distribution among cells, therefore, is not responsible for the observed changes in the specific activity of V1 ATPase.

The immunocytologic measurements indicate that V1 specific activity continues to increase throughout the first year of life. Because this technique measures myosin isozyme composition and ATPase activity in serial sections, these estimates of V1 specific activity should be more accurate than those derived from gel measurements of V1 concentration.

V3 is the predominant isozyme in neonatal hearts,
Effect of cAMP on Myosin ATPase Activity

Previous studies have shown that the maximum calcium-activated force produced by hyperpermeable cardiac cells can be increased by cAMP and myosin ATPase activity in sections of quickly frozen hearts can be increased by the same second messenger. The enhancement of ATPase activity from exposure of the tissue section to cAMP is age dependent, exhibiting a threefold increase in magnitude over the first 2 months of life (Figure 9). Because the relative amount of V1 remains near 100% from 1 to 2 months, the increase in the effect of cAMP on myosin ATPase activity during this period probably reflects maturation of a system that regulates the specific activity of V1 myosin. The time course of the development of the cAMP-activated system correlates well with the age-dependent changes in V1 specific activity measured under control conditions. Both of these developmental processes appear to be incomplete in animals younger than 1.5–2 months. Therefore, we investigated the myosin isozyme dependence of the effect of cAMP in animals 1.5 months and older.

Serial sections of hearts from animals between 1.5 and 12 months old were used for measurement of total myosin ATPase activity and relative concentration of V1. Total myosin ATPase activity was measured either under control conditions or in the presence of cAMP. The lack of a correlation between the relative amount of a-HC and total myosin ATPase activity during control measurements of enzymatic activity is converted to a positive correlation when cAMP is included in the enzymatic assay (Figure 10). In contrast to control conditions, the results indicate that in the presence of cAMP, the specific activities of V1 and V3 are constant and independent of age or concentration (Figure 10). Some additional interesting inferences can be made from the regression line relating relative amounts of a-HC to cAMP-stimulated myosin ATPase activity. The value extrapolated to 0% V1 or 100% V3 is approximately 40% of the maximum value seen with 100% V1. This ratio of V3: V1 ATPase activity has been indicated in studies with more traditional techniques of measuring ATPase activity. The linear relation between a-HC% and ATPase activity during cAMP stimulation suggests that the contribution of V3 to the total ATPase activity during cAMP stimulation...
is minimal in adult rat hearts. This finding is consistent with the observation that cAMP inhibits V3 activity, which has been reported in more detail elsewhere.\(^\text{11}\)

**Discussion**

**Specific Activity of VI and V3: Age and cAMP Dependence**

During the first year of the rat’s life, there is a change in the specific activity of calcium-activated myosin ATPase in the heart that is independent of alterations in isozyme composition. Direct measurements of the ATPase activity of VI indicate an increase in its specific activity of 100% during this period. It appears likely that the specific activity of V3 is also regulated in as much as the value for total myosin ATPase in the neonates, in which most of the myosin is V3, is about half of that in hearts from thyroidec tomized rats, in which all or almost all of the myosin is also V3. As these experiments were performed on rapidly dissected and frozen tissue with a minimum of experimental manipulation, the age-dependent change in VI specific activity should reflect changes in the properties of the enzyme in the intact heart.

Between 1 and 2 months after birth, the increase in ATPase activity produced by cAMP triples even though myosin isozyme composition remains constant at 100% VI. Apparently, the cAMP-dependent system for regulating the specific activity of myosin is not completely developed before 2 months. The rise in specific activity of VI, measured under control conditions, follows a time course similar to that of the cAMP effect, increasing after birth and reaching its maximum level at 2.0 months. The close temporal correspondence between change in the specific activity of VI and development of a response to cAMP applied exogenously suggests that both phenomena are reflections of the postnatal development of a physiologic system for regulation of VI. (Response of the ATPase activity of VI to endogenous cAMP has already been shown in isolated rat hearts perfused with isoproterenol prior to freezing.) The decline in the amplitude of the cAMP effect on total ATPase after 2–3 months of age should be the result of the increasing concentration of V3 at the expense of V1. cAMP has been shown not only to increase the ATPase activity of V1 but also to decrease the ATPase activity of V3.\(^\text{11}\)

Although it has been customary to focus on the regulation of cytoplasmic calcium concentration for modulation of contractility in cardiac muscle, many instances of changes in contractile proteins themselves have been reported. The two now generally accepted mechanisms for modification of contractile proteins, phosphorylation of the inhibitory subunit of troponin to produce a decrease in calcium sensitivity\(^\text{33–35}\) and change in the myosin isozyme composition of myocardial cells, are inadequate to explain observations in the literature. For instance, Martin et al\(^\text{17}\) have found that injection of thyroid hormones into rabbits containing primarily V3 causes an increase in the relative amount of VI present in the cells and a parallel rise in calcium-activated ATPase activity of myosin over at least 10 days. However, the actin-activated ATPase activity of isolated myofibrils rises for only 2–3 days and then remains constant. Because the calcium- and actin-activated ATPase of pure myosin increase in parallel with the increase in VI, there must be an additional factor dissociating this relation in the intact cell or in the myofibril even after its removal from the intact cell. Diabetic rats have a reduced amount of VI and a decreased myosin ATPase activity, both measured in frozen hearts.\(^\text{36}\) When thyroid hormone is administered to these rats, the decrease in myosin ATPase is prevented, but the change in the myosin isozymes is not prevented. In larger mammals, pressure overload hypertrophy of hearts produces a decrease in myosin ATPase activity with no change in myosin isozymes because the animals already contain almost entirely or entirely V3.\(^\text{37}\) Similar results have been found in hearts from animals suffering from congestive failure.\(^\text{38,39}\)

There are also reports in the literature that show a dissociation between myosin isozyme shift and myosin ATPase activity in hearts from animals at different ages. Alpert et al\(^\text{40}\) have found a decrease in myosin ATPase activity of about 15–25% as rats age from 100 to 1,000 days, but the decline in the relative amount of V1 over this period is at least twice that amount.\(^\text{10,19}\) At a recent meeting of the European Conference on Muscle and Motility, Capelli et al,\(^\text{41}\) using myofibrils isolated from the free wall and the left ventricles of rats by the technique of Solaro et al,\(^\text{42}\) measured myofibrillar ATPase activity. They found that the ATPase activity increased by almost 50% between 10 and 20 days (no measurements were made at ages younger than 10 days), and between 60 and 240 days, there was a small but statistically insignificant decline in ATPase activity. Several laboratories have shown that a significant
decrease in V1 occurs between 60 and 240 days and very little increase occurs between 10 and 20 days. Therefore, the results of Capelli et al confirm the findings first reported in 1984 by Horowits and Winegrad. These studies also showed that additional, necessary features of the putative regulatory system include a phosphorylation reaction and the release of a 21,000-dalton "active factor." 

In this report, we have presented evidence that this same cAMP-dependent regulatory system is probably responsible for the changes in the specific activity of myosin that are observed during the first year of life. Demonstration of these changes depends on preservation of the in vivo regulatory state of the contractile proteins in as much as they do not survive extraction of the proteins. Techniques such as quick freezing as used in this study and by Garber and the Solaro technique as used in the work of Martin et al and Capelli et al appear to preserve the in vivo regulatory state. Loss of the regulatory state with time after removal of the heart from the animal has been observed in our laboratory (N. Kato, A. Weisberg, and S. Winegrad, unpublished observations).

In summary, there appears to be a cAMP-dependent system for regulating cardiac myosin that is absent at birth and which appears during the first 1–2 months of age. It seems to be important in the normal regulation of the properties of the contractile proteins in the adult rat. An analogous system has also been demonstrated in the rabbit heart (N. Kato, A. Weisberg, and S. Winegrad, unpublished results).
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924
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