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MYOSIN AND ELECTROPHYSIOLOGICAL HETEROGENEITY  
IN CARDIAC MUSCLE

A thesis submitted to the University  
of Glasgow  
in Candidature for the degree of  
DOCTOR OF PHILOSOPHY  
in the Faculty of Science

by

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from

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CONTENTS

|   | Page |
|---|------|
| <u>Acknowledgements</u> ... ..                              | (i)  |
| <u>Contents</u> ... ..                                      | (ii) |
| <u>Summary</u> ... ..                                       | (v)  |
| <u>Introduction</u>   |      |
| General introduction ... ..                                 | 1    |
| Contractility changes: the role of myosin ... ..            | 4    |
| Contractility changes: sarcoplasmic reticulum function ...  | 10   |
| Contractility changes: electrophysiological properties ...  | 15   |
| The present work ... ..                                     | 19   |
| <u>Methods</u>  |      |
| Animals ... ..  | 20   |
| Preparation of histochemical and histological sections ...  | 20   |
| ATPase staining ... ..                                      | 21   |
| Metabolic stains ... ..                                     | 23   |
| Other stains ... ..   | 24   |
| Photometric methods ... ..                                  | 24   |
| Fibre cross-sectional area measurement ... ..               | 26   |
| Sympathectomy ... ..  | 28   |
| Formaldehyde induced fluorescence of adrenergic endings ... | 29   |
| Electrophysiological methods ... ..                         | 31   |
| <u>Results</u>  |      |
| Inter-species comparison ... ..                             | 34   |
| Workload: fibre metabolic capacity ... ..                   | 48   |
| Workload: fibre cross-sectional area ... ..                 | 51   |



|                                 |     |     |     |     |     |     |     |     |
|---------------------------------|-----|-----|-----|-----|-----|-----|-----|-----|
| Staining methods                | ... | ... | ... | ... | ... | ... | ... | 152 |
| Results                         | ... | ... | ... | ... | ... | ... | ... | 154 |
| Discussion                      | ... | ... | ... | ... | ... | ... | ... | 158 |
| <u>Supplementary Chapter 3</u>  |     |     |     |     |     |     |     |     |
| <u>Isolated Myocytes</u>        |     |     |     |     |     |     |     |     |
| Introduction                    | ... | ... | ... | ... | ... | ... | ... | 162 |
| Methods                         | ... | ... | ... | ... | ... | ... | ... | 164 |
| Isolation techniques            | ... | ... | ... | ... | ... | ... | ... | 164 |
| Single cell staining techniques | ... | ... | ... | ... | ... | ... | ... | 165 |
| Results                         | ... | ... | ... | ... | ... | ... | ... | 169 |
| Discussion                      | ... | ... | ... | ... | ... | ... | ... | 173 |
| <u>References</u>               | ... | ... | ... | ... | ... | ... | ... | 177 |

SUMMARY

The work presented in this thesis is involved in the main with myosin types in the rabbit ventricle. These have been investigated using calcium-activated myosin ATPase staining. Considerable heterogeneity of myosin ATPase staining intensity, and hence myosin isoform distribution, is shown to exist in the ventricle of the rabbit. This is compared with the situation in the ventricles of several other mammalian species.

The heterogeneity found in the rabbit ventricle can be divided into two categories: regional and local. The most commonly found example of regional heterogeneity was between endocardial and epicardial fibres. Lower intensity staining (and hence a higher proportion of  $V_{\alpha}$  myosin) was always found in endocardial fibres relative to epicardial fibres. Local heterogeneity refers to differences in staining intensity between neighbouring cells. These local differences have been examined photometrically and the possibility of distinct fibre types is discussed.

Workload has been investigated as a possible cause of both regional and local myosin heterogeneity; metabolic capacity and cross-sectional area have been used as indirect measures of workload. It was found that it was possible to explain transmural myosin heterogeneity in terms of workload but that local heterogeneity could not be wholly explained in such terms.

The possibility that the sympathetic nervous system might have a role in the production of myosin heterogeneity in the rabbit heart

was investigated by examining distribution of nerve endings and the effect of chemical sympathectomy. It was concluded that neither regional nor local heterogeneity was dependent on sympathetic nerve endings, as both forms of heterogeneity survived their complete removal.

Another aspect of heterogeneity investigated in this work was electrophysiological and the possibility of a correlation of this and myosin distribution was investigated. Muscles from various regions of the heart were compared in terms of electrophysiology and myosin ATPase activity and it was found that in general longer action potentials were associated with lower myosin ATPase activity (and hence a higher proportion of  $V_3$  myosin). The possibility that changes in electrophysiology might act as a trigger to change myosin distribution is discussed.

Three self-contained chapters are also included. These deal with: differences between rat atrial and ventricular myosin heavy chains; differences between reptilian atrial and ventricular myosins; and methods of staining individual isolated myocytes for myosin ATPase activity. The first of these shows, using histochemical and immunohistochemical techniques, that differences do exist between rat atrial ( $HC_{\alpha}$ ) and ventricular ( $HC_{\beta}$ ) myosin heavy chains. The chapter on reptilian hearts shows that differences in myosin ATPase staining between atrium and ventricle, similar to those found in mammalian hearts, can also be demonstrated in reptilian hearts. These differences appear to be attributable to differences in workload (as shown by metabolic capacity) as is the case in mammalian hearts.



In the chapter on isolated myocytes are described two techniques for myosin ATPase staining of these cells, one of which could be used to cut and stain single, identified myocytes. There is also a discussion of the fact that these cells were electrophysiologically non-viable.

DECLARATION

I hereby declare that this thesis embodies the results of my own special work, that was carried out in the Institute of Physiology, The University of Glasgow, between August, 1983 and December, 1986.

This thesis does not include work forming part of a thesis presented successfully for a degree in this or another University.

## INTRODUCTION

It is well known that myosin composition of skeletal muscle fibres can be altered by changes in the pattern of fibre activation. This has been demonstrated by means of cross-reinnervation experiments where for example, a slow muscle nerve is made to innervate a fast muscle. This leads to the re-innervated muscle producing slow myosin and becoming a slow muscle (Buller et al., 1960)\*. Myosin type can also be changed in skeletal muscle fibres by chronic stimulation at higher than normal frequencies for slow skeletal muscle and at lower than normal frequencies for fast skeletal muscle (Salmons & Vrbova, 1969)\*. Clearly, therefore, electrophysiological interventions can have an influence on myosin gene expression in skeletal muscle fibres.

In cardiac muscle, it is known that atrial action potentials are shorter and have a less pronounced plateau phase than ventricular action potentials (e.g. Hume & Uehara, 1985). Since the first demonstration of Sartore et al. (1978) in chick heart it has also become apparent that atrial and ventricular myosins are distinct molecules in most species (Sartore et al., 1981; Syrový et al., 1979; Yazaki et al., 1979). It is obviously not possible for different activation frequencies of the atria and ventricles to be the cause of these differences in myosin type but the difference in action potential configuration is another obvious candidate.

Watanabe et al. (1983) have shown that, in the intact rat ventricle and in myocytes isolated from the rat ventricle, considerable heterogeneity of action potential duration exists; there being three distinct groups of action potentials. The earlier

discovery of three distinct isoforms (distinct molecules having the same enzymatic function) of ventricular myosin in the rat (Hoh et al., 1978) appeared to make it possible that a relationship between electrophysiology and myosin gene expression, not unlike that existing both within skeletal muscle and between atrial and ventricular muscles, could also exist within ventricular muscle.

In skeletal muscle Barany (1967) has shown that the ATPase activity of myosin is directly proportional to the speed of shortening of the muscle from which it came. The same has been shown for cardiac muscle by Hamrell & Low (1978), Carey et al. (1979) and Schwartz et al. (1981). It seems reasonable to suppose from this that chronic changes in cardiac contractility might be a consequence of changes in the proportions present in the ventricles of the different myosin isoforms discovered by Hoh et al. (1978).

I propose below to summarise some of the evidence for the involvement of myosin isoform distribution in chronic alterations of cardiac contractility and other evidence which suggests a role for changes of electrophysiological properties. Sarcoplasmic reticulum function will also be discussed in this regard. First however, it will be necessary to discuss briefly the structure of myosin.

Myosin consists of six sub-units: two heavy chains (molecular weight about 200,000 Da) and two pairs of light chains (molecular weights between 16,000 and 27,000 Da). The enzymatic activity (i.e. the ATP hydrolysis site) of myosin is situated on the heavy chains. The role of the light chains is not yet clear it may be that they

play a part in the regulation of the heavy chain ATPase activity (Chantler 1981).

In rat cardiac muscle Hoh et al. (1978), using a non-dissociating electrophoretic technique, identified five myosin isoforms. These they named  $A_1$ ,  $A_2$  (atrial forms),  $V_1$ ,  $V_2$  and  $V_3$  (ventricular forms). As this technique only allows whole myosins (i.e. heavy chains + light chains) to be compared, the differences between the electrophoretic mobilities of these myosin isoforms could be due to either light and/or heavy chain heterogeneity. Hoh et al. concluded that the differences between the ventricular myosin isoforms were located on the heavy chains as each of the isoforms had identical light chain complements. Similarly the atrial isoforms differed only in their heavy chains. It has since been shown that only two distinct heavy chain types exist in the ventricles of the rat. These heavy chain isoforms have been named  $\alpha$  and  $\beta$ , and they combine as a heterodimer ( $\alpha\beta$ ) to form  $V_2$  with  $V_1$  and  $V_3$  being the homodimers  $\alpha\alpha$  and  $\beta\beta$  respectively. The  $\alpha$  and  $\beta$  heavy chains have been shown to be different in their primary structures by peptide maps (Hoh et al., 1979), and immunological properties (Chizzonite et al., 1982) and their respective genes have been isolated and characterized (Madhavi et al., 1982).

The ATPase activities of  $V_1$  and  $V_3$  myosins have been shown to be different by Pope et al. (1980). They found that  $V_1$  myosin had a higher  $Ca^{2+}$ -activated ATPase activity than  $V_3$  myosin and that the activity of  $V_1$  was more alkali stable than that of  $V_3$  myosin, resembling fast twitch muscle and atrial myosins in this respect. As ATPase activity is a heavy chain property (Chantler, 1981) these

differences in activity between  $V_1$  and  $V_2$  must be due to differences between the  $\alpha$  and  $\beta$  heavy chains.

Contractility changes: the role of myosin

In cardiac muscle, changes in contractility are known to be caused by a variety of disease states e.g. thyrotoxicosis and hypertension. It has been shown recently that these changes can be at least partially explained by changes in the type of myosin present in the cardiac cells.

There have been many studies which have looked at the myosin composition of the heart in conditions which alter cardiac contractility. The earliest of these was by Hoh et al. (1978) who looked at the effect of hypophysectomy and thyroid hormone. They found that hypophysectomy (which has been shown to reduce cardiac contractility; Buccino et al., 1977) led to a decrease in the proportion of  $V_1$  myosin (the high ATPase activity isoform) and its replacement with  $V_2$  (the low ATPase activity isoform). This overall reduction of myosin ATPase activity in the heart provides a possible explanation for the decreased contractility, as evidenced by reduced maximum rate of tension production (maximum rate of rise of twitch) and decreased maximum speed of shortening. These changes in contractility following hypophysectomy have been shown to be reversible on administration of thyroxine (Beznak, 1963) and Hoh et al. showed that thyroxine administration also reverses the changes in myosin isoform distribution caused by hypophysectomy.

It appears therefore that the changes in cardiac contractility which follow removal and replacement of thyroid hormone can be at least partly explained by changes in the distribution of

ventricular myosin isoforms and the resultant changes in myosin ATPase activity.

Recently the mechanism of action of thyroxine has been investigated by Lompre et al. (1984). They found that thyroid hormone directly affects the transcription rates of the cardiac myosin heavy chain genes. When increased levels of thyroxine were present they found the transcription rate of the  $\alpha$  myosin heavy chain gene was increased whereas that of the  $\beta$  myosin heavy chain gene was decreased. The idea of thyroxine directly affecting gene transcription rates in cardiac muscle is, of course, in keeping with the general view of  $T_3$  and  $T_4$  actions; specifically to the present tissue it is supported by the identification of chromatin-associated  $T_3$  receptors (Samuels, 1978). These changes in gene transcription rates, of course, lead to increased production of  $\alpha$ -myosin heavy chain and decreased production of  $\beta$ -myosin heavy chain, so the proportion of  $V_1$  myosin in the ventricle increases and the proportion of  $V_2$  myosin decreases.

Another condition leading to altered cardiac contractility which has received a great deal of attention recently is cardiac overload. Many studies have followed that of Spann et al. (1967) reporting that mechanical overload causes a reduction in the "intrinsic contractility" of the heart i.e. that time to peak isometric tension is increased and maximal speed of unloaded shortening is decreased (Maughan et al., 1979). Other studies have shown that these changes in cardiac contractility can, in small mammals at least, be explained by changes in myosin isoform distribution. For example Lompre et al., (1979) have shown that,



pressure overload in the rat leads to an increase in the proportion of  $V_3$  myosin, the low activity isoform. The cardiac response to pressure overload in larger mammals (such as man), where  $V_3$  myosin predominates normally, does not seem to involve any redistribution of myosin isoforms. This has been demonstrated by the use of anti- $V_1$  and  $V_3$  selective antibodies (Mercadier et al., 1983). Reduced  $Ca^{2+}$ -activated ATPase activity has, however, been reported in severely stressed human hearts (Leclercq & Swynghedauw, 1976). Swynghedauw et al. (1982) have suggested that these changes in ATPase activity could be due to changes in some other sarcomeric protein e.g. troponin.

Redistribution of myosin isoforms is not the only adaptation made by the mechanically overloaded heart. Hypertrophy of the ventricles is an attempt by the heart to restore wall stress to normal by 'spreading the load'. However it is not always accompanied by an increase in the proportion of  $V_3$  myosin in the affected ventricle. For example in the case of the thyrotoxic heart, where hypertrophy often occurs,  $V_1$  myosin predominates (Morkin, 1979). In addition, not all kinds of mechanical overload lead to increased levels of  $V_3$ . Volume overload does not always lead to a change in myosin distribution in either direction, whereas pressure overload appears always to produce an increased proportion of  $V_3$  in the affected ventricle.

Swynghedauw et al. (1982) have suggested a reason for this difference between pressure and volume overloads. They have argued that a decline in contraction efficiency is brought about by a pressure overload but does not necessarily follow a volume

overload. This in turn, they say, is due to the different effects of these two conditions on the speed of contraction of the myocardium. An increase in end-diastolic volume (as would be seen in a volume overload) moves the myocardium onto a higher force-velocity curve (Pollack, 1970). This means that for a given tension production a higher speed of shortening is attained. As cardiac muscle achieves its maximal contraction efficiency at the speeds of shortening used in exercise (which are of course higher than those at resting heart rates) it appears unlikely that volume overload will lead to a decrease in contraction efficiency. Pressure overload slows the speed of contraction of the myocardium, by requiring more tension on the same length-tension curve, moving it further from its peak efficiency speed.

Studies on different types of cardiac work have been done which support this proposed difference between pressure and volume overloads. In 1914 Evans & Matsuoka found that for equivalent increases in stroke work an elevation of blood pressure was more costly, in terms of increased oxygen consumption, than an increase in stroke volume. Similar results have been reported by Pool et al. (1968) who found that an increase in external work (i.e. work against a volume) was less costly in terms of changes in high energy phosphates than an increase in internal work (where work is done to produce tension rather than shortening i.e. a pressure overload).

Clearly if pressure overload leads to a decline in contraction efficiency, by slowing the rate of contraction, then it is to the advantage of the myocardial cells to produce contractile proteins

which are more efficient under these conditions. This is indeed what happens: Alpert & Mulieri (1982) have shown that rabbit cardiac muscle, hypertrophied in response to a pressure overload, contracts more economically than normal cardiac muscle. They suggest that at least part of this improved efficiency is due to the decrease in myosin ATPase activity which has been shown to occur as a result of pressure overload (Carey et al., 1978). This decreased ATPase activity is, of course, due to the increased proportion of  $V_3$  myosin which is known to follow a pressure overload.

If the increased proportion of  $V_3$  myosin in pressure overloaded ventricles is brought about in order to improve contraction efficiency, then it is clear that a volume overloaded ventricle will not benefit from such an adaptation (cases of volume overload where  $V_3$  does increase are normally complicated by a pressure overload component; Moalic et al., 1981).

The changes in cardiac myosin isoform distribution in response to either thyroid hormone imbalance or mechanical overload are obviously adaptations to pathological conditions. However, more physiological influences on cardiac myosin distribution do exist e.g. maturation and exercise. As the principles involved in these cases are essentially the same as in thyroid imbalance and mechanical overload they will be dealt with more briefly.

Capasso et al., (1983) have reported a study in which they looked at the mechanical properties of papillary muscles of rats of different ages, ranging from 3 to 24 months. They noticed that peak unloaded shortening velocity decreased and time to peak isometric

tension increased with age i.e. that the intrinsic contractility of the heart decreased with age. Prior to this Hoh et al., (1978) in the rat and Chizzonite et al., (1982) in the rabbit showed that after birth the proportion of  $V_3$  in the ventricles steadily increased with age. As with hypothyroidism and pressure overload the decreased contractility which progresses with age appears to be at least partly explained by a change in myosin isoform distribution.

The increase of  $V_3$  with age seems to be related to the size of the animal. The myosin redistribution appears to be an attempt to maximise the efficiency of the slower contraction required of the larger heart of a larger (older) animal. A slower contraction is required in larger hearts because the mass (volume) of blood increases out of proportion with the cross-sectional area and hence the strength of the ventricular wall. If a larger heart tried to contract as quickly as a smaller heart it would rupture as the ventricular walls would not be strong enough to withstand the tension developed. A slower contraction will, of course, be done more efficiently by a slower myosin, hence the increased proportion of  $V_3$  myosin in the ventricles of older and larger animals. The same increase of  $V_3$  myosin with animal size can be seen between species of different size. In large species such as man,  $V_3$  myosin predominates in the ventricles (Cummins, 1984) whereas in the rat ventricle little  $V_3$  is present (Hoh et al., 1978).

The effect of exercise on cardiac myosin distribution is similar to the effect of hyperthyroidism i.e. an increase in the proportion of  $V_1$  myosin present in the ventricle. Indeed, thyroid hormone may

be involved in this response as thyroidectomy abolishes the increase in  $V_1$  in the rat due to swimming exercise, although it does not effect the exercise-induced hypertrophy (Pagani & Solaro, 1983). Paradoxically thyroxine levels have been shown to fall in response to exercise training (Leblanc et al., 1982), a fact which seems to disagree with the observed increase in  $V_1$ . It is far from clear therefore exactly how the myosin change in response to exercise training is brought about. Neither is it clear why an increase in  $V_1$  is desirable, as maximal cardiac contraction efficiency is achieved, with the normal isoform distribution, at exercise contraction rates (Moalic et al., 1981). Perhaps the decrease in contraction efficiency postulated by Swynghedauw et al. (1982) occurs only with severe exercise where an increased proportion of  $V_1$  would be appropriate due to the high contraction rates that would be required for very high heart rates. This may explain the different responses to different training programmes (e.g. Tibbits et al., 1978; Schaible et al., 1979).

Other conditions known to influence cardiac myosin isoform distribution are: diabetes (Belcastro et al., 1985); castration (Malhotra et al., 1983)\* and sympathectomy (Rupp et al., 1983). All three of these conditions lead to an increase of  $V_3$  myosin, but as yet mechanisms have not been found for these changes.

#### Contractility changes: sarcoplasmic reticulum function

A clue that myosin isoform changes do not constitute a complete explanation for changes in contractility in response to the sort of stimuli discussed above comes from the work of Korecky & Beznak, (1971). They noticed that, in the rat, moderate thyroxine

administration led to an increased rate of tension production and decreased time to peak tension but that peak tension itself was unchanged. When they increased thyroxine levels further, time to peak tension decreased further and as a result of this peak tension was decreased. In the rat, where V<sub>1</sub> myosin predominates normally, thyroxine can have little effect on myosin isoform distribution. It appears from the work of Korocky & Beznak that the active state of the cardiac myocytes has been abbreviated beyond the point which myosin isoform changes can accommodate i.e. the rate of rise of tension cannot, in the rat ventricle, be increased by producing a 'faster' myosin.

Changes in the duration of the active state as well as changes in rates of tension development and relaxation have been reported in thyroid imbalance (Buccino et al., 1967) and it has been shown by Suko (1973) that the function of cardiac sarcoplasmic reticulum changes in parallel with changes in the thyroid state. He found that sarcoplasmic reticulum isolated from hyperthyroid and hypothyroid rabbits showed higher and lower rates respectively of Ca<sup>2+</sup> accumulation and Ca<sup>2+</sup> activated ATPase activities when compared with euthyroid rabbit sarcoplasmic reticulum. This he suggested was the biochemical correlate of changes in rates of relaxation and development of tension and in the duration of the active state.

It seems, therefore, that sarcoplasmic reticulum function is changed in altered thyroid states. Is sarcoplasmic reticulum function changed in the other conditions discussed above, which alter contractility and myosin isoform distribution ?

The effect of pressure overload on sarcoplasmic reticulum function has been studied by Suko et al. (1970). They found that the rate of  $\text{Ca}^{2+}$  uptake into, and the  $\text{Ca}^{2+}$ -activated ATPase activity of sarcoplasmic reticulum isolated from chronically failing calf hearts was significantly reduced. Similar results have since been found in the rabbit by Sordahl et al. (1973). More recently Gwathmey & Morgan, (1985) looked at  $\text{Ca}^{2+}$  handling in the papillary muscles of ferret hearts which had been subject to a pressure overload. Using the  $\text{Ca}^{2+}$ -sensitive photoprotein aequorin, they noticed that the increased duration of contraction in hypertrophied muscle was correlated with an increased duration of the calcium transient (i.e. the free calcium concentration was elevated for longer during activation). This, they concluded, was due to the rate of uptake and possibly release of calcium by sarcoplasmic reticulum being reduced in chronic pressure overload. They also suggested that the decreased rate of relaxation they found in hypertrophied muscle could be due, at least in part, to this prolongation of the calcium transient.

Another of the pathological conditions affecting cardiac contractility, in which sarcoplasmic reticulum has been implicated, is diabetes. Penpargkul et al. (1981) found a reduced rate of calcium uptake into sarcoplasmic reticulum vesicles of hearts from rats made diabetic by administration of streptozotocin, as were  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}/\text{Mg}^{2+}$  activated ATPase activities. They concluded that the reduced rate of relaxation of cardiac muscle from diabetic rat hearts (Fein et al., 1980) is due to depressed sarcoplasmic reticulum activity.

From the above it appears that pathological influences on cardiac contractility have an effect not only on the myosin isoform distribution but also on the functional properties of the sarcoplasmic reticulum. Studies looking at the function of sarcoplasmic reticulum in physiological conditions which affect cardiac contractility e.g. ageing and exercise have also been done although with conflicting results.

Capasso et al. (1983) showed that the ageing process in rats leads to a progressive increase in the duration of the active state and decreases in the rates of tension development and relaxation. Froehlich et al. (1978) had previously shown over a similar ageing period (6-8 months to 24-25 months) that calcium transport activity in rat cardiac sarcoplasmic reticulum was reduced and that this correlated well with changes in contraction duration and relaxation time they found in the same hearts.

Again changes in cardiac contractility, this time under a physiological influence, appear to be accompanied by changes in both myosin and sarcoplasmic reticulum. However for training-induced changes in cardiac contractility, the other physiological influence we have discussed, there is evidence both for and against changed sarcoplasmic reticulum function.

Bersohn & Scheuer (1977) showed that exercise training produced increased rates of tension development and relaxation in the rat heart. The increased rate of relaxation they suggested could be due to increased rates of  $Ca^{2+}$  uptake by the sarcoplasmic reticulum as seen by Penpargkul et al. (1977) and Malhotra et al. (1981). However Sordahl et al. (1977) in dogs and Pagani & Solaro (1984) in



rats (using a different sarcoplasmic reticulum preparation) could demonstrate no difference in terms of  $\text{Ca}^{2+}$  uptake rate into cardiac sarcoplasmic reticulum between trained and sedentary animals. It is therefore unclear as yet whether changes in sarcoplasmic reticulum function are involved in the exercise-induced change in cardiac contractility. It should be remembered, however, that the myosin response to exercise is by no means entirely predictable either. In general it appears that sarcoplasmic reticulum function changes in parallel with the chronic changes of cardiac contractility due to thyroid imbalance, pressure overload, diabetes, ageing and possibly also exercise training.

The view that a reduced rate of relaxation of cardiac muscle is due solely to depressed sarcoplasmic reticulum activity has been questioned by Lopaschuk et al. (1983). They found that sarcoplasmic reticulum activity could be restored to normal in diabetic rat hearts by the administration of carnitine but that this did not return the rate of relaxation of diabetic hearts to normal. Further doubt is cast on the role of sarcoplasmic reticulum in cardiac relaxation by Rossmanith et al. (1986)\* who investigated the effect of isomyosin changes on the mechanical characteristics of rat papillary muscles. They concluded that, particularly under isometric conditions, so few cross-bridge cycles took place in a contraction (as few as a single cycle, they suggest) that the detachment rate of cross-bridges must be the rate limiting step of cardiac relaxation, not the uptake of  $\text{Ca}^{2+}$  into the sarcoplasmic reticulum.

Contractility changes: electrophysiological properties

Another important property of cardiac myocytes, which appears to be altered in conditions which chronically change cardiac contractility, is their electrophysiology. Changes in electrophysiological properties, as evidenced by changes in transmembrane action potential duration, are known to occur in response to pressure overload, maturation and diabetes.

Gulch (1980) and Keung & Aronson (1981) have shown that transmembrane action potential recordings from the hypertrophied hearts of rats which had been subjected to a pressure overload are longer than those of control hearts (no differences were found in resting potential, upstroke velocity or action potential amplitude). The possibility existed, of course, that these differences in action potential duration were due to changes in the syncytial connections between cells rather than to changes in the electrophysiological properties of the cells themselves. This was ruled out by Aronson & Nordin in 1984. They showed that cardiac myocytes isolated from pressure overloaded rat hearts had longer action potential durations than myocytes isolated from control hearts. Since isolated myocytes have no syncytial connections, differences in action potential duration between myocytes isolated from hypertrophied and control hearts have to be due to changes in cell membrane electrical properties. The longer action potential seen in pressure overloaded hearts correlates well with the longer contraction and slower  $Ca^{2+}$  accumulation by the sarcoplasmic reticulum in these tissues.

Diabetes, another of the pathological conditions which causes increased contraction duration and decreased sarcoplasmic reticulum activity, has also been shown to change the electrophysiological properties of cardiac cells. Fein et al. (1983) showed that the action potentials of papillary muscles from hearts of diabetic rats were longer and resting potential and action potential amplitude were reduced. These changes in action potential duration are similar to those found by Gulch (1980) and Keung & Aronson (1981) for pressure overloaded hearts; however, diabetes also has effects on the upstroke velocity, the amplitude of the action potential and the cell resting potential which are not seen in pressure overload.

The electrophysiological effects of thyroid hormone in atrial muscle have been investigated by Freedberg et al. (1970)\*. They found that hyperthyroidism caused a shortening and hypothyroidism a prolongation of the atrial action potential. Resting membrane potential and action potential amplitude they found to be unaffected by thyroid state. Similar work on ventricular muscle appears not to have been done but the work of Adams (1964)\*, which showed that the Q-T interval of the ECG was lengthened by hypothyroidism in humans, indicates that at least hypothyroidism has a similar effect in both atria and ventricles. It remains to be seen if, in ventricular muscle, action potential effects of thyroid hormone are independent of stimulation frequency as they are in atrial muscle.

The effect of ageing on the electrophysiology of cardiac myocytes has been investigated by Capasso et al. (1983), who looked at the mechanical and electrical performance of rat myocardium at

various ages. They found a gradual lengthening of both action potential and contraction durations of papillary muscles with age, however action potential amplitude and resting membrane potential were found to be similar in all age groups. It is interesting to note that similar changes in the electrophysiological properties of rat atrial muscle have also been found (Cavato et al., 1974) i.e. increased action potential duration with no changes in action potential amplitude or resting potential.

It appears therefore that in all the cases where myocardial contractility is chronically reduced the action potential duration is increased. Capasso et al. (1983) have put forward two alternative views of this change in action potential duration which appear to be applicable not only to changes in electrophysiology induced not only by maturation but also by pressure overload and diabetes. They suggest that a slower release and uptake of  $Ca^{2+}$  by the sarcoplasmic reticulum would lead to slower inactivation of the trans-sarcolemmal calcium current and slower activation of potassium conductance, both of which are to some extent under the influence of myoplasmic calcium concentration (Marban & Tsien, 1981; Isenberg, 1975). Both of these influences of a slower increase of myoplasmic calcium concentration would lead to slower repolarisation and hence a longer action potential. They also suggest that a longer action potential could be necessary to allow the slower contractile mechanism (myosin and sarcoplasmic reticulum) to reach normal tension.

One other example of action potential duration differences which is important to the work of this thesis is the regional differences

to be found within one heart. In 1980 Gulch reported different action potential durations in different regions of rat and cat hearts. He found that in normal and hypertrophied hearts left ventricular action potentials were longer than those from the right ventricle. He also found that within a ventricle of the normal rat heart sub-endocardial action potentials were longer than those of the sub-epicardium but that this difference was larger in hypertrophied hearts. Since then similar differences have been found between epicardial and endocardial cells in rats by Keung & Aronson (1981) and in the dog by Sekiya et al. (1983). These differences in action potential duration have been related to chronic wall stress by Gulch (1980) who found that longer action potentials were to be found in areas of the heart where wall stress was chronically greatest. He had been led to look for these differences in action potential duration by the lengthened action potential of the pressure overloaded heart. Following a similar logic for myosin (i.e. that pressure overloaded hearts contain a higher proportion of  $V_{\alpha}$  myosin) I have looked for differences in myosin distribution in different regions of individual hearts and for a correlation of these differences with electrophysiology.

The Present Work

The work in this thesis deals, to a large extent, with the rabbit heart where, due to a degree of myosin heterogeneity not seen in many mammals, the relationship between myosin type and electrophysiological properties can be investigated in individual hearts. An attempt has been made to explain this myosin heterogeneity in terms of workload and sympathetic innervation. A comparison between cat and rabbit hearts has also been made for the same reason.

Three self contained chapters are also included. In the first two of these differences between atrial and ventricular myosins are demonstrated in the rat and in reptiles respectively. The third self contained chapter contains results of a pilot study on the histochemistry of isolated myocytes from rabbit hearts.

## METHODS

### Animals

All animals were kept in the departmental animal house before and, in the case of sympathectomized animals, during experiments. Rats (Sprague-Dawley), rabbits (New Zealand White) and guinea-pigs were killed by a blow to the back of the head. Ferrets and cats were obtained from colleagues after their experiments, as available. Of the larger animals used bovine hearts were obtained from a local abattoir and human cardiac muscle was surgical biopsy tissue obtained courtesy of Dr. D.J. Miller.

### Preparation of Histochemical and Histological Sections

All of the histochemical techniques described below were used on fresh, frozen sections. I will describe first how these were prepared. Cardiac muscle samples were collected from freshly killed animals. These samples were then mounted on a cork base in Tissue-Tek O.C.T. compound, prior to freezing in liquid-nitrogen-cooled isopentane. The frozen blocks were then rapidly transferred to a cryostat (Bright Instruments Ltd.) where they were stored until they had warmed to the cryostat temperature (about  $-25^{\circ}\text{C}$ ), at which sections could be cut. Sections were cut  $10\mu\text{m}$  thick and picked up on glass coverslips, they were then allowed to dry in air at room temperature for 15-20 min before staining. Sections were sometimes stored overnight in the cryostat before staining.

When the samples used were either papillary muscles or trabeculae it was possible to mount them in frozen blocks such that most fibres would be cut transversely, as the orientation of the fibres is predictable in these cases. Ventricular wall fibre



orientation is much more complex, so ventricular wall samples were mounted such that endocardial trabeculae would be cut transversely. This ensured that not all fibres were cut obliquely. Fibre orientation is important when photometric readings are required, as only transversely cut fibres can be used for photometry (see Photometric Methods p.24).

#### ATPase Staining

The methods used for ATPase staining were identical to system A of Snow et al. (1982). Fresh, frozen sections were pre-treated in an alkaline medium before incubation in an ATP containing solution. These pre-treatments selectively inhibit different myosins so that differences in myosin type can be demonstrated as differences in the ability of the myosin to hydrolyse ATP. The ability to hydrolyse ATP is demonstrated by the accumulation of inorganic phosphate in the muscle fibre sections. Phosphate is trapped and visualised by a series of inorganic reactions.

Alkaline pre-treatment consisted of a 15 min immersion in the following solution: Solution A

0.075M      Na-barbital

0.07M      Na-acetate

0.1M      CaCl<sub>2</sub>

pH adjusted to 10.0-11.0 with NaOH

Alkali pre-treated sections were then washed briefly in a solution identical to the alkaline pre-treatment solution except that its pH was set to 9.45. Sections were then incubated in the following solution: Solution B

0.075M Na-barbital

0.07M Na-acetate

0.1M CaCl<sub>2</sub>

1.5mg/ml Na<sub>2</sub>ATP

pH adjusted to 9.45 with acetic acid

Alkali pre-treated sections were incubated in solution B for 30 min.

After incubation sections were taken through the following steps for visualisation of phosphate deposits (the product of myosin hydrolysis of ATP)

1 min 1%(w/v) CaCl<sub>2</sub>

wash distilled water

1 min 1%(w/v) CoCl<sub>2</sub>

wash several times distilled water

1 min 1%(w/v) (NH<sub>4</sub>)<sub>2</sub>S

wash several times distilled water

Sections were then dehydrated by immersion in progressively more concentrated ethanol, starting at 70% and grading to 100%. Dehydration was followed by immersion in xylol or histosol to remove the ethanol, immediately after which sections were mounted, on glass slides, in the synthetic resin, Piccolyte. This final group of processes will henceforth be referred to as 'dehydration, clearing and mounting'.

Metabolic Stains

Two metabolic enzyme stains have been used in this work: succinate dehydrogenase (SDH; an oxidative marker) and  $\alpha$ -glycerophosphate dehydrogenase ( $\alpha$ GPDH; a glycolytic marker). Both reactions involve the reduction of the tetrazolium salt nitro blue tetrazolium (NBT) to produce coloured, insoluble diformazans. In SDH staining the electrons for this reduction are made available by the oxidation of succinate to fumarate. In the case of  $\alpha$ GPDH staining, the electrons for the reduction of the tetrazolium salt are made available by oxidation of  $\alpha$ -glycerophosphate. In the latter reaction, the electron transfer from the enzyme to the tetrazolium is facilitated by the presence of menadione.

The incubation media for these reactions were prepared immediately before use from refrigerator-stored stock solutions.

The solution for SDH staining is:

0.9ml NBT buffer + 0.1ml 2.5M Na-succinate

The solution for  $\alpha$ GPDH staining is:

0.9ml NBT buffer + 0.1ml 1M Na-glycerophosphate

+ 0.04ml 0.05% menadione in acetone

The NBT buffer is listed below:

|                       |       |
|-----------------------|-------|
| 5mg/ml NBT            | 2.5ml |
| 0.1M phosphate buffer | 2.5ml |
| 5mM MgCl <sub>2</sub> | 1.0ml |
| Dist. water           | 3.0ml |

Sections to be stained were placed on a moist filter paper in a Petri-dish and a drop of SDH or  $\alpha$ GPDH incubation medium was placed on the section so that it was completely immersed. The Petri-dish

was then covered and put in an oven at 37°C. The progress of the reaction could be followed under a microscope. The reactions (both SDH and  $\alpha$ GPDH) were stopped by immersion in distilled water after about 30 min for SDH and 50 min for  $\alpha$ GPDH depending on the strength of the reaction. Sections were then dehydrated, cleared and mounted.

#### Other Stains

One histological staining method was used: haematoxylin and eosin. Fresh, frozen sections were fixed by a brief (5 min) incubation in formol saline. Sections were then immersed for 1 min in 0.5% haematoxylin, washed in tap water for 30s before immersion in 0.5% eosin for 1 min. After another brief wash in tap water sections were quickly (to avoid loss of alcohol-soluble eosin) dehydrated, cleared and mounted.

#### Photometric Methods

Photometric readings were made on a Leitz Orthoplan microscope, fitted with a stabilised light source (Kingshill Electronic Products Ltd.), using a Leitz MPV Compact photometer. Readings were taken at a total magnification of x1000, which allowed 3-4 readings to be made in each fibre without overlap. In this way the effect of variations of stain intensity within one fibre could be minimised. Readings were taken after a 'warming-up period' of several hours, during which the lamp filament and photometer circuitry reached a stable temperature. With the photometer reading set to '100' on the background (i.e. glass slide, coverslip and mounting medium taken as 100% transmission), the fraction of light transmitted through a particular fibre was measured and directly fed into a PDP-11

computer which converted this transmission reading to absorbance, using the relation below:

$$\text{Absorbance} = -\log_{10}(\% \text{transmittance}/100)$$

The mean value of the absorbance readings from each fibre was stored on the PDP-11 for later analysis.

The potential affect on the readings of light scattered from other parts of the section was prevented by a 0.2mm aperture placed between the light source and the microscope condenser; this masked all parts of the section, other than those to be measured and a small surrounding annulus, from the incident beam. The wavelength of light used when making measurements depends on the nature of the deposit on the section and is altered by the inclusion of narrow band interference filters . For ATPase stained sections a 482nm filter was used, for diformazan deposits (metabolic stains) a 592nm filter was used. These wavelengths were chosen in order to reduce the contribution to fibre absorbance of endogenous pigmentations, which as they are not necessarily present in proportion to the reaction product tend to reduce contrast between fibres.

When attempts were made to correlate different stains with each other it was necessary to make 'maps' of corresponding areas on serial sections. Photographs of each area were taken on the Leitz Orthoplan microscope with a camera attachment using an Orthomat 1 automatic exposure meter. Fibres were identified and numbered on both photographs and these were then used as maps to identify fibres on each section. In this way photometric readings of two stains could be taken from one fibre.

Photometric data was normalised by division of all readings by the geometric mean of the sample, thus all results were expressed in terms of the relative deposit-density in one fibre as against another, and excluded any irrelevant differences between thickness and reaction conditions which would otherwise have complicated comparisons between one section and another.

#### Fibre Cross-sectional Area Measurement

Increased chronic workload in the heart leads to hypertrophy of cardiac myocytes (Gulch, 1980). An equivalent phenomenon also appears to be present within the wall thickness of a normal heart; in the rat heart, endocardial myocytes have greater cross-sectional areas than epicardial myocytes (Gerdes et al., 1979) and chronic wall stress is known to be lower in the epicardial layers of the ventricle (Gulch, 1980). On these bases cross-sectional area of cardiac myocytes was used as a measure of chronic workload.

Measurements of cross-sectional area of endocardial and epicardial fibres were made on haematoxylin and eosin stained sections of rabbit hearts.

Fibre cross-sectional area was measured on a Wild microscope which had a drawing tube attachment using a Terminal Display Systems LC 12 digitising tablet. Light from the tablet cursor lamp was projected via the drawing tube onto the specimen image. The spot of light seen on the image was tracked around the perimeter of the object to be measured by movement of the tablet cursor. Movement of the cursor over the tablet generates a series of x,y-coordinates corresponding to the shape of the object being measured. These coordinates are converted to an area by an Apple

The computer according to a UCSD Pascal program written by Dr. V. Moss. The principle used is described in detail in Moss (1981); the diagram below is adapted from this article and helps to explain how area is calculated.

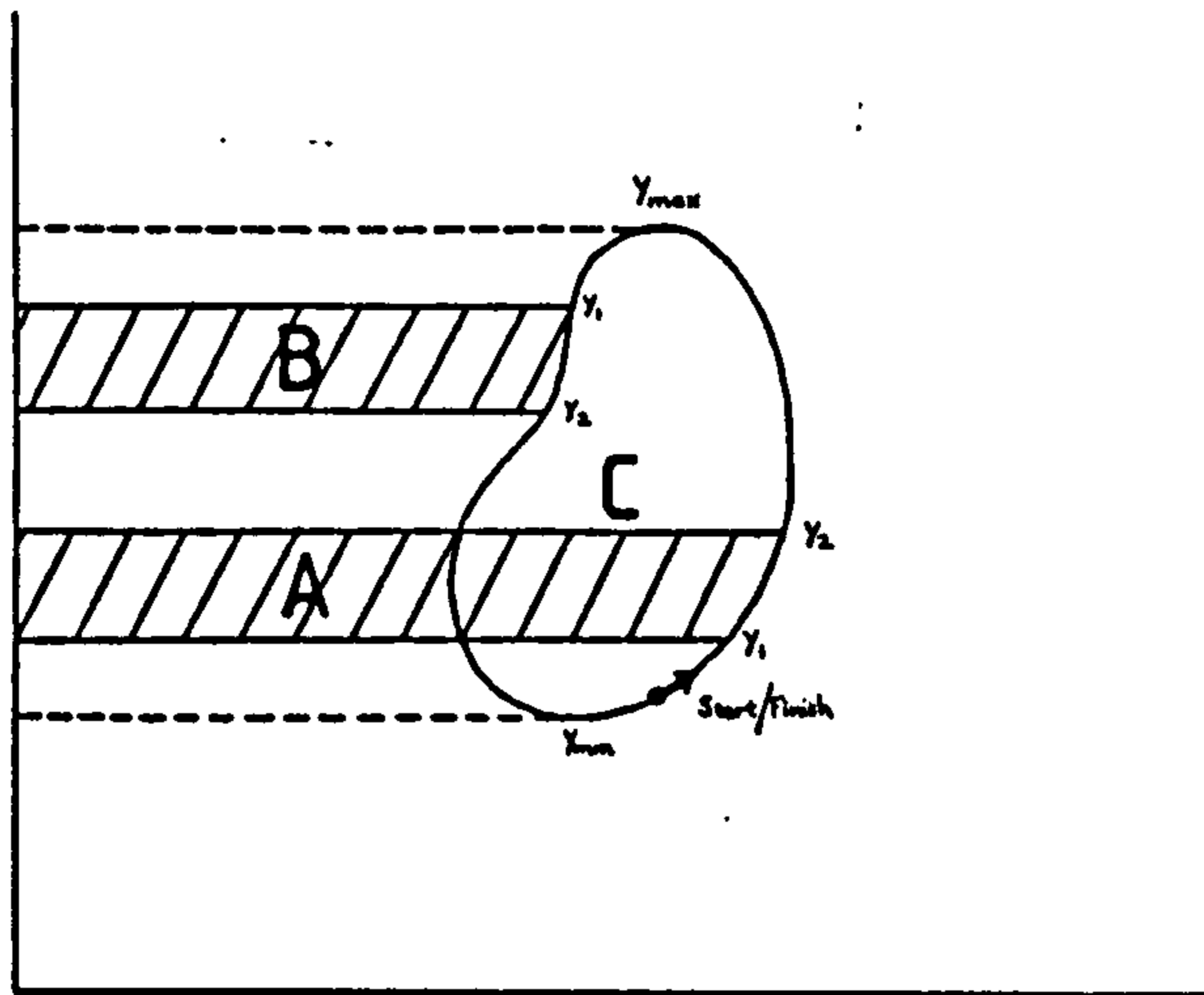


Fig.1 Illustration of how fibre cross-sectional area is measured. The area C is calculated by summing all the areas A and B between  $y_{min}$  and  $y_{max}$ .

If the areas of A and B above are calculated according to the relation :

$$\text{Area} = x \cdot \delta y \quad \text{where } x = \text{mean } x\text{-coordinate} \\ \text{between } y_1 \text{ and } y_2$$

$$\delta y = y_2 - y_1$$

then it is clear that area A will be a positive figure whereas area B will be a negative figure. If all the areas between  $y_{max}$  and  $y_{min}$  are calculated in this way and summed the result will be the area of C; the object which was measured i.e.

$$\text{Area} = \sum x \cdot \delta y$$

The step size (the distance between coordinates) is set such that very small movements (e.g. hand tremor) are not measured and yet corners of objects are not significantly cut off (as would happen if too large a step size were used). One other important point is how the measurement start/finish point is recognised. As the cursor is moved to start the measurement, the distance from the start point is monitored. Once the distance from the start point begins to decrease, within a set distance of the start (four times the step size) an increase in distance is interpreted as the cursor having passed the start point and the measurement is terminated. This procedure has two slight disadvantages; if the object to be measured is very small it may be that the cursor never leaves the set distance circle around the start point so the measurement cannot be terminated, or if the object has an irregular shape a movement away of the cursor can occur within the circle before the start point has been reached. These difficulties can be overcome, respectively by reducing the step size and by starting the measurement away from irregularities in the perimeter of the object.

#### Sympathectomy

Chronic chemical sympathectomy was achieved using a protocol similar to that of Fronck (1980) in which repeated intravenous injections of 6-hydroxydopamine hydrobromide (6-OHDA) are given. This compound causes a selective degeneration of peripheral adrenergic nerve terminals. Eight New Zealand White rabbits weighing between 2 and 2.5kg were used in these experiments. The required amount of 6-OHDA was dissolved in 2ml of a solution of



0.9% saline and 1% ascorbic acid which had been gassed with 100% N<sub>2</sub>. Ascorbic acid and N<sub>2</sub> were included to slow the oxidative inactivation of 6-OHDA. Control animals received injections of saline and ascorbic acid (with no 6-OHDA). Injections were made into an ear vein.

On day 1 four animals were given injections containing 42mg/kg 6-OHDA, these injections were repeated 24 hours later. Injections of 75mg/kg 6-OHDA were given one week, two weeks, four weeks and six weeks after the first injection. In all, this treatment lasted six weeks.

Control and sympathectomized animals were killed by a blow to the back of the head two days after the final injection. Their hearts were removed and composite blocks of right and left ventricles and atria made for ATPase staining. Samples of cardiac muscle were also taken to check the extent of sympathectomy by formaldehyde-induced fluorescence of sympathetic endings.

#### Formaldehyde-Induced Fluorescence of Adrenergic Endings

Two methods for the visualisation of adrenergic endings were used with similar results. The first of these was the technique of Falck (1962). For this method samples of cardiac tissue were quench-frozen, as described above. The frozen tissue samples were then freeze-dried overnight before being exposed to paraformaldehyde vapour at 80°C for 60 min. After vacuum embedding in paraffin wax, sections of the tissue were cut, mounted in paraffin oil and examined in a microscope under ultra-violet illumination. Under these conditions nerve endings which contained catecholamines fluoresce due to the presence of the compound

dihydroisoquinoline. This molecule is formed by the condensation of formaldehyde with the catecholamines in the nerve endings to form tetrahydroquinoline which is then converted to the more strongly fluorescent dihydroisoquinoline.

As this technique was time consuming the method of Azhyalis et al. (1984) was preferred. This is similar to that of Falck but is suitable for use on fresh, frozen sections and so does not require the tissue to be freeze-dried. Azhyalis et al. find that diffusion of catecholamines (and hence the loss of fluorescent nerve endings) is a problem with most fluorescent techniques adapted for the demonstration of adrenergic endings in fresh, frozen sections. Diffusion of catecholamines is prevented, they find, by pre-treatment of sections in an aluminium containing Tyrode solution. The solution they use is listed below:

|                                  |        |
|----------------------------------|--------|
| NaCl                             | 137mM  |
| KCl                              | 2.9mM  |
| CaCl <sub>2</sub>                | 1.36mM |
| MgCl <sub>2</sub>                | 0.25mM |
| NaHCO <sub>3</sub>               | 11.9mM |
| NaH <sub>2</sub> PO <sub>4</sub> | 0.26mM |
| Glucose                          | 5.5mM  |

18ml of 150mM Al<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> solution is added to 100ml of the above solution.

Immediately after cutting, sections are placed for 30 sec in the above solution which has been cooled to 0-4°C. Sections are then air dried for 10-15 min before being placed for two hours in a desiccator containing phosphorus pentoxide for further drying.

After this the sections are exposed to paraformaldehyde vapour at 80°C for 60 min, mounted in paraffin oil and examined by eye in a microscope under ultra-violet illumination.

Electrophysiological Methods

New Zealand White rabbits (2-2.5kg) were killed by a blow to the back of the head. The chest wall was opened and the heart quickly removed and placed in oxygenated solution of the following

NaCl 137

NaH<sub>2</sub>PO<sub>4</sub> 1.8

MgCl<sub>2</sub> 0.5

KCl 2.7

CaCl<sub>2</sub> 2.7

HEPES 5.0

Glucose 5.5

pH to 7.2 with NaOH

Papillary muscles and trabeculae of the left and right ventricles were dissected and mounted with small stainless steel pins on a wax board. Tissue was stored at room temperature in the above continuously oxygenated solution until required for recordings. Recordings were made in a perspex chamber, the bottom of which was covered with a layer of Sylgard transparent resin. Pieces of muscle were mounted in the chamber on the Sylgard with small stainless steel pins and superfused at a rate of 10ml/min with the above solution warmed to 37-37.5°C. The effective volume of the chamber was 9ml.

Pieces of muscle were stimulated at 1 sec. intervals through Teflon coated silver wires. Stimuli were rectangular pulses 2msec

in duration and 0.25V in amplitude from a Devices Isolated Stimulator triggered by an AEL Laboratory Stimulator. The muscles were viewed using a Carl Zeiss Dissecting Microscope and impalements made using Carl Zeiss Jena Micro-manipulators.

Action potentials were recorded with 3M KCl-filled glass microelectrodes (tip resistance 15-30M $\Omega$  ; tip potential less than 2mV) connected through a Ag/AgCl junction to a high input impedance pre-amplifier designed and built by Mr. J. Sinclair of the Institute of Physiology, Glasgow. DC potentials were backed off and membrane potentials measured using a micro-electrode amplifier (frequency response better than 1kHz) also designed and built by Mr. J. Sinclair. The return electrode from the bath was a chlorided silver wire inserted in 2% agar dissolved in 3M KCl. Recordings were displayed on a recording oscilloscope (Tektronix 5103N) and either photographed with a Polaroid camera or traced directly from the screen. The quality of an impalement was assessed by the rate of change of potential as seen on the oscilloscope screen.

Action potential characteristics recorded were: resting membrane potential; action potential amplitude and action potential durations at 50%, 75% and 90% of repolarisation.

Recordings were taken from a small region of each piece of tissue which could be identified afterwards e.g. the mid point of a papillary muscle. This allowed the regions from which recordings had been taken to be mounted close to each other in a frozen block; thus making the comparison of myosin ATPase activities easier.

RESULTS

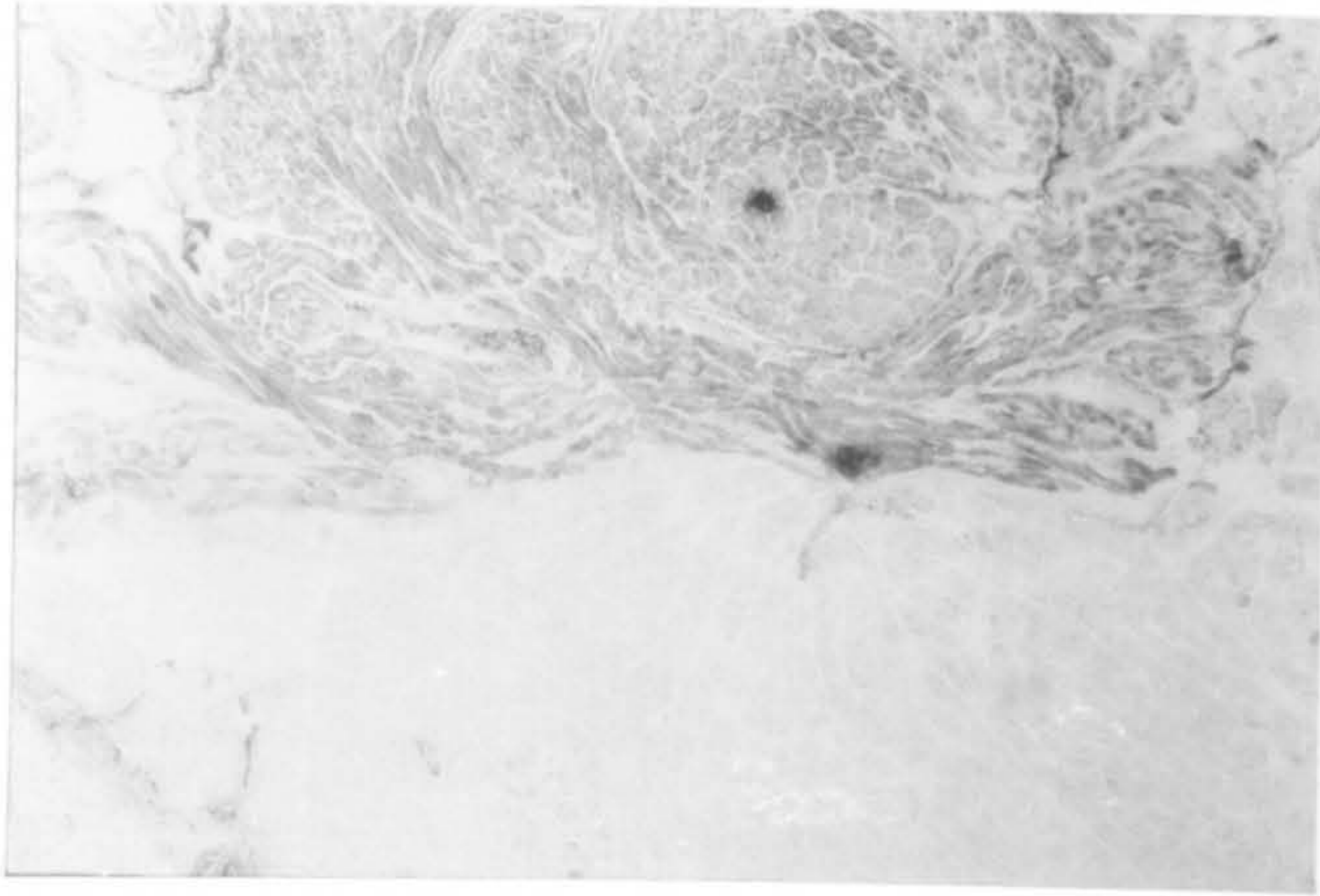


Fig.2 Rat atrial (top) and ventricular (bottom) muscle stained for myosin ATPase activity after pre-treatment at pH 10.55 (x100).

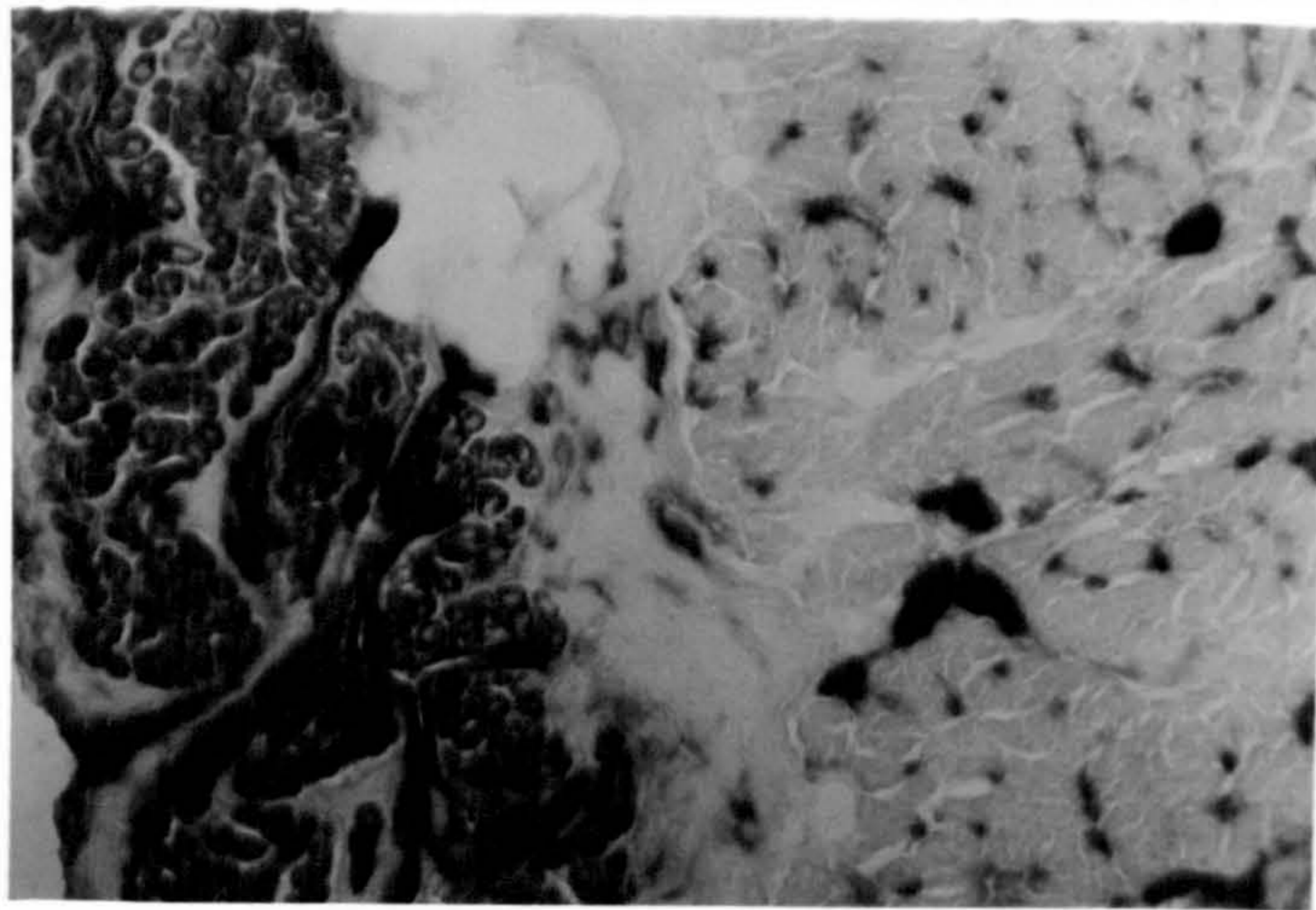


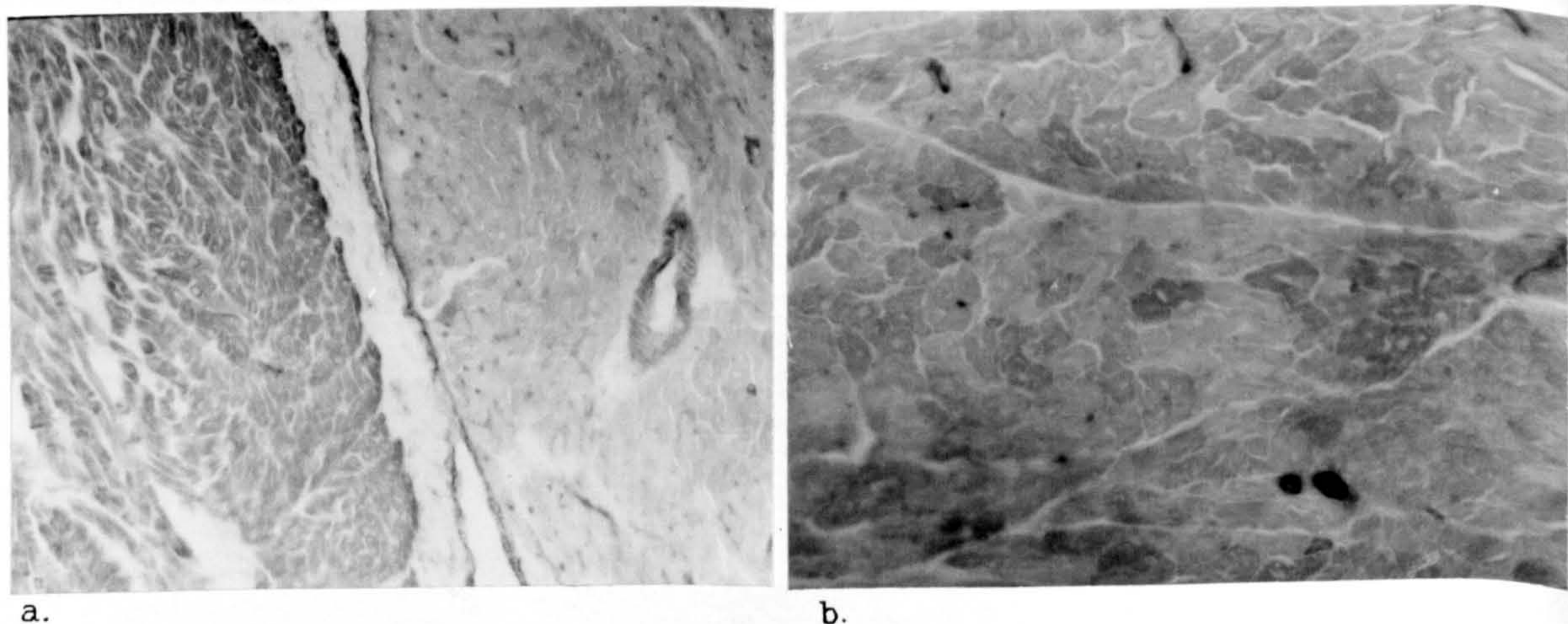
Fig.3 Cat atrial (left) and ventricular (right) muscle stained for myosin ATPase activity after pre-treatment at pH 10.6 (x250).

### Inter-Species Comparison

All myosin ATPase results presented below are for alkaline pre-treated sections, as it was found that acid pre-treatment demonstrated myosin heterogeneity much less well than alkaline pre-treatment. The hearts of several species were investigated in terms of myosin ATPase activity early in this work, in an attempt to find a species with suitable heterogeneity of myosin isoforms. The species investigated were: rat, cat, ferret, guinea pig, beef, human and rabbit. Figs. 2-7 show examples of myosin ATPase staining after alkaline pre-treatment in the first six of these species. After this rabbit heart is dealt with in more detail.

Fig.2 shows rat atrial and ventricular muscle stained for myosin ATPase activity after alkaline pre-treatment. The atrial muscle (top) stains more strongly than the ventricular muscle (bottom). However atrial and ventricular muscle each appear to stain homogeneously i.e. atrial fibres are uniformly dark and ventricular fibres uniformly pale. This indicates that different myosins are present in the atria and ventricles but suggests that all atrial fibres contain the same myosin(s), as do all ventricular fibres. It is not possible to say from these results whether only one or more isoforms of myosin are present in the atria or ventricles. More on the subject of rat atrio-ventricular differences can be found on p.135.

Fig.3 shows a similar phenomenon in cat cardiac muscle i.e. cat atrial muscle stains more darkly for myosin ATPase activity after alkaline pre-treatment than does ventricular muscle. Fig.3 emphasises this difference between atrial and ventricular muscle,

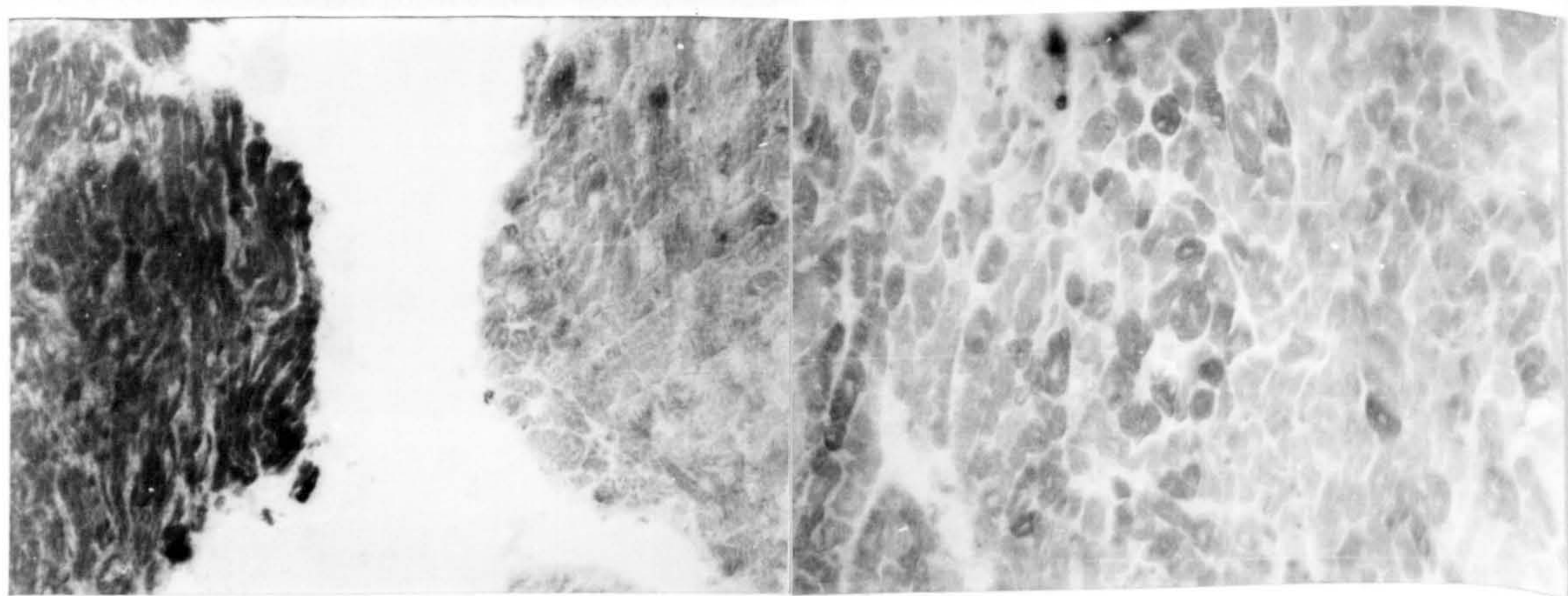


a.

b.

Fig.4a. Ferret atrial (left) and ventricular (right) muscle stained for myosin ATPase activity after pre-treatment at pH 10.4 (x160).

b. Ferret ventricular muscle stained for myosin ATPase activity after pre-treatment at pH 10.4 (x160).



a.

b.

Fig.5a. Guinea-pig atrial (left) and ventricular (right) muscle stained for myosin ATPase activity after pre-treatment at pH 10.2 (x250).

b. Guinea-pig ventricular muscle stained for myosin ATPase activity after pre-treatment at pH 10.4 (x160).



as the alkaline pre-treatment was so severe in this case that no ventricular myosin ATPase activity remained. However, even if less severe pre-treatments were used, still only the atrio-ventricular difference was readily apparent i.e. as with the rat, no heterogeneity of staining can be seen between ventricular fibres. (The very darkly stained objects in the ventricle in Fig.3 are blood vessel smooth muscle cells which contain very alkali-stable myosin).

Ferret heart muscle is shown in Fig.4a, where it can be seen that here too atrial muscle (left) myosin ATPase activity is more alkali-stable than that of ventricular muscle (right). As in the rat, atrial muscle of the ferret appears to stain homogeneously, however, Fig.4b shows this is not the case for ventricular fibres. In Fig.4b ventricular fibres stain heterogeneously for myosin ATPase activity after alkaline pre-treatment. This indicates that more than one form of ventricular myosin exists in ferret hearts and that ventricular fibres can contain different proportions of these myosins (Again, the very darkly stained objects in Fig.4b are blood vessel smooth muscle cells).

Fig.5a shows that a similar pattern of staining to that found in the ferret is present in the guinea pig heart. As with all the species so far investigated, atrial muscle myosin ATPase activity after alkaline pre-treatment is higher than that of ventricular muscle. However, ventricular fibres appear to stain heterogeneously, Fig.5b; as in the ferret this indicates that more than one ventricular myosin exists and that these are distributed

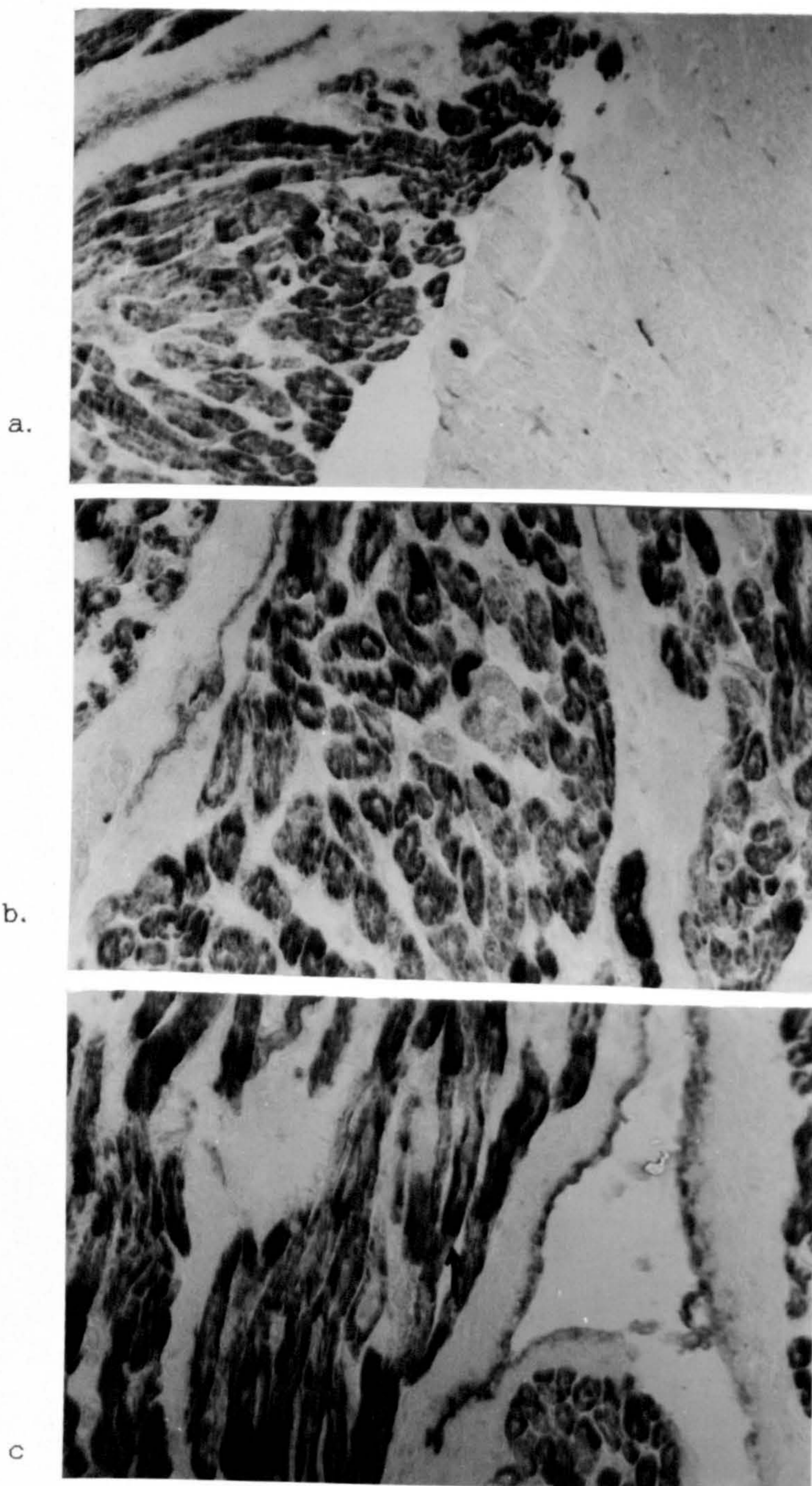


Fig.6a. Bovine atrial(left) and ventricular (right) muscle stained for myosin ATPase activity after pre-treatment at pH 10.4 (x160).

b. Bovine atrial muscle stained for myosin ATPase activity after pre-treatment at pH 10.4 (x250).

c. Longitudinal section of bovine atrial muscle stained for myosin ATPase activity after pre-treatment at pH 10.4 (x250). Note the sudden change in the stain intensity (arrowed).

differently in different fibres. Atrial fibres of the guinea pig heart stain homogeneously.

Figs.2-5 show that two types of myosin heterogeneity exist in the hearts of small mammals i.e. an atrio-ventricular difference in all cases and in some cases one between neighbouring fibres of the ventricle.

In larger mammals atrio-ventricular differences can also be demonstrated using myosin ATPase activity after alkaline pre-treatment. Fig.6a shows bovine atrial (left) and ventricular muscle (right) stained in this way; the atrial muscle has stained more darkly than the ventricular muscle. As in Fig.3 the ventricular myosin in Fig.6a is completely inhibited, however, less severe alkaline pre-treatment does not reveal any heterogeneity of staining within the ventricles. On the other hand, Fig.6b shows that bovine atrial fibres do stain heterogeneously. This indicates that, as in the ferret and guinea pig ventricles, more than one myosin isoform exists in bovine atrium and that these are distributed differently among individual atrial fibres. Fig.6c shows another aspect of this heterogeneity. Here atrial fibres are cut longitudinally and stained in the same way as in Figs.6a & b. The arrow indicates the point of interest, the sudden change in stain intensity suggests that fibres connected by an intercalated disc can contain myosins of different alkaline labilities.

In Fig.7 it can be seen that human cardiac muscle appears to be similar to bovine cardiac muscle, with respect to the heterogeneity of myosin ATPase staining. Fig.7a shows human atrial (left) and ventricular muscle (right) stained in the same way as the bovine

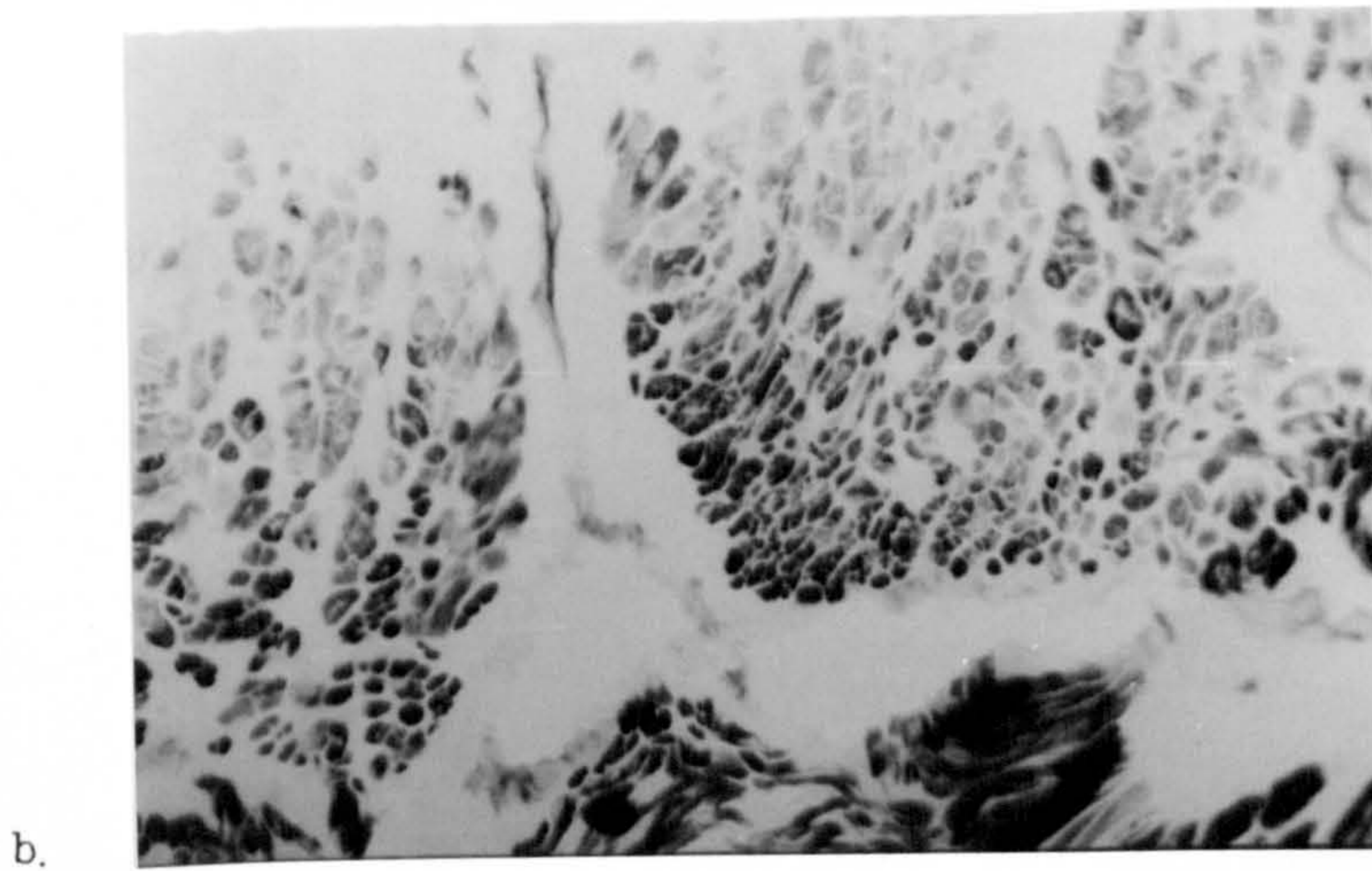
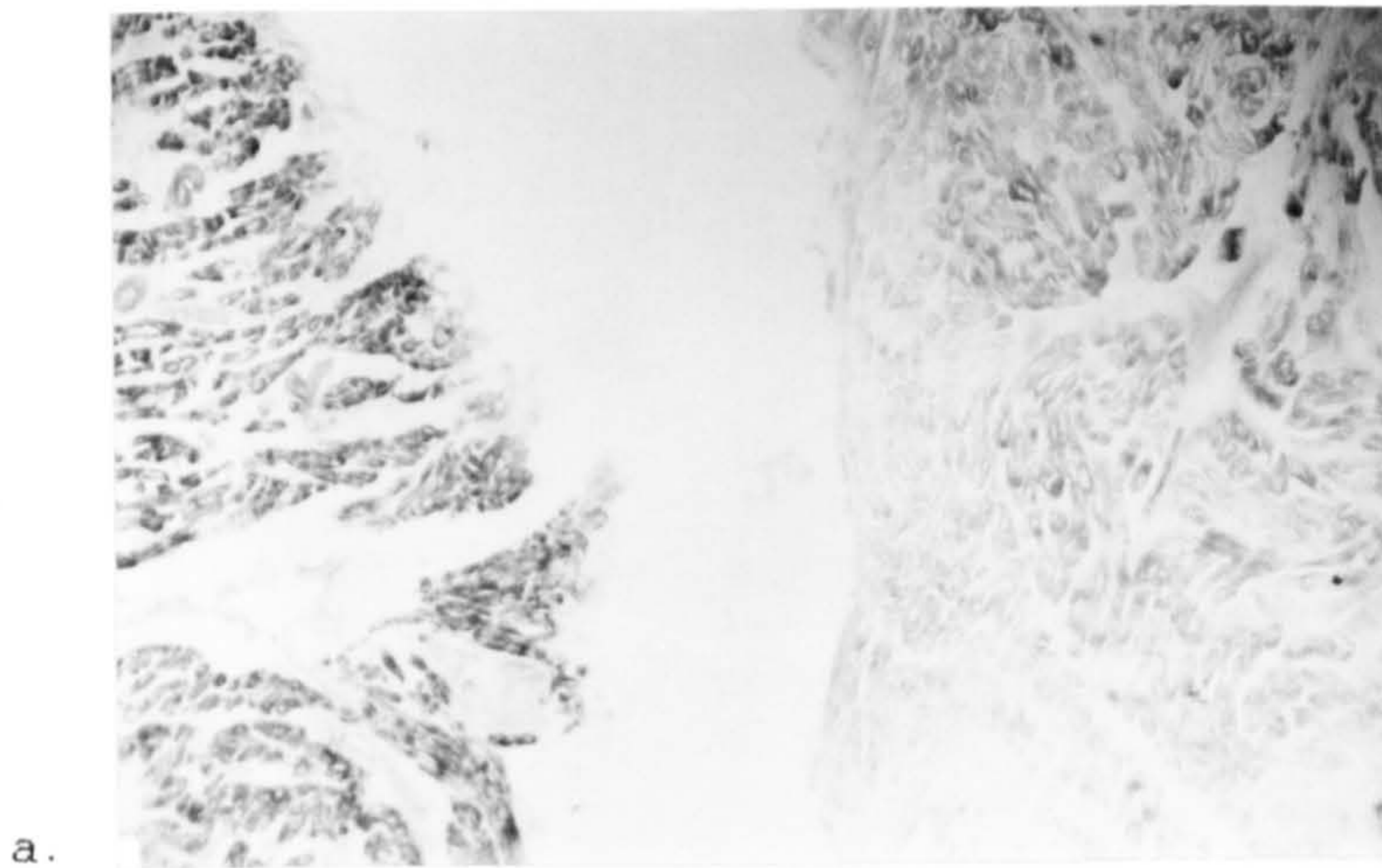


Fig.7a. Human atrial (left) and ventricular (right) muscle stained for myosin ATPase activity after pre-treatment at pH 10.6 (x100).

b. Human atrial muscle stained for myosin ATPase activity after pre-treatment at pH 10.6 (x250).

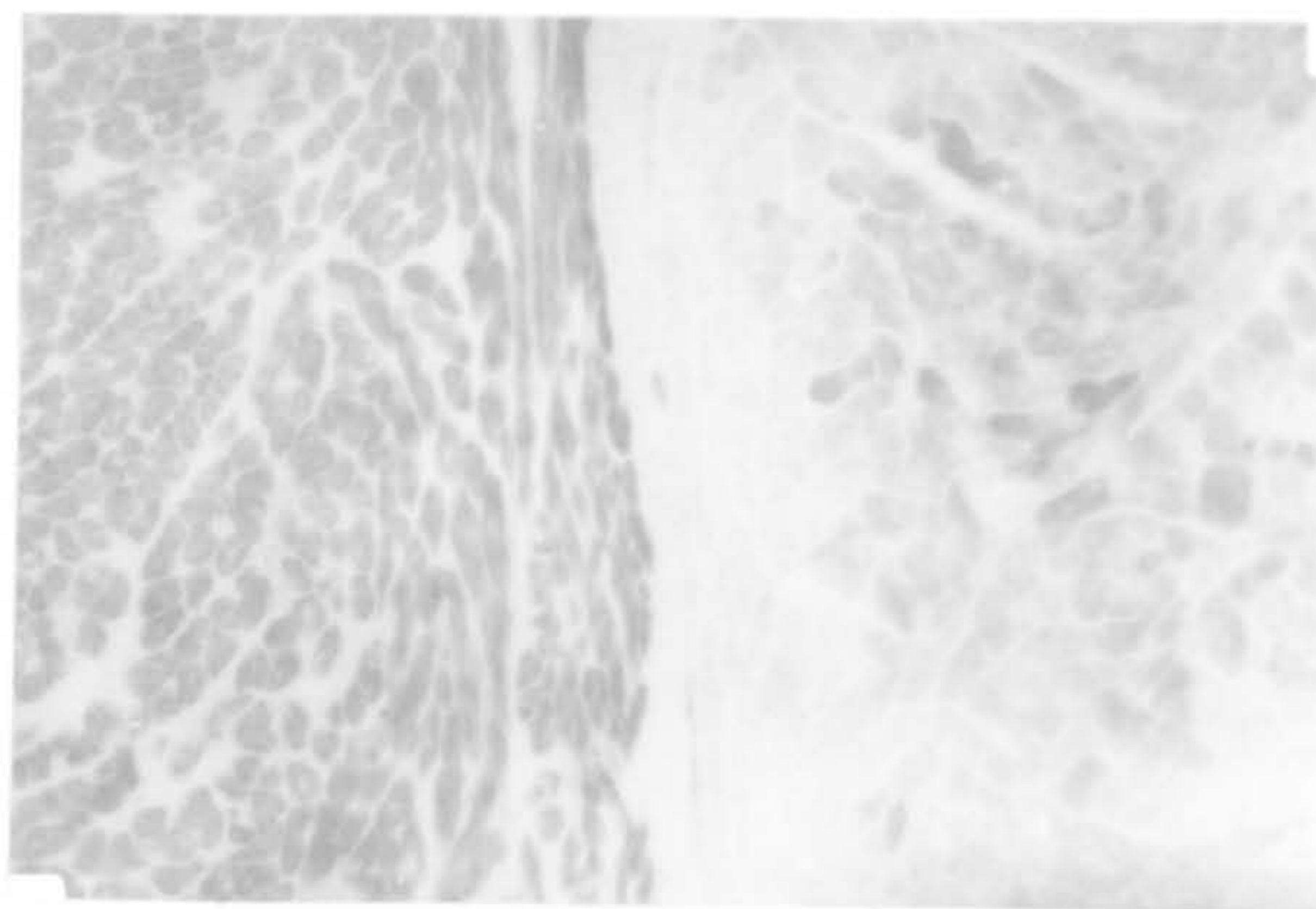


Fig.8 Rabbit atrial(left) and ventricular (right) muscle stained for myosin ATPase activity after pre-treatment at pH 10.4 (x160).

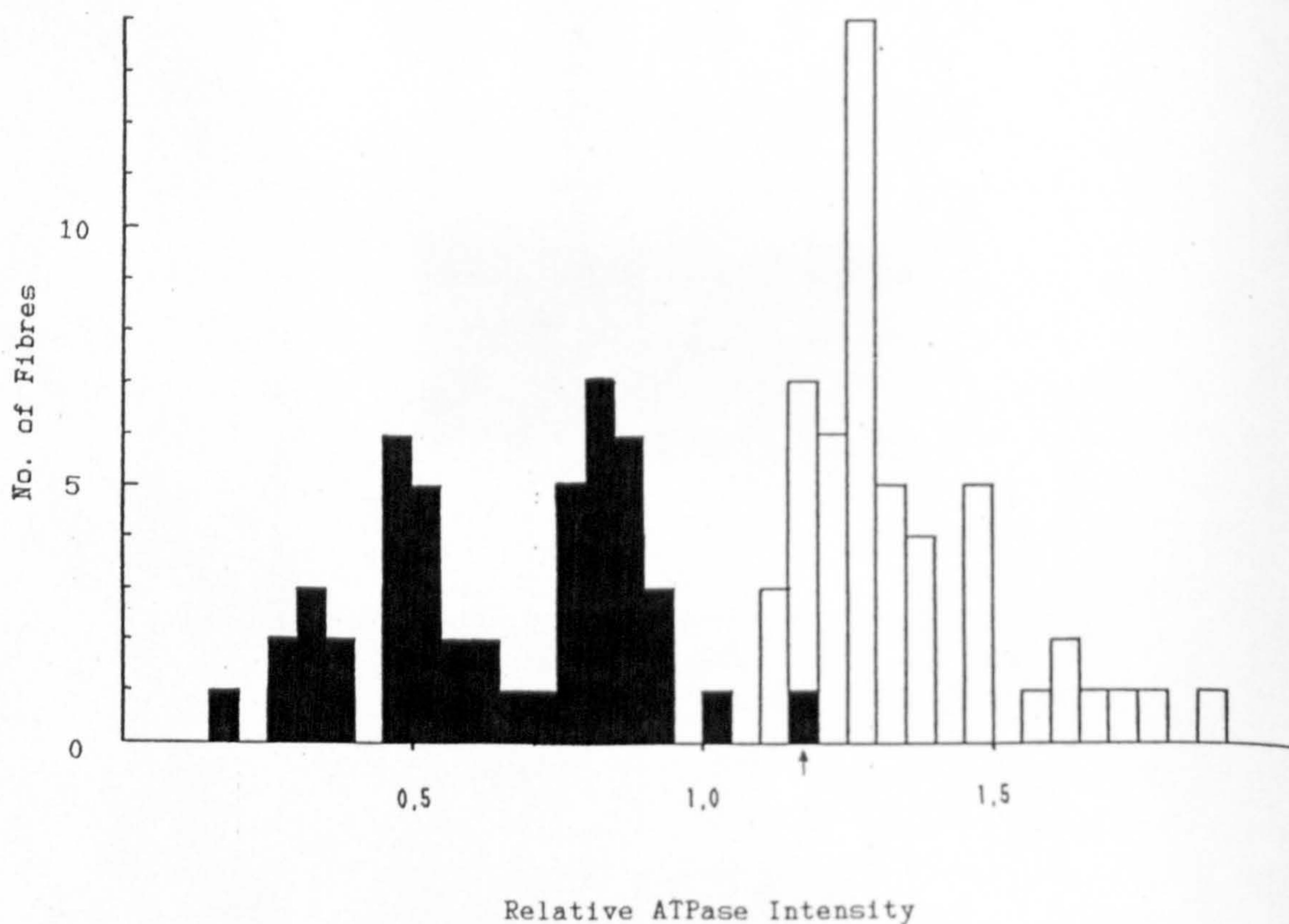


Fig.9 Histograms of photometric readings taken from rabbit cardiac muscle stained for myosin ATPase activity after pre-treatment at pH 10.4. Readings taken from atrial muscles are shown in white, those taken from ventricular fibres in black.

muscle in Fig.6. Although the difference is less clear than in Fig.6a it does appear that the atrial muscle is more darkly stained than the ventricular muscle. Fig.7b shows that human atrial fibres also stain heterogeneously. It seems, therefore, that bovine and human cardiac muscles are similar in that atrial and ventricular muscles contain different myosins and more than one myosin isoform is present in the atrium.

Most of the work in this thesis has been done on rabbit cardiac muscle as it was found, from the above inter-species comparison, that the degree of myosin heterogeneity which can be demonstrated using myosin ATPase staining techniques is much greater than in any of the species presented in Figs.2-7. The results obtained from rabbit cardiac muscle in this comparison are presented below.

Fig.8 shows an example of rabbit atrio-ventricular differences in myosin ATPase staining after alkaline pre-treatment. As in all the species mentioned above, atrial muscle (left) stains more darkly than ventricular muscle (right). The histogram below (Fig.9) shows a quantitative analysis of the staining intensities of atrial and ventricular fibres. In Fig.9 ventricular fibres are shown in black, atrial fibres in white. Columns appearing completely white or black contain only atrial or ventricular fibres respectively, those which have both black and white portions contain both atrial and ventricular fibres. The column indicated by the arrow in Fig.9, therefore, contains one ventricular fibre and six atrial fibres. Of the fifty ventricular fibres measured, only this one fibre falls within the staining intensity range of the atrial fibres. This objectively confirms the visual impression that

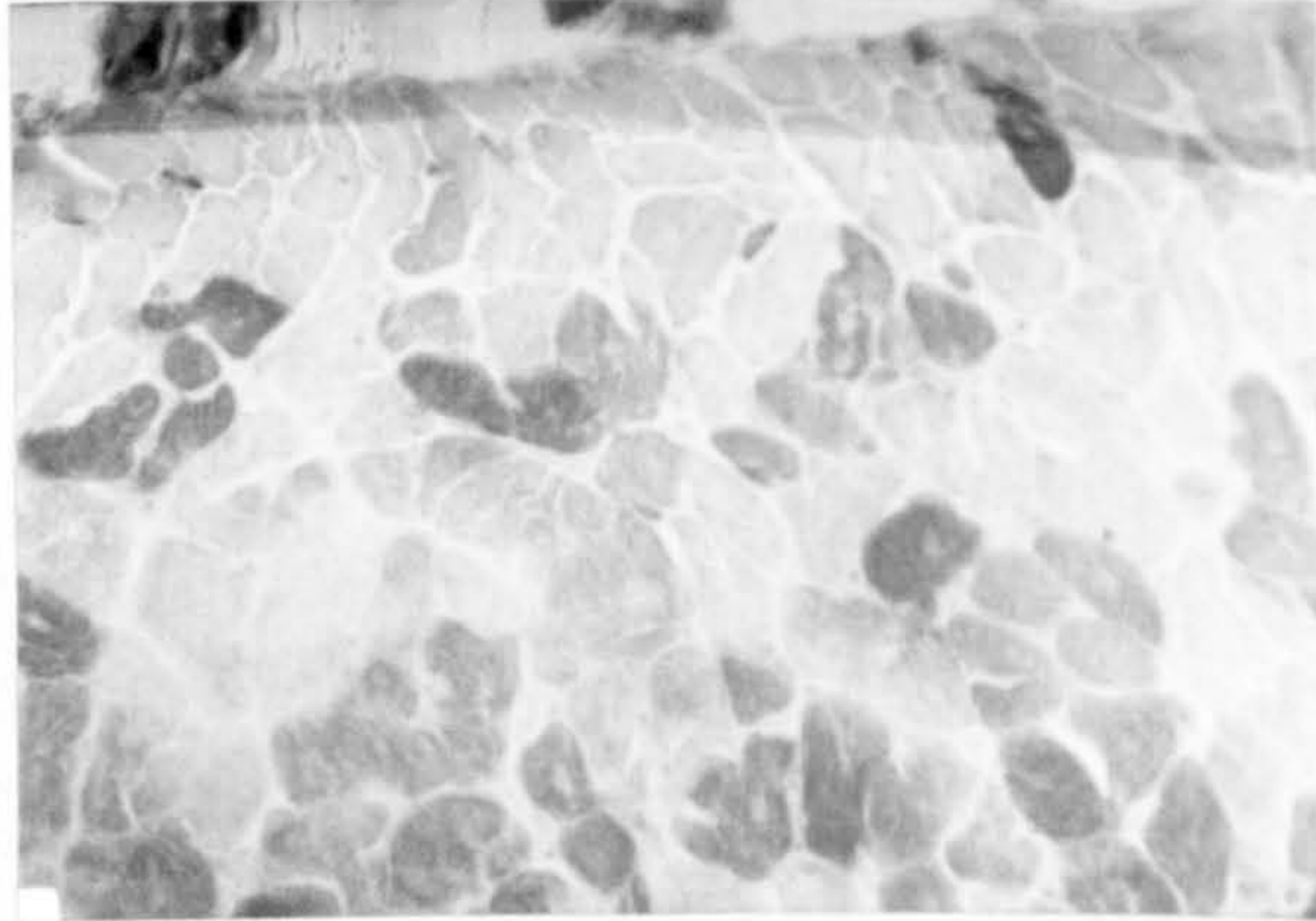


Fig.10 Rabbit ventricular muscle stained for myosin ATPase activity after pre-treatment at pH 10.6 (x1000).

ventricular fibres stain less darkly than atrial fibres for myosin ATPase activity after alkaline pre-treatment.

It is possible to demonstrate more myosin ATPase staining heterogeneity within rabbit ventricles than in those of any of the species shown in Figs.2-7. A good example of this is shown in Fig.10, where left ventricular muscle has been stained for myosin ATPase activity after alkaline pre-treatment. The range of fibre staining intensity is greater than has been seen in either ferret (Fig.4) or guinea pig (Fig.5). This range is quantified in Fig.11a where the staining intensity distribution is skewed towards the paler fibres. Such a skewing towards pale fibres is a constant feature seen in this sort of analysis of rabbit ventricular fibres (c.f. Fig.11b-d.) Fig.11c & d show that peaks within this skewed distribution are sometimes more apparent than in Figs.11a & b. However several analyses of this sort have not produced a consistent number of peaks.

Rabbit ventricular muscle shows one other type of staining heterogeneity which is not found in the species mentioned before. This heterogeneity can be seen in Figs.12a & b. Fig.12a is a montage showing how staining intensity varies across the left ventricular wall. Myosin ATPase activity is inhibited less by alkaline pre-treatment in sub-epicardial fibres than in sub-endocardial fibres, indicating that myosin distribution differs in these regions. Fig.12b shows that the same differences are present in the right ventricular wall.

In the rabbit, therefore, two types of ventricular myosin isoform heterogeneity can be demonstrated with the myosin ATPase



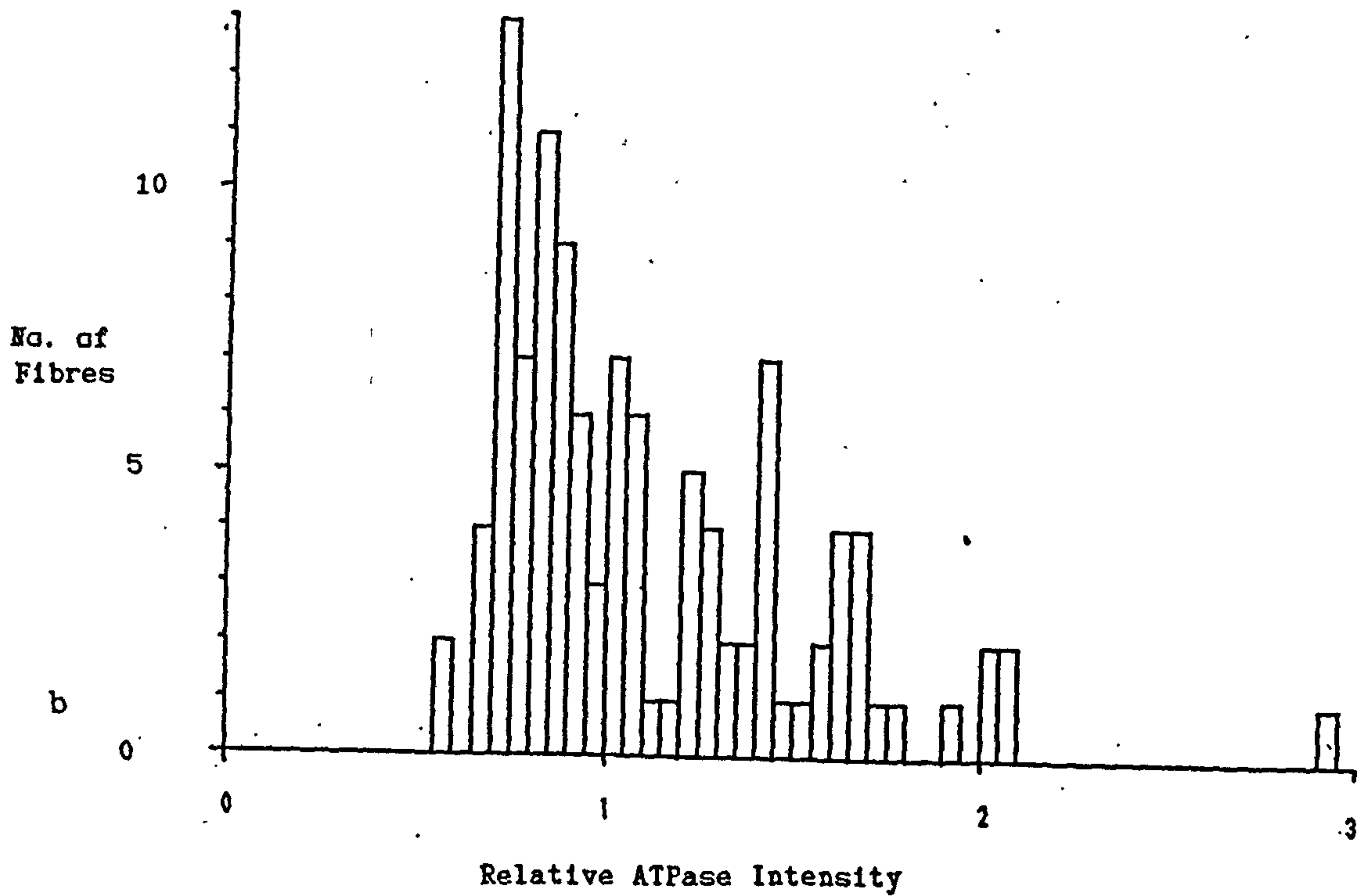
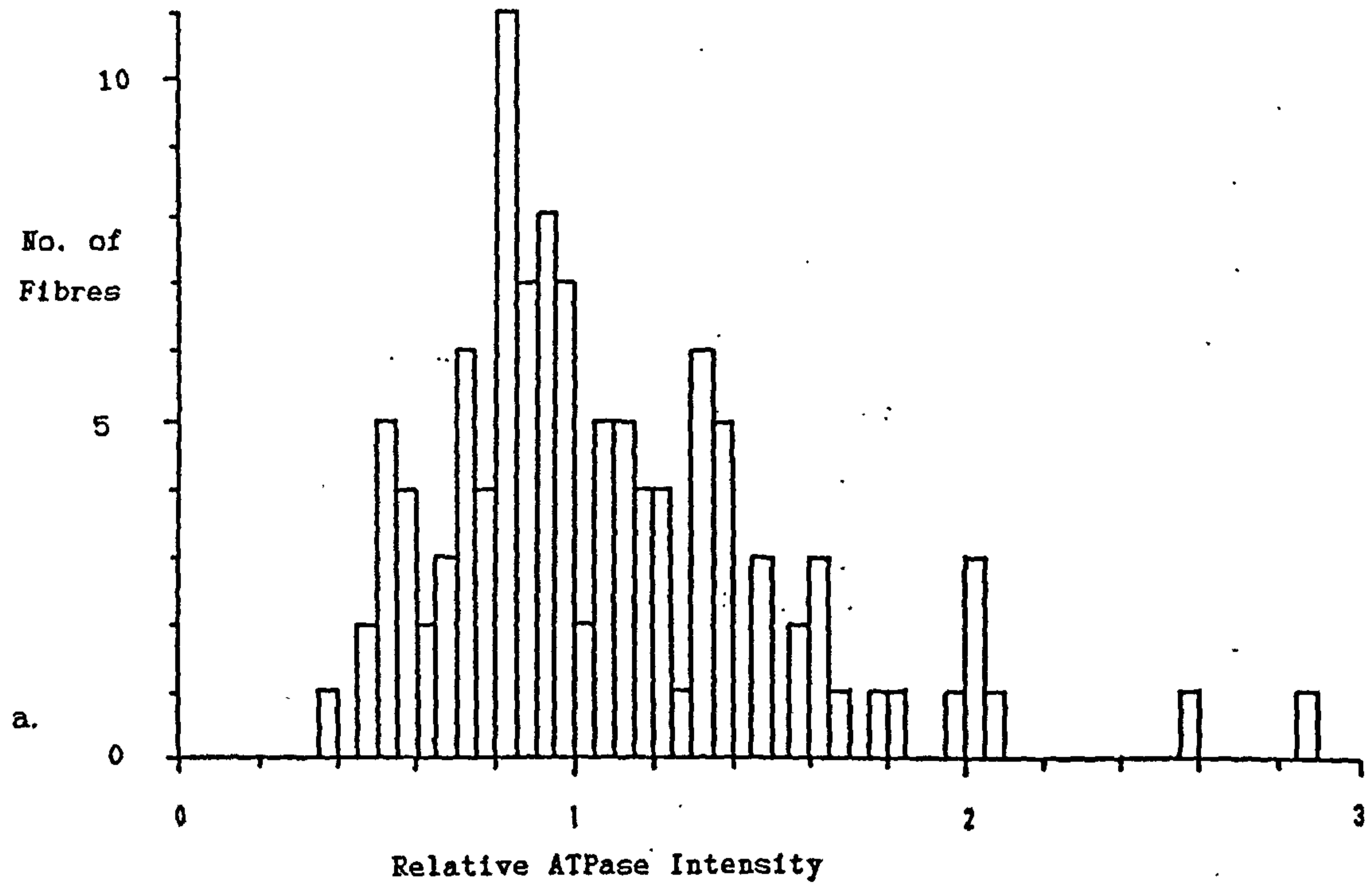
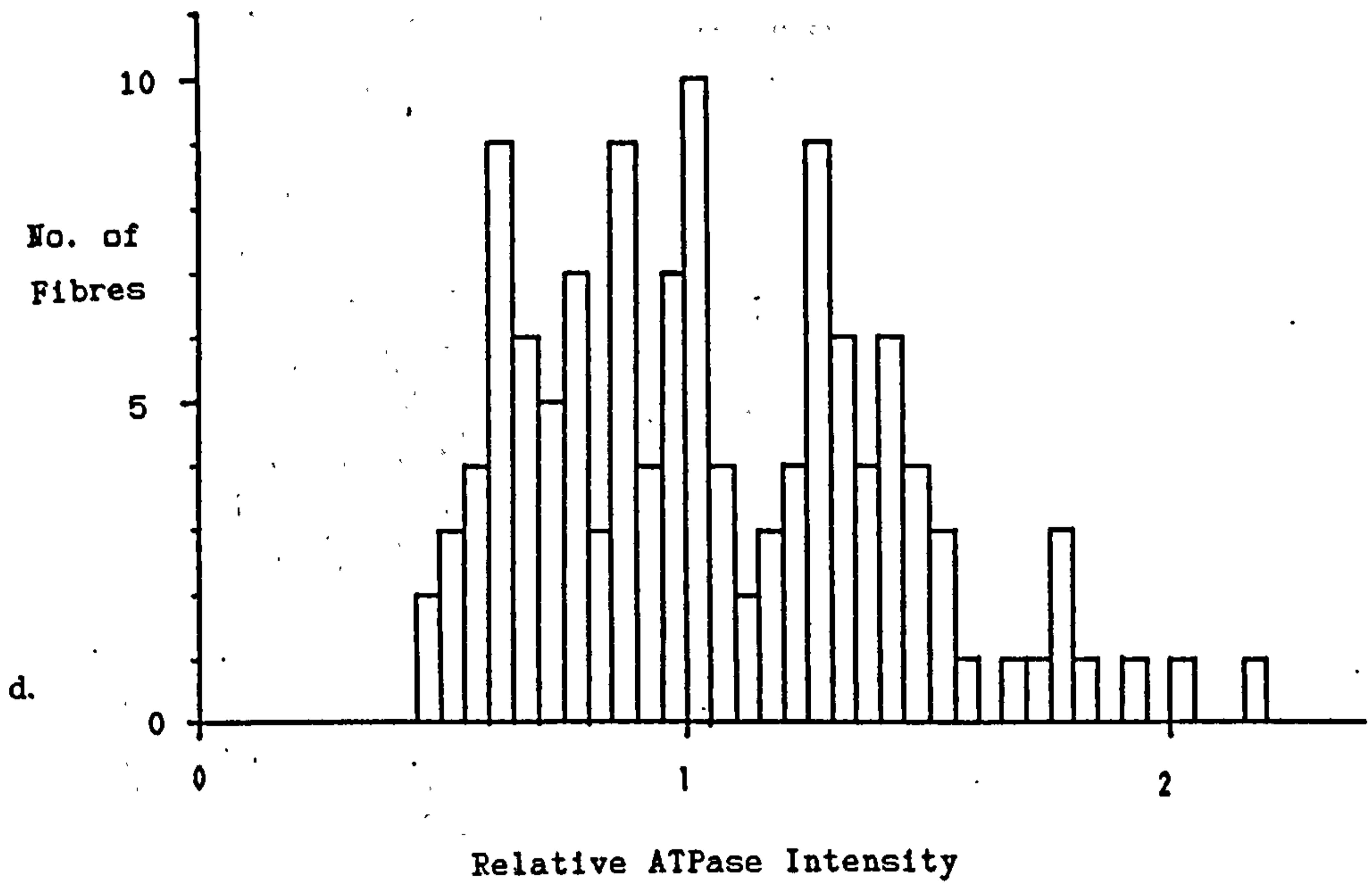
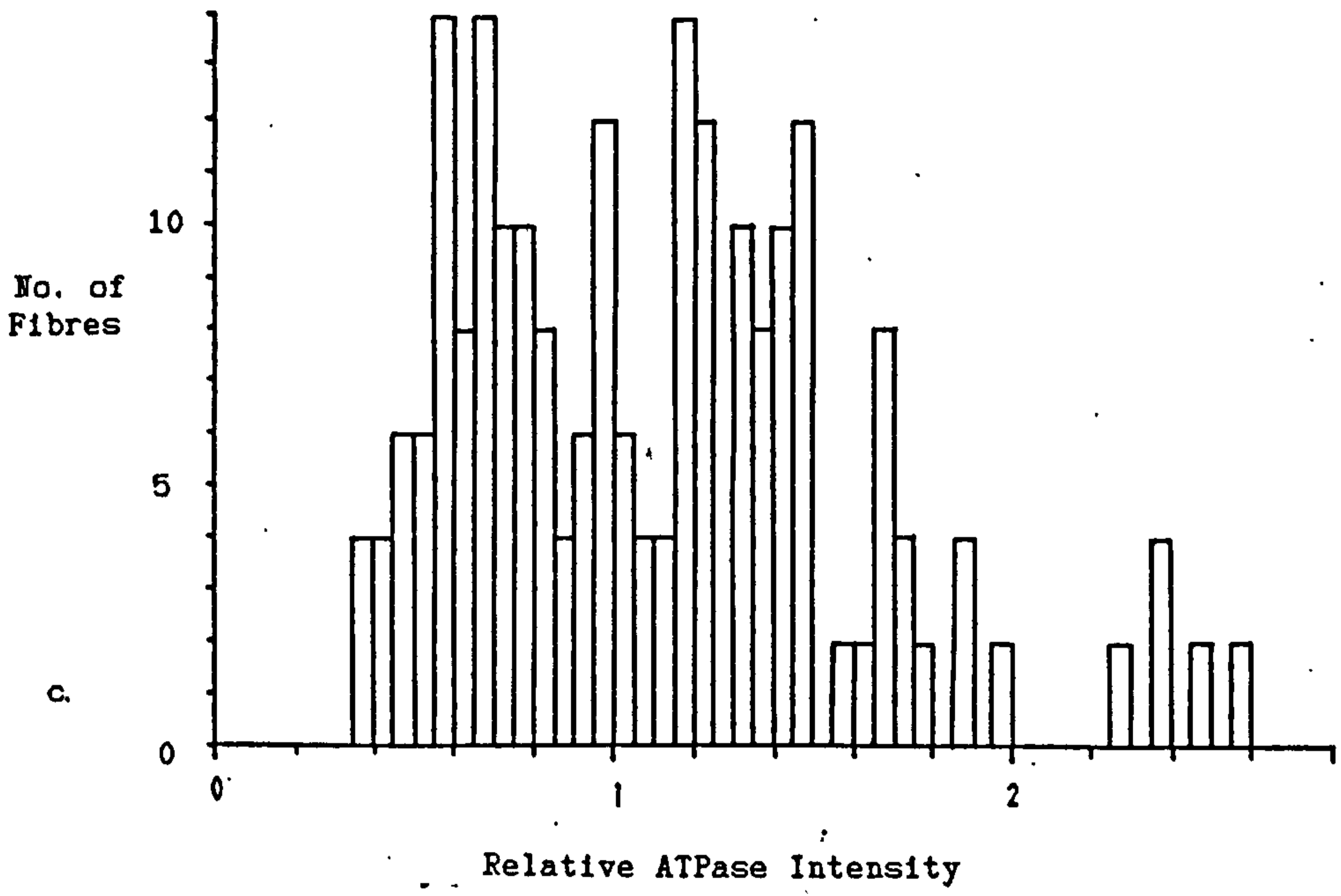
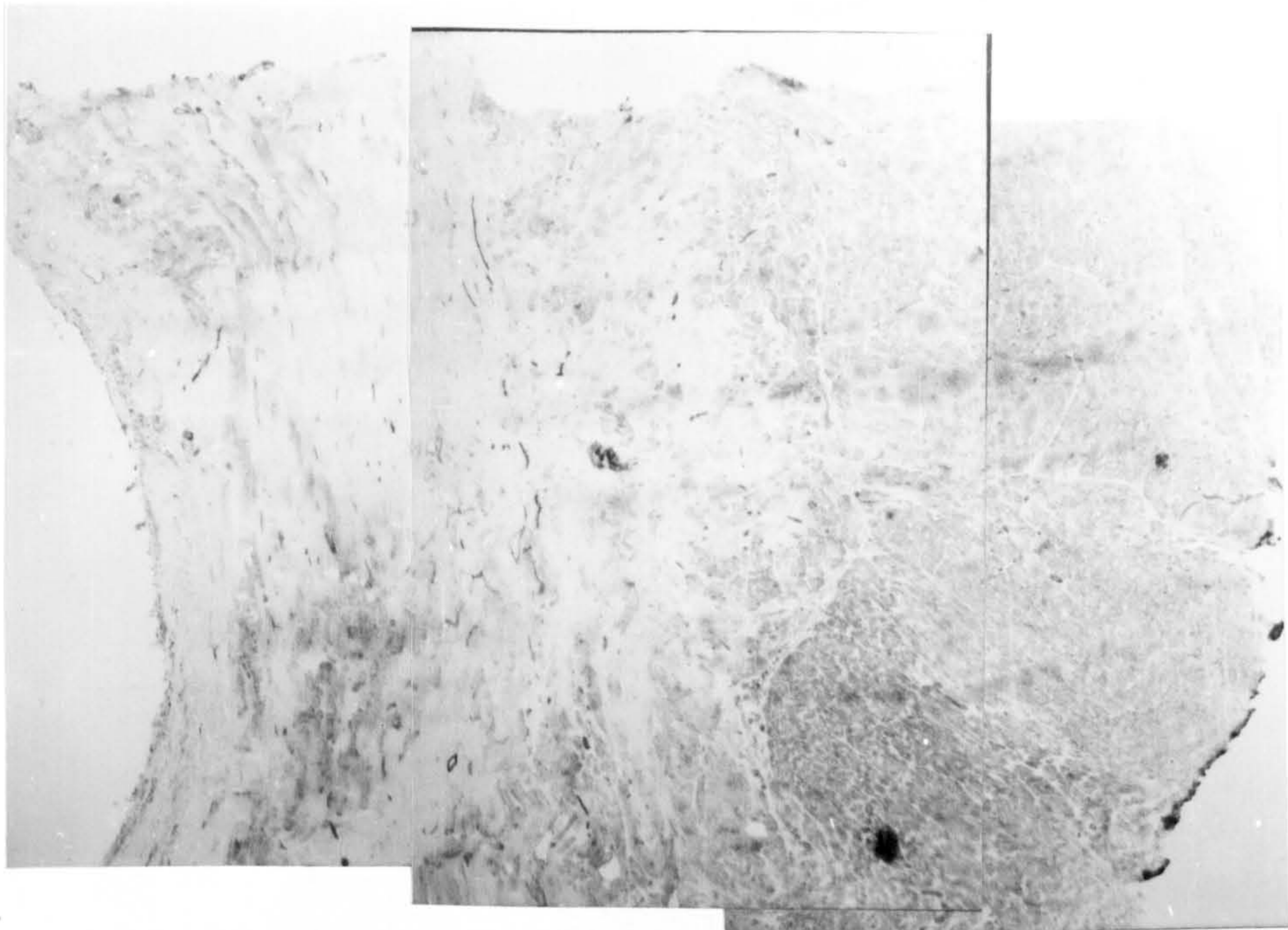
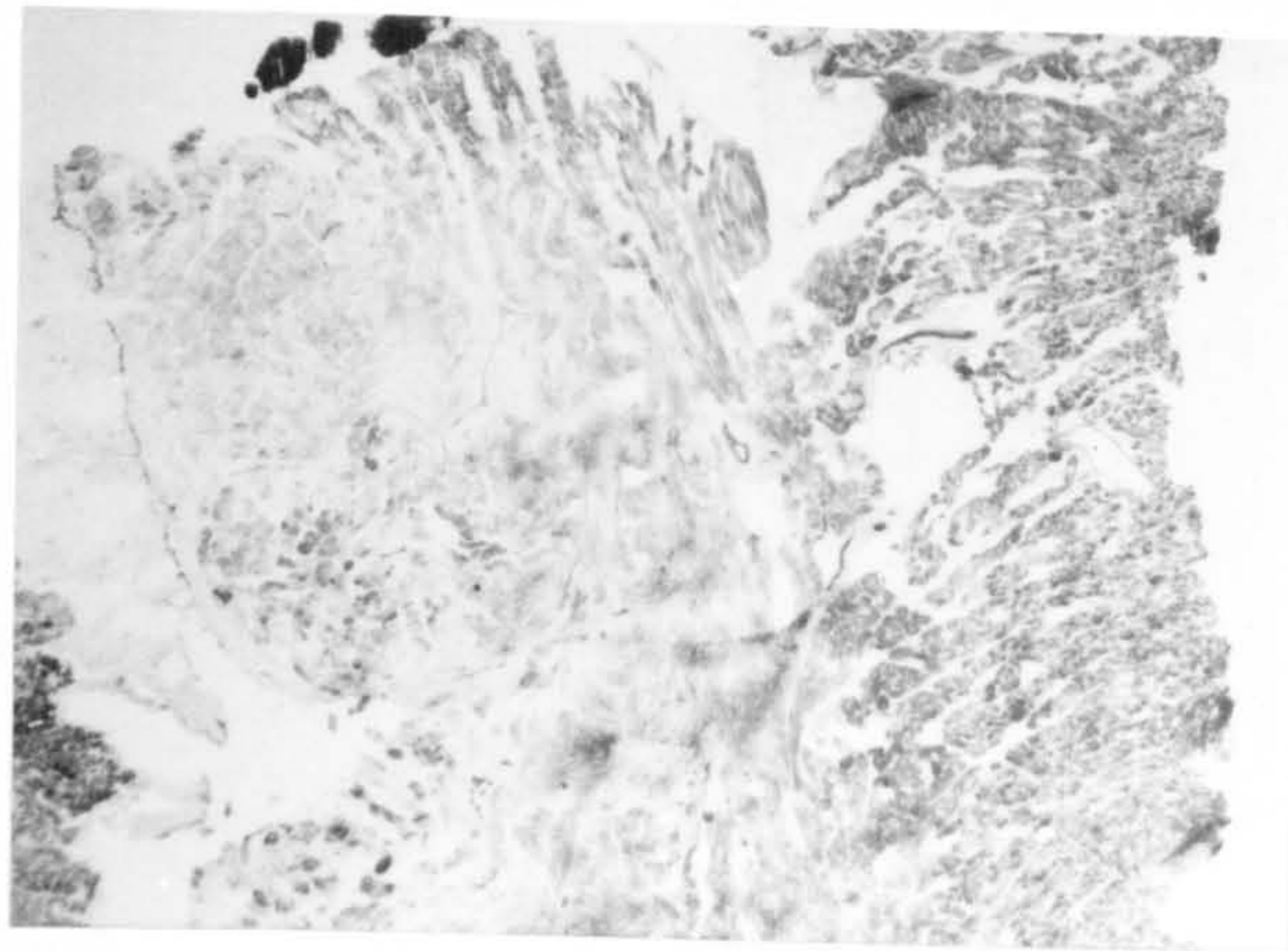


Fig.11a-d. Histograms of readings taken from ventricular muscle of four rabbit hearts, stained for myosin ATPase activity after pre-treatment at pH 10.2 (a, b & d) or 10.4 (c).





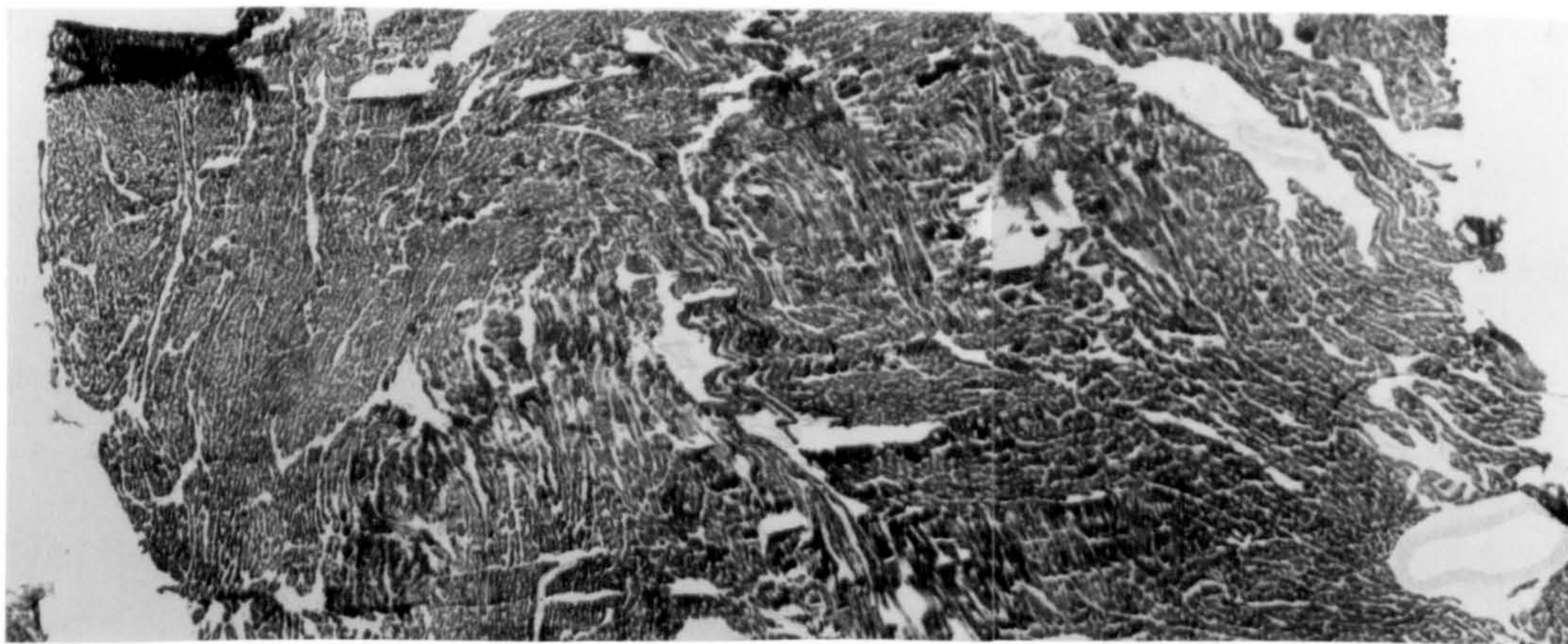
a.



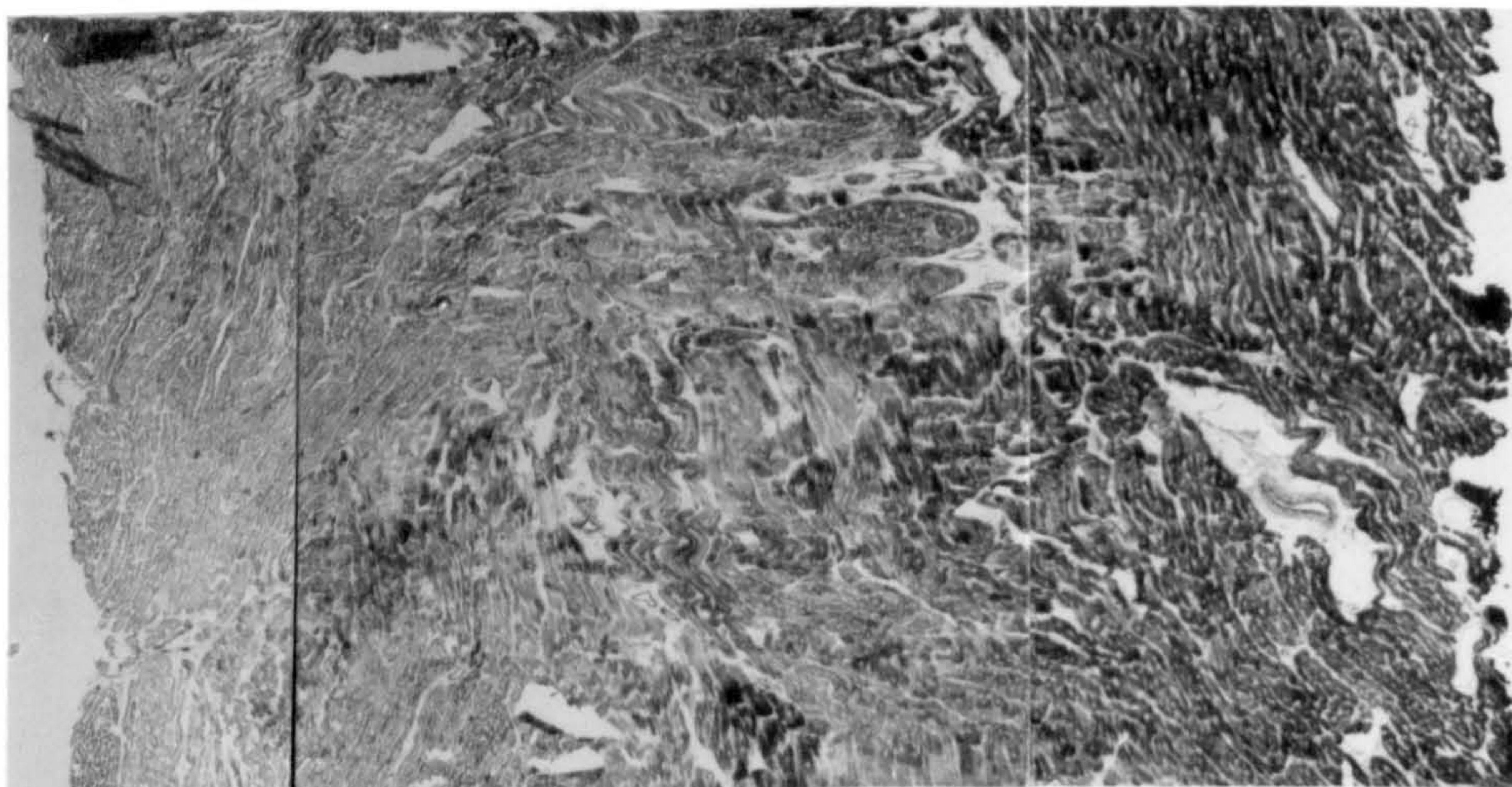
b.

Fig,12a. Montage showing rabbit left ventricular muscle stained for myosin ATPase activity after pre-treatment at pH 10.6 (x40). The endocardial surface is to the left, the epicardial surface to the right.

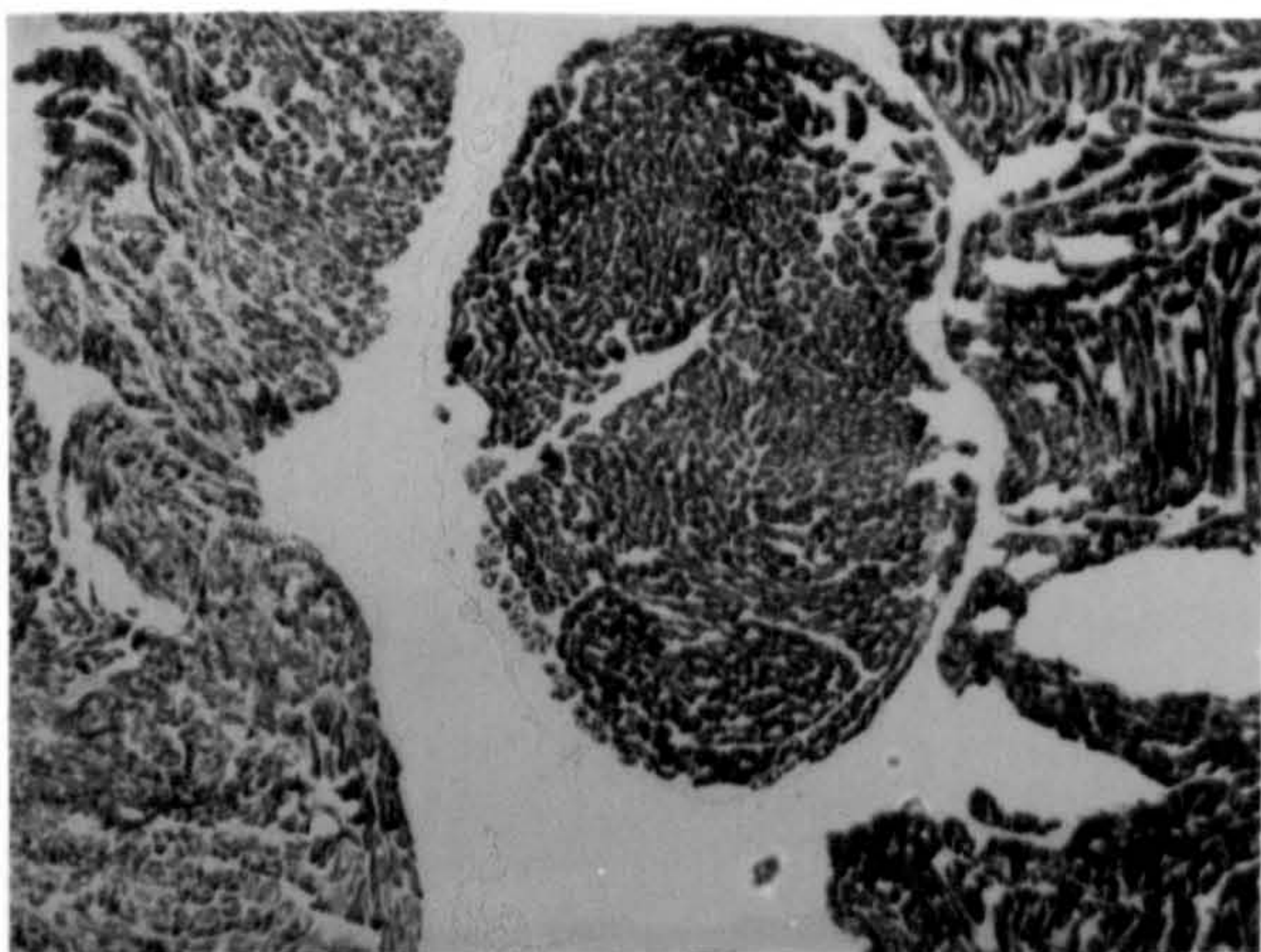
b. Rabbit right ventricular muscle stained for myosin ATPase activity after pre-treatment at pH 10.2 (x40). The endocardial surface is to the left, the epicardial surface to the right.



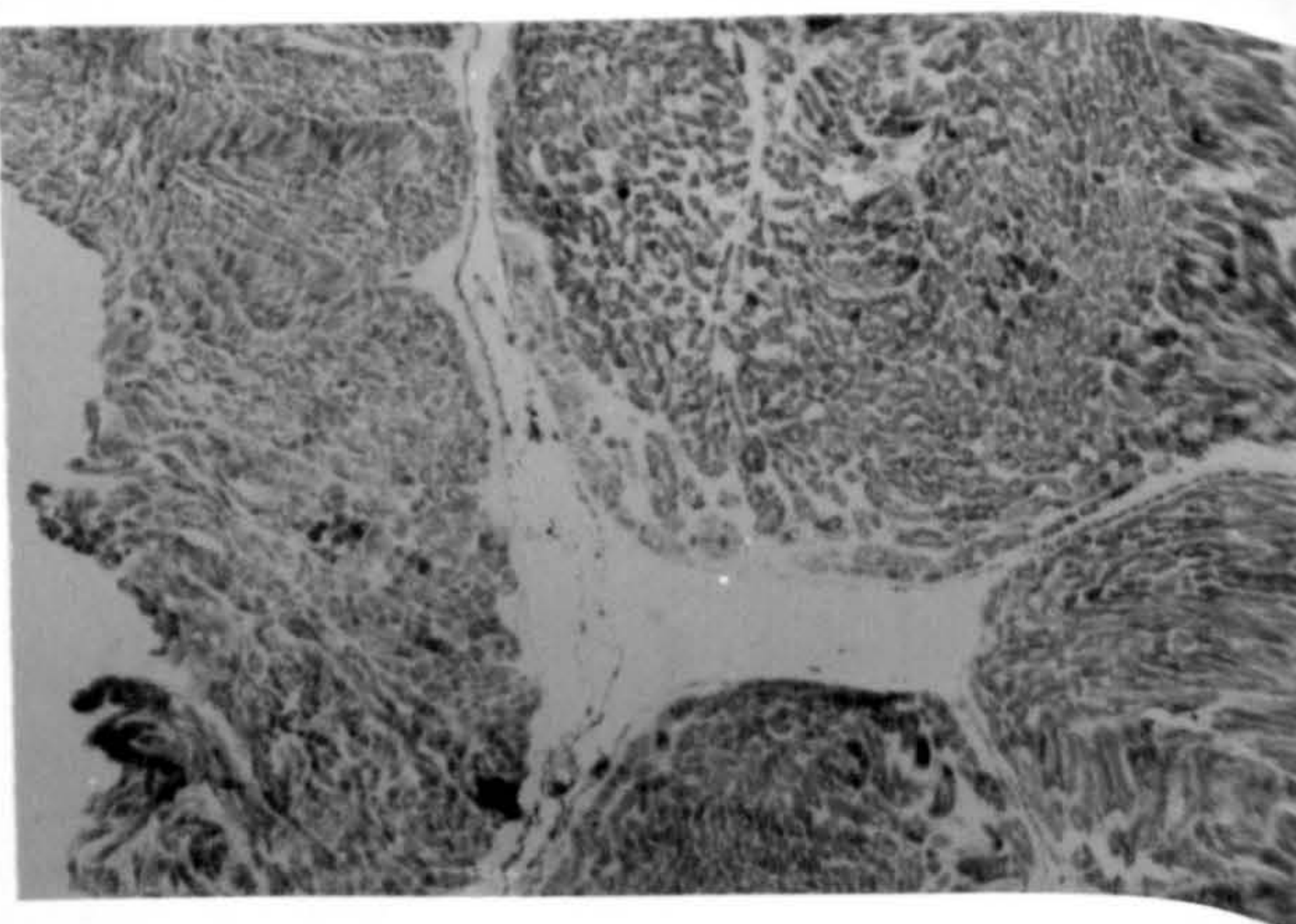
a.



b.



c.



d.

Fig.13a. Montage showing rabbit left ventricular muscle stained for succinate dehydrogenase activity. The endocardial surface is to the left, the epicardial surface to the right.

b. Montage showing rabbit left ventricular muscle stained for  $\alpha$ -glycerophosphate dehydrogenase activity. The endocardial surface is to the left, the epicardial surface to the right.

c. Rabbit atrium (left) and left ventricle (right) stained to show succinate dehydrogenase activity.

d. Rabbit atrium (left) and left ventricle (right) stained to show  $\alpha$ -glycerophosphate dehydrogenase activity.

technique used here i.e. heterogeneity between neighbouring fibres (Fig.10) and regional differences (examples of which are shown in Fig.12). The results presented below were collected in an attempt to find the cause of these differences.

Workload: Fibre Metabolic Capacity

Chronic increased workload leads to hypertrophy of ventricular myocytes and an increased proportion of  $V_3$  myosin within them (Lompre et al., 1979). Similar adaptations to altered workload in skeletal muscle, are accompanied by changes in the metabolic capacity of muscle fibres<sup>Salmons (1980)</sup> I have therefore looked at fibre cross-sectional area and the metabolic capacities of ventricular fibres to see if these are correlated with myosin type. Such a correlation would suggest that differences in workload are responsible for the heterogeneity of myosin ATPase staining in the rabbit ventricle.

Fig.13 shows how succinate dehydrogenase (SDH) and  $\alpha$ -glycerophosphate dehydrogenase ( $\alpha$ GPDH) activities vary across the left ventricular wall (a. and b. respectively) and how SDH and  $\alpha$ GPDH activities (c. and d. respectively) differ between atrium and ventricle. In both a. and b. the epicardium is to the left; in c. and d. the atrium is to the left. It appears that SDH staining varies very little through the wall, with only slightly darker staining in the mid-wall region. The variations of SDH staining in Fig.13a are, however, much smaller than those seen for  $\alpha$ GPDH staining in Fig.13b. Here  $\alpha$ GPDH staining is seen to be strongest in the epicardial layers, getting progressively weaker as the endocardial layers are approached. When one compares the atrio-ventricular distributions of these enzymes, it appears that SDH

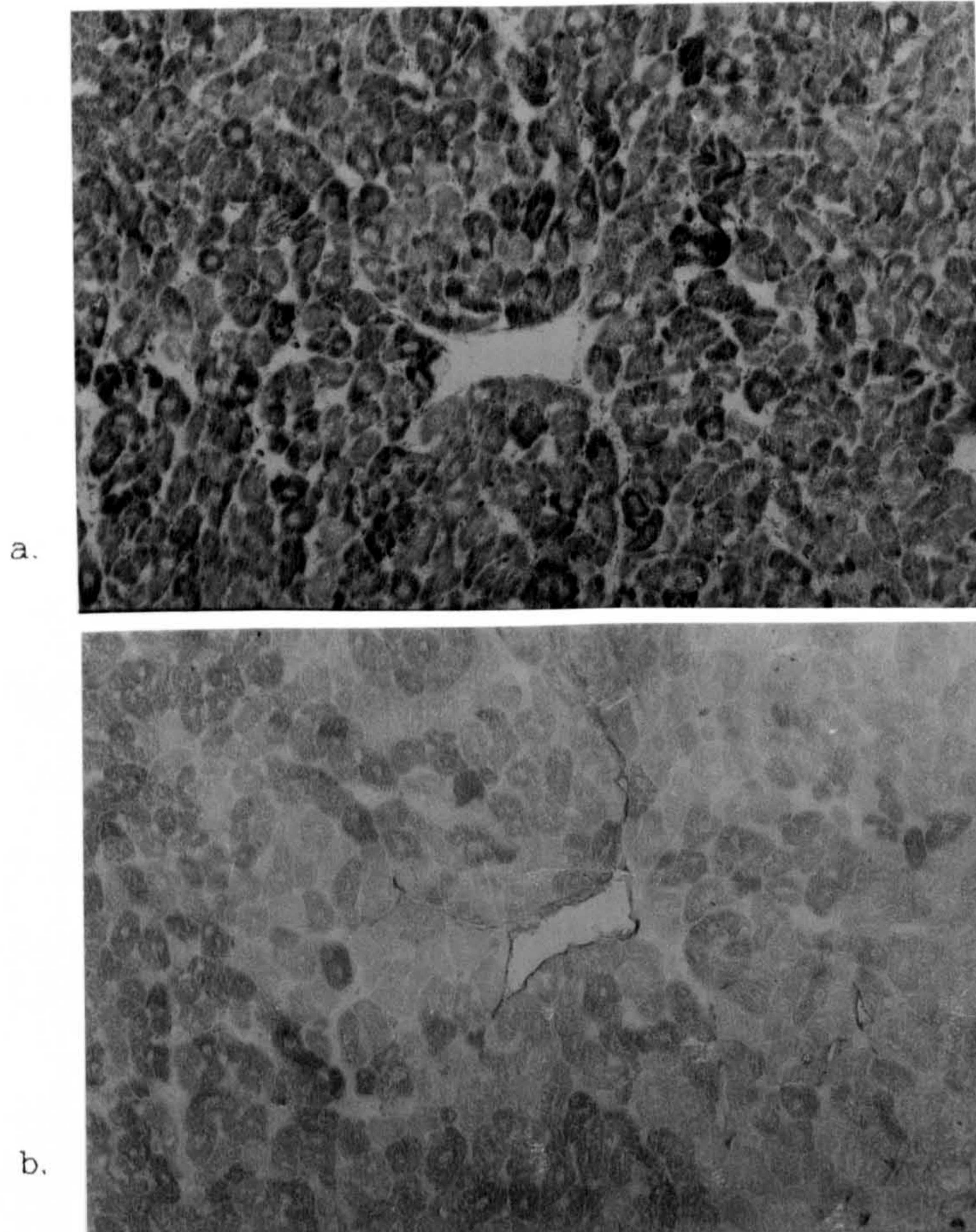


Fig.14a. Rabbit left ventricular papillary muscle stained for  $\alpha$ GPDH activity (x160).

b. Rabbit left ventricular papillary muscle stained for myosin ATPase activity after pre-treatment at pH 10.2 (x160).

Sections a. and b. are serial sections of the same muscle.

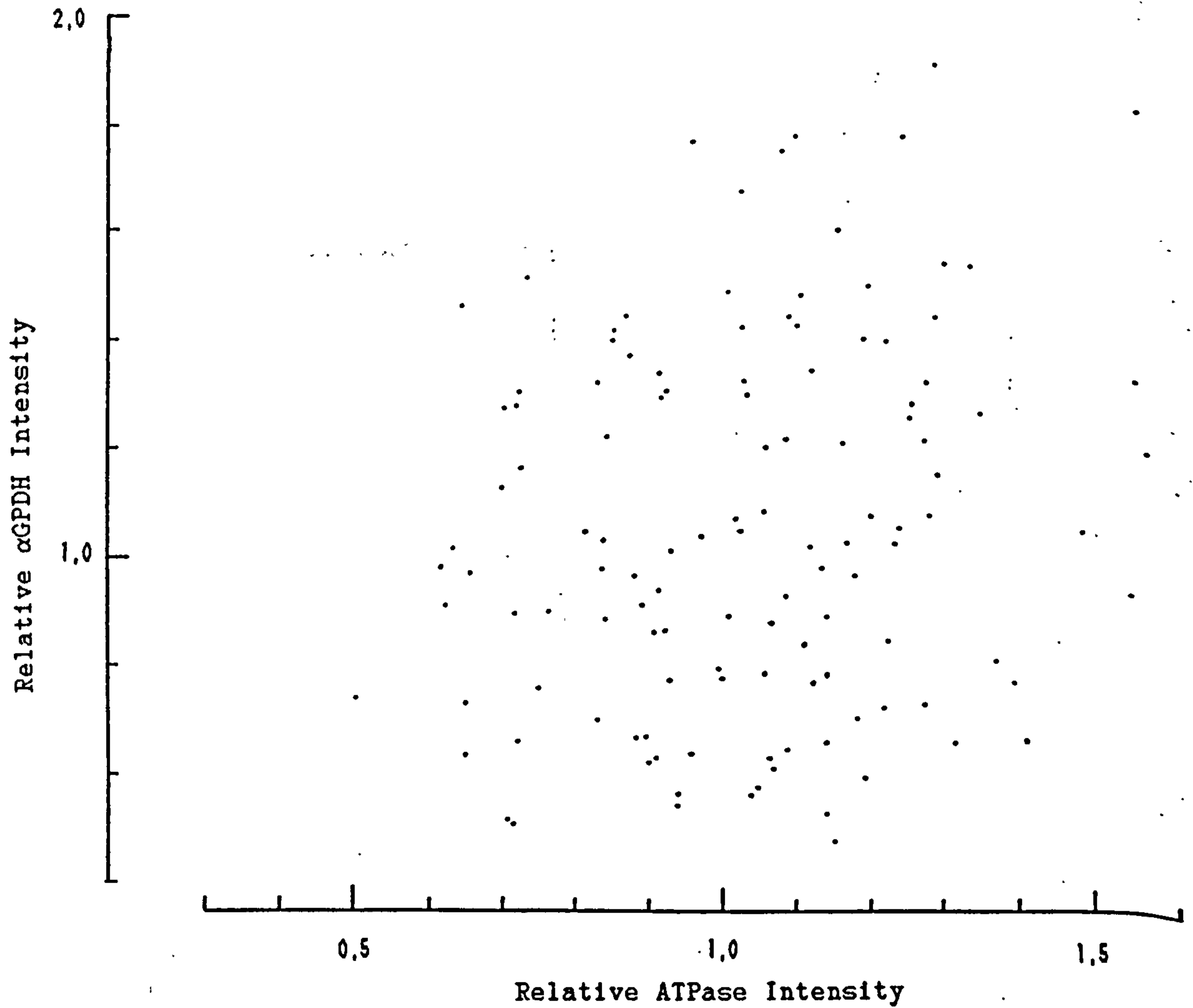


Fig.15 Scattergram of relative  $\alpha$ GPDH activity plotted against myosin ATPase activity after alkaline pre-treatment. Each point represents a reading of each stain in serial sections of one fibre. Correlation coefficient,  $r = 0.15$

(which shows little variation across the ventricular wall) activity differs whereas  $\alpha$ GPDH does not. As the differences in  $\alpha$ GPDH staining within the ventricle were greater than those for SDH and because they correlated with the trans-mural differences in myosin ATPase staining after alkaline pre-treatment (Fig.12) this stain was used for the second part of this work on individual fibres.

Fig.14 shows two differently stained serial sections. Section a. has been stained for  $\alpha$ GPDH. Section b. is stained for myosin ATPase activity after alkaline pre-treatment. Differences in staining intensity between neighbouring fibres can be seen in both sections.

124 fibres were identified in each of these sections using photographic 'maps' and photometric readings were taken of the two stains in each fibre. Fig.15 shows a scattergram of relative  $\alpha$ GPDH staining intensity plotted against relative myosin ATPase staining intensity. There appears to be little correlation between  $\alpha$ GPDH and myosin ATPase staining intensities although it does appear that the distribution of points is somewhat triangular in shape. The prima facie indication from  $\alpha$ GPDH staining is therefore that workload has an influence on myosin distribution where regional differences are concerned but is not much involved in neighbouring cell heterogeneity. This conclusion will, however, be considered further in the Discussion.

#### Workload: Fibre Cross-Sectional Area

Table 1 shows results, from two rabbits, of fibre cross-sectional area measurements in sub-epicardial and sub-endocardial regions. In both cases endocardial fibre cross-sectional areas were found to be significantly greater than those of epicardial fibres



|              | Rabbit 1     | Rabbit 2     |
|--------------|--------------|--------------|
| Endocardial  | 65.09 ± 2.72 | 62.84 ± 2.14 |
| Epicardial   | 54.29 ± 3.65 | 52.13 ± 1.72 |
| Significance | p < 0.05     | p < 0.001    |

Table 1 Cross-sectional areas (arbitrary units) of sub-endocardial and sub-epicardial fibres. Significance levels were calculated using Student's t-test.

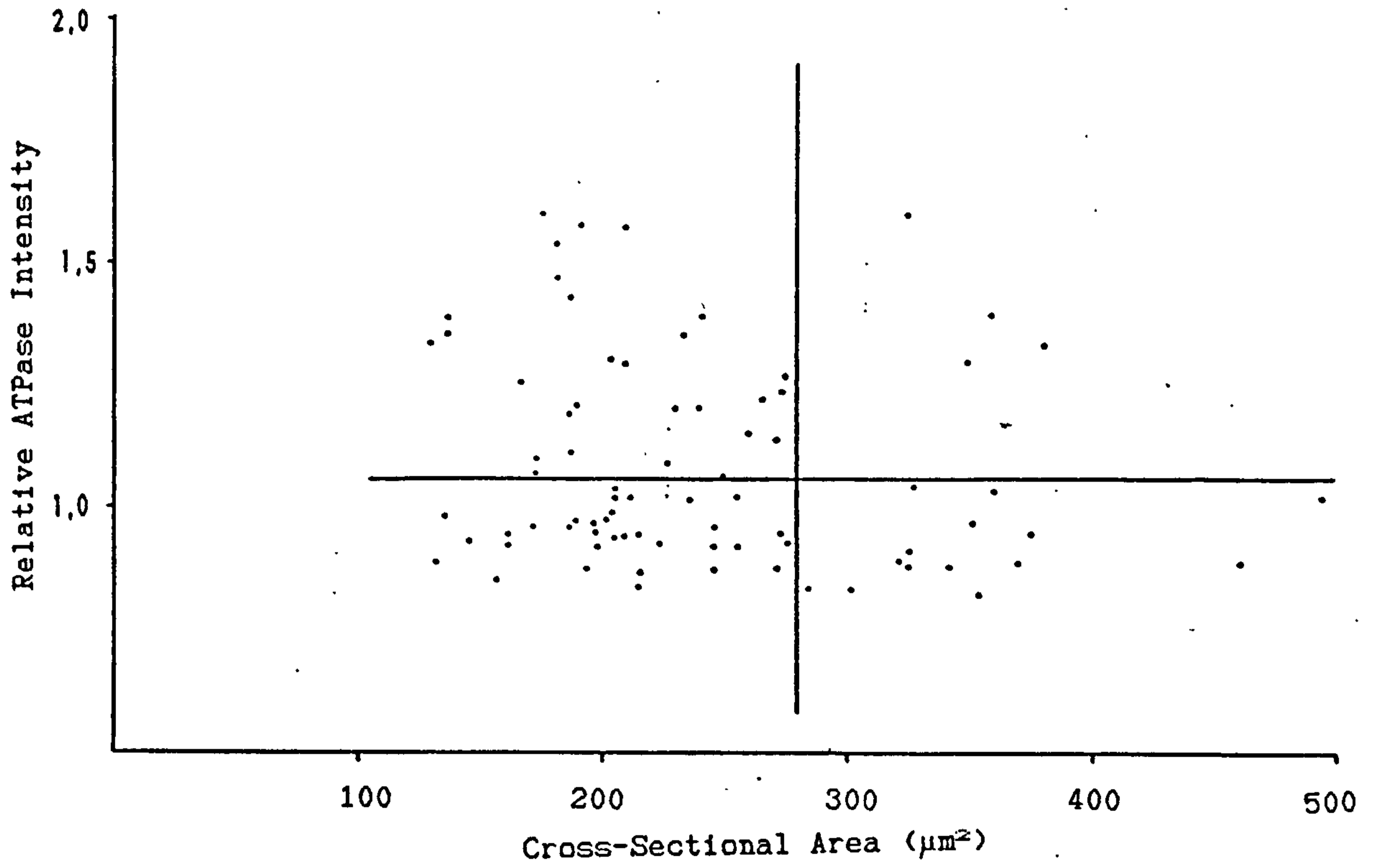


Fig.16 Scattergram of fibre cross-sectional area ( $\mu\text{m}^2$ ) against relative myosin ATPase activity after alkaline pre-treatment.

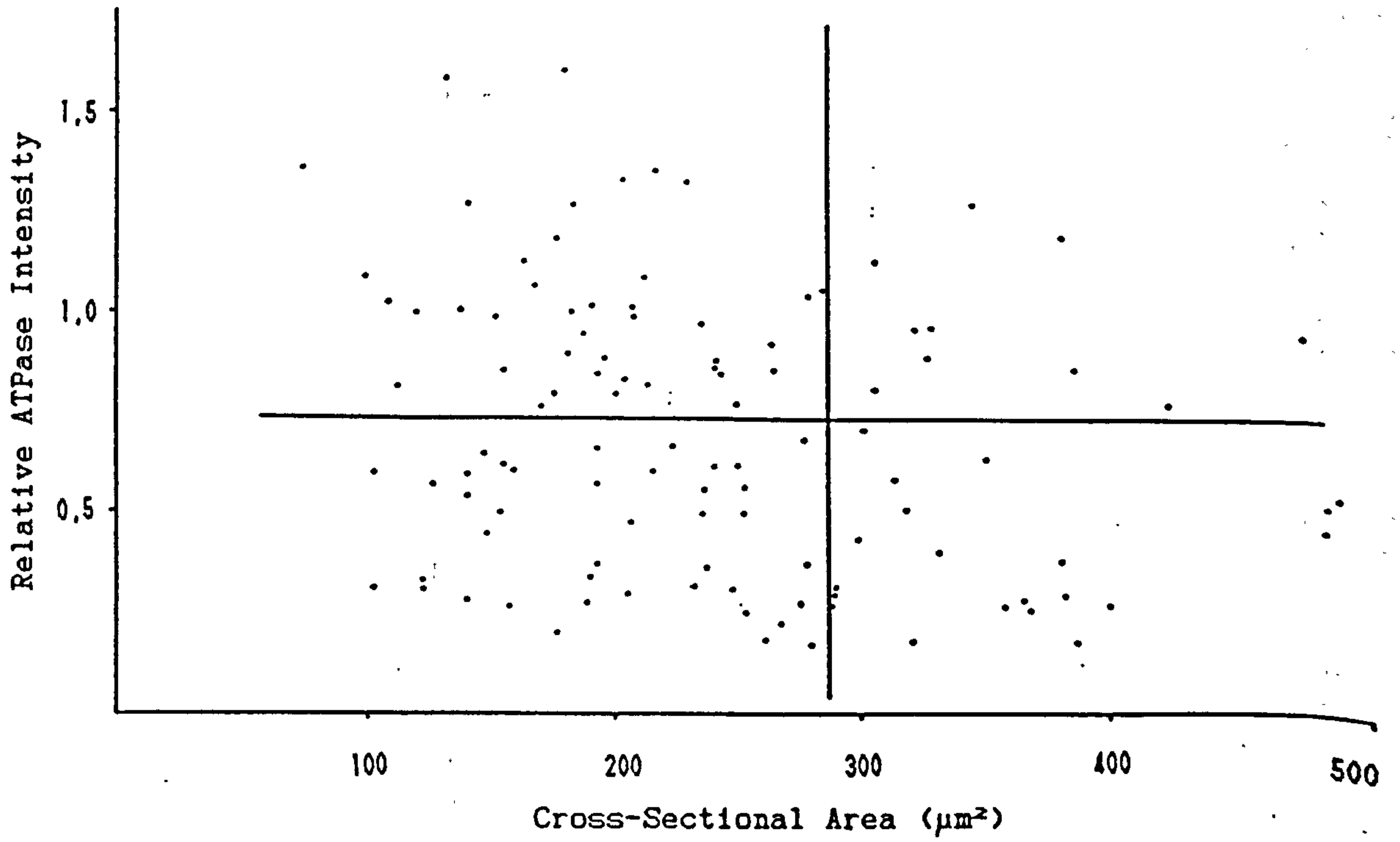


Fig.17 Scattergram of fibre cross-sectional area ( $\mu\text{m}^2$ ) against relative myosin ATPase activity after alkaline pre-treatment. Variations in fibre width have been controlled for in this case.

(by about 20% in both cases;  $p < 0.05$ ,  $p < 0.001$ ). As with the other index of chronic workload,  $\alpha$ GPDH staining intensity, fibre cross-sectional area appears to correlate with the trans-mural difference in myosin distribution i.e. larger (and therefore harder working) endocardial fibres contain more alkali-labile myosin than do the smaller epicardial fibres.

Fibre cross-sectional area was then used as an index of workload for individual fibres, to see if it correlated with alkali pretreated myosin ATPase activity. The results of this are shown in Fig.16, where fibre cross-sectional area is plotted against relative ATPase staining intensity. The relation between these variables is not a linear correlation, yet it does appear that the upper right quadrant contains disproportionately few points. The horizontal line in Fig. 16 (and Fig.17) was chosen such that roughly equal numbers of points would be in the two left hand quadrants, the vertical line was positioned to maximise the difference between the two right hand quadrants. Table 2 shows the number of points in each of the quadrants.

As cardiac myocytes are rarely perfectly rectangular, often having large changes in diameter along their length (see chapter on isolated myocytes p. 162), it is important to know whether the value of cross-sectional area for a fibre has been measured at a thick or thin point on the fibre. A measurement of a mainly thick fibre made at a thin portion would be misleading and might help explain the large number of fibres showing small measured cross-section.

|                  |                   |
|------------------|-------------------|
| Upper Left<br>41 | Upper Right<br>10 |
| Lower Left<br>40 | Lower Right<br>20 |

Table 2 Number of points in each of the quadrants of Fig. 17.

|                  |                   |
|------------------|-------------------|
| Upper Left<br>29 | Upper Right<br>4  |
| Lower Left<br>33 | Lower Right<br>14 |

Table 3 Number of points in each of the quadrants of Fig. 16.

Using isolated myocytes (see p.162) it was possible to measure the thin portions of fibres. Of 100 fibres only two were found to have thin portions over 30 $\mu$ m in length. To control for the more typical thinner portions, four serial 10 $\mu$ m sections were cut and stained for myosin ATPase activity after alkaline pre-treatment. Individual fibres were then indentified in each section and compared. If the size of a fibre changed it was not used for cross-sectional area measurements. Only those cells whose profiles remained more or less unchanged from the first to the fourth section were used (all measurements of cross-sectional area for correlation with myosin ATPase were made in the first of the four sections). The result of this procedure is shown in Fig.17. The number of points in each quadrant is shown in Table 3. Again the upper right quadrant contains fewer points than does the lower right quadrant, whereas the two left quadrants contain equal numbers of points. This is, of course, very similar to Fig.16 and indicates that the random sampling of different positions along the lengths of fibres had not significantly influenced the shape of the earlier scattergram.

From Figs.16 & 17 therefore, it appears that whereas small fibres are just as likely to have high as low activity myosin, larger fibres are more likely to have the low activity isoform. Like  $\alpha$ GPDH staining intensity, fibre cross-sectional area correlates with trans-mural differences in myosin ATPase staining but does not correlate well with differences between neighbouring fibres.

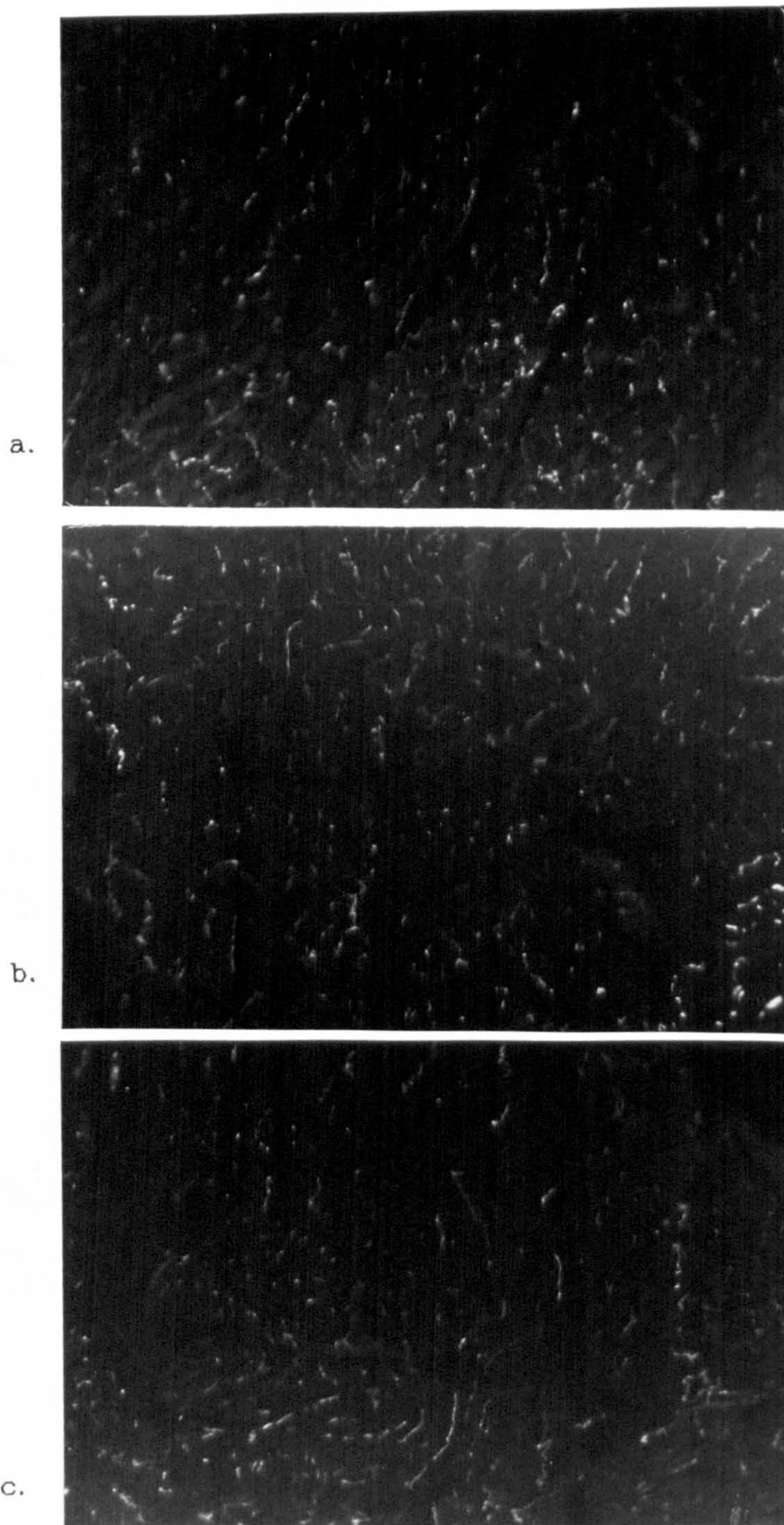


Fig.18a. Cat left ventricular muscle treated to show fluorescence of sympathetic endings.

b. Rabbit left ventricle (epicardium) treated to show fluorescence of sympathetic endings.

c. Rabbit left ventricle (endocardium) treated to show fluorescence of sympathetic endings.

### Sympathetic Endings

Chemical sympathectomy has been shown to change myosin distribution in rat ventricles (Rupp et al., 1983). For this reason I have looked at sympathetic innervation of rabbit and cat ventricle to see if this can explain the differences in ventricular heterogeneity between these species. As cat ventricle shows no myosin ATPase heterogeneity, a finding that there were different distributions of sympathetic nerve endings in cat and rabbit ventricles might suggest a possible reason for the differences in myosin distribution between these two species.

In Fig.18 are examples of a. cat ventricle ,b. rabbit left ventricle (epicardium) and c. rabbit left ventricle (endocardium) showing formaldehyde-induced fluorescence (FIF) of sympathetic endings. There is no apparent difference in the distribution of endings in these species or within the rabbit ventricle, innervation appearing to be uniform in all three cases. No differences in distribution were seen in three cats and three rabbits which were compared in this way. The reason for the differences between cat and rabbit and within the rabbit ventricle, in terms of myosin heterogeneity, are not likely therefore, to be sympathetic ending distribution. However, this does not exclude sympathetic endings from having some influence. To investigate this further, I looked at the effect of chemical sympathectomy on rabbit ventricular myosin ATPase heterogeneity.



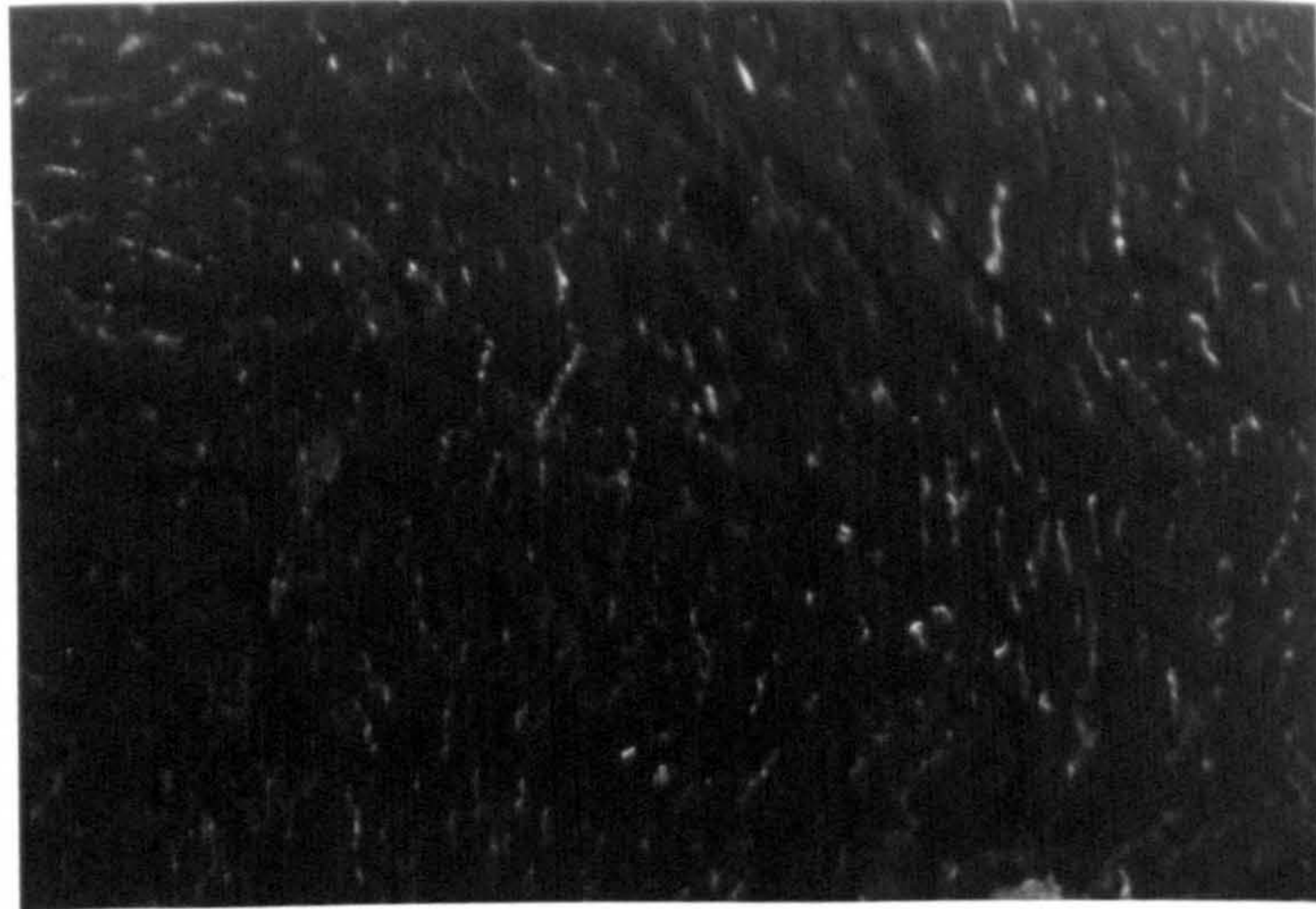


Fig.19 Control sympathectomized rabbit left ventricular muscle treated to show fluorescence of sympathetic endings. No fluorescent endings were visible in the ventricle of the sympathectomized rabbits.

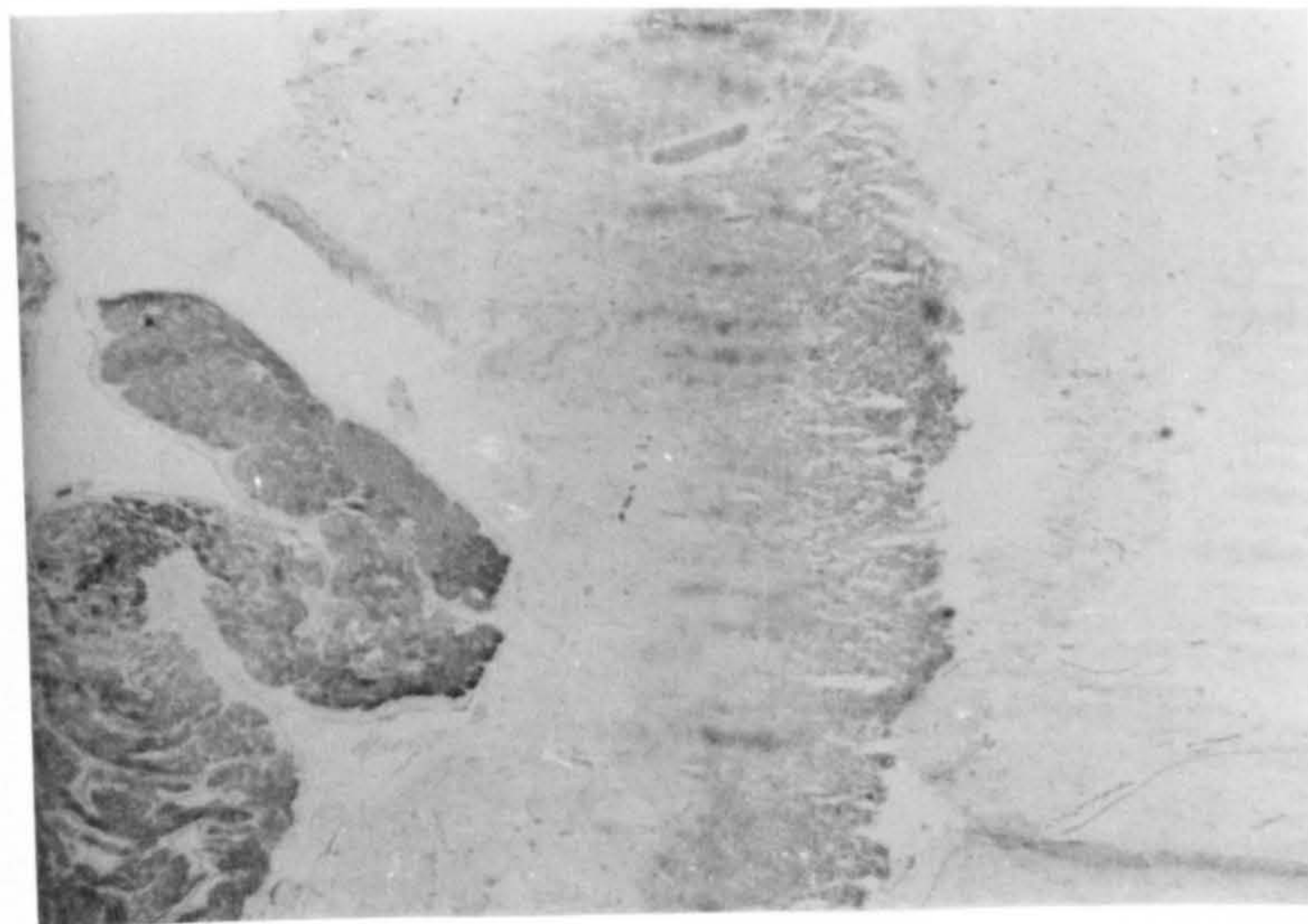


Fig.20 Sympathectomized rabbit atrial (left), right ventricular (middle) and left ventricular (right) muscle stained for myosin ATPase activity after pre-treatment at pH 10.2 (x25).  
The epicardium of the right ventricle is arrowed above.

Chemical Sympathectomy

Of the four rabbits which were sympathectomized, two died after only one week. As this was thought to be too short a time for any effects of sympathectomy on myosin ATPase staining to be seen, no results from these animals are included. All four control animals survived the six week course of injections. Fig.19 shows FIF sections of left ventricle of a control rabbit. No fluorescent endings were visible in the sympathectomized rabbit ventricles, however as there was practically nothing to be seen on these sections it proved impossible to photograph this satisfactorily. (It can be assumed that sympathectomy was complete throughout at least five of the six weeks as neither of the two animals which died after the third injection showed any evidence of sympathetic endings in the left ventricle).

In Fig.20 is shown a composite block of sympathectomized rabbit atrium, right ventricle and left ventricle. The atrium appears to the left of the photograph and is the most darkly stained piece in the section. To the right of the photograph can be seen the endocardium of the left ventricle. The right ventricular piece is 'sandwiched' between these pieces and shows that the epicardial layers of sympathectomized ventricles contain a more alkali-stable myosin isoform than do the endocardial layers.

Fig.21 shows examples of left ventricular muscle from each sympathectomized rabbit, stained for myosin ATPase activity after alkaline pre-treatment. Comparison with Fig.22, where left ventricular muscle from a control rabbit is shown, confirms that

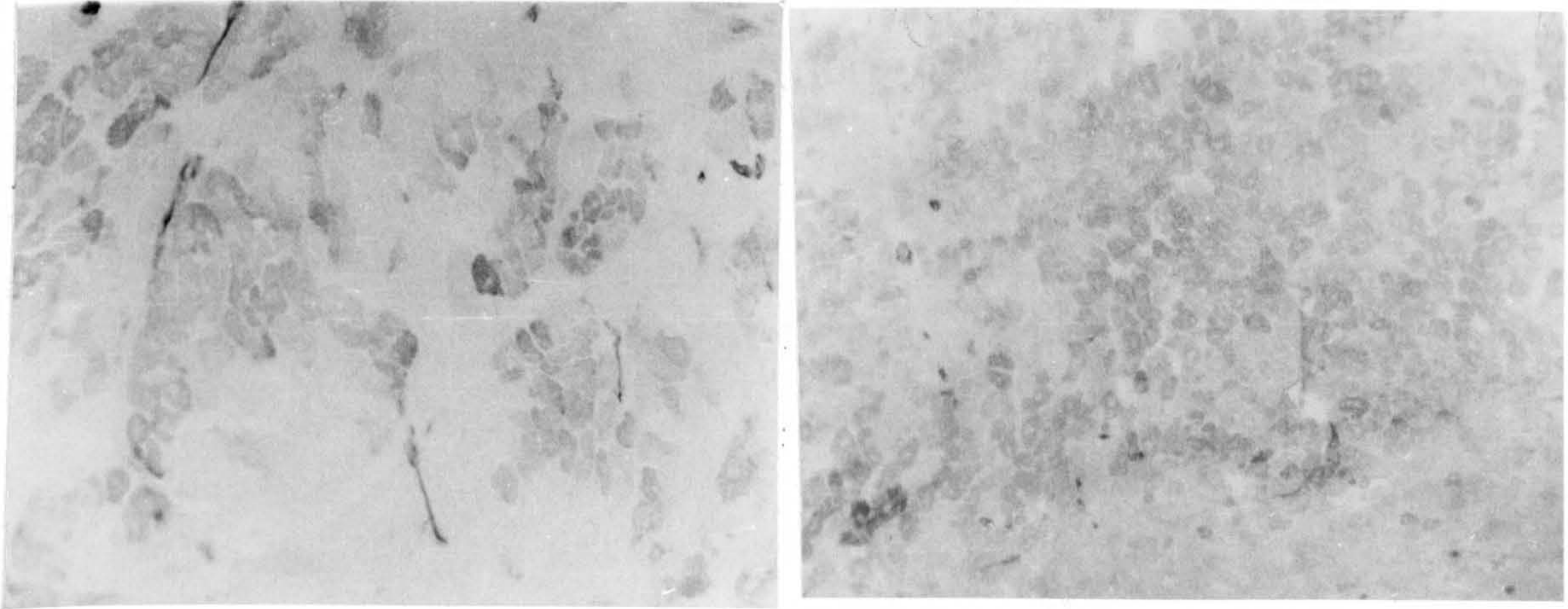


Fig.21 Left ventricular muscle from each of the two sympathectomized rabbits, stained for myosin ATPase activity after pre-treatment at a. pH 10.0 (x100) and b. pH 10.2 (x160).

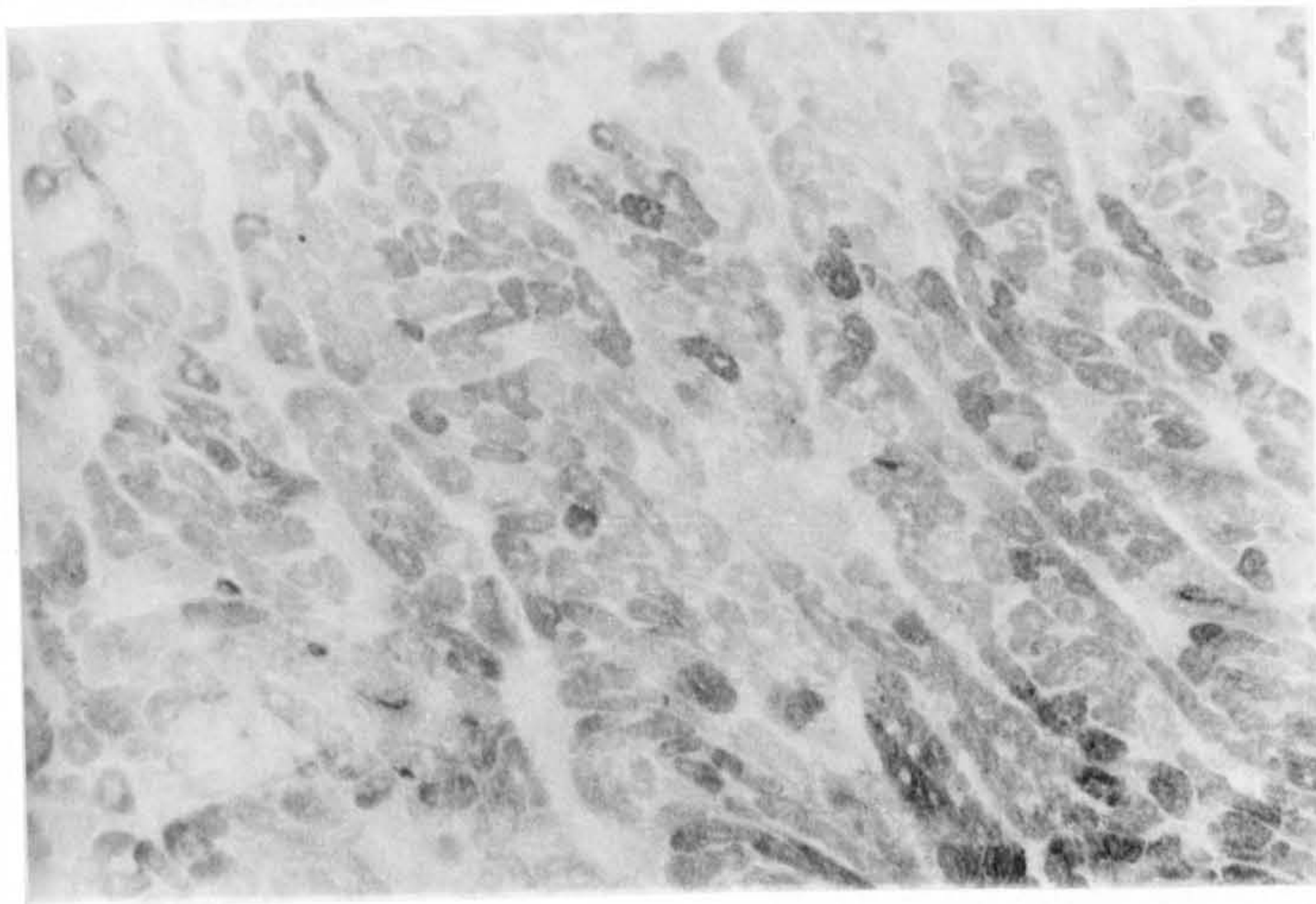


Fig.22 Control rabbit left ventricular muscle stained for myosin ATPase activity after pre-treatment at pH 10.4 (x160).

|                    | APD <sub>50</sub> ms | APD <sub>75</sub> ms | APD <sub>90</sub> ms | E <sub>m</sub> mV | APA mV    |
|--------------------|----------------------|----------------------|----------------------|-------------------|-----------|
| Expt. 1            |                      |                      |                      |                   |           |
| RVPAP              | 68,5±5,4             | 120,9±5,5            | 140,4±4,2            | -82,2±0,8         | 106,7±2,9 |
| LVPAP              | 113,9±4,5            | 148,3±3,2            | 171,8±3,6            | -82,2±0,4         | 103,7±2,6 |
| Expt. 2            |                      |                      |                      |                   |           |
| RVPAP <sub>1</sub> | 120,5±3,3            | 150,7±3,3            | 170,6±3,2            | -78,4±1,2         | 106,3±1,7 |
| RVPAP <sub>2</sub> | 131,6±5,0            | 157,8±5,8            | 171,8±4,6            | -81,2±0,9         | 103,8±2,0 |
| Expt. 3            |                      |                      |                      |                   |           |
| RVPAP <sub>1</sub> | 99,1±2,0             | 124,6±1,9            | 141,6±2,5            | -78,6±1,1         | 97,2±1,1  |
| RVPAP <sub>2</sub> | 53,7±2,8             | 95,9±3,0             | 113,6±4,5            | -76,2±0,8         | 93,8±1,4  |
| Expt. 4            |                      |                      |                      |                   |           |
| RVTRAB             | 123,8±3,3            | 153,1±4,9            | 172,1±5,3            | -78,1±0,9         | 92,4±1,6  |
| RVPAP              | 81,2±3,1             | 120,9±5,8            | 157,9±5,5            | -77,8±2,0         | 94,1±1,8  |
| Expt. 5            |                      |                      |                      |                   |           |
| RVPAP              | 127,4±7,2            | 152,4±7,1            | 179,1±5,7            | -78,8±0,7         | 96,5±1,1  |
| LVPAP              | 176,0±7,2            | 209,1±6,8            | 227,9±5,6            | -77,0±0,8         | 96,1±0,9  |
| Expt. 6            |                      |                      |                      |                   |           |
| RVPAP              | 136,0±2,8            | 170,1±2,4            | 190,7±3,2            | -80,4±1,0         | 99,4±1,9  |
| LVPAP              | 246,3±18,5           | 277,1±18,4           | 292,5±18,5           | -77,6±0,6         | 99,7±1,3  |
| Expt. 7            |                      |                      |                      |                   |           |
| LVTRAB             | 158,6±2,7            | 182,2±1,9            | 195,5±3,0            | -78,8±0,8         | 97,0±1,2  |
| RVPAP              | 119,4±1,7            | 151,8±1,4            | 167,3±1,4            | -80,9±0,6         | 106,3±1,8 |
| Expt. 8            |                      |                      |                      |                   |           |
| RVPAP              | 131,9±2,3            | 152,4±2,1            | 164,1±2,0            | -80,8±0,4         | 100,2±2,0 |
| LVTRAB             | 156,2±6,0            | 174,2±5,4            | 193,5±5,1            | -79,6±0,3         | 96,1±1,9  |
| Expt. 9            |                      |                      |                      |                   |           |
| RVPAP              | 120,9±2,6            | 152,3±2,8            | 166,9±3,5            | -82,3±0,7         | 123,5±3,2 |
| LVTRAB             | 152,1±2,5            | 174,2±3,2            | 187,0±3,6            | -79,2±0,4         | 116,9±2,5 |
| Expt. 10           |                      |                      |                      |                   |           |
| LVTRAB             | 126,8±3,2            | 151,6±2,7            | 165,1±2,8            | -79,9±0,7         | 98,0±2,4  |
| RVPAP <sub>1</sub> | 153,6±4,9            | 179,2±3,2            | 190,6±3,8            | -82,0±0,9         | 98,8±1,5  |
| RVPAP <sub>2</sub> | 152,7±5,3            | 172,5±2,7            | 190,9±3,5            | -83,7±1,8         | 94,3±0,9  |
| Expt. 11           |                      |                      |                      |                   |           |
| LVPAP              | 133,5±5,2            | 166,4±4,0            | 190,7±7,3            | -78,2±1,3         | 103,4±1,7 |
| RVTRAB             | 131,5±3,6            | 163,1±1,8            | 175,4±1,5            | -82,6±1,2         | 119,4±4,9 |
| RVPAP              | 164,6±5,1            | 197,2±4,1            | 208,2±3,9            | -82,6±0,8         | 109,1±1,9 |

Table 4. Means and standard errors of the mean of electrophysiological measurements. Abbreviations: APD- action potential duration; E<sub>m</sub>-membrane potential; APA-action potential amplitude; RVPAP-right ventricular papillary muscle; LVPAP- left ventricular papillary muscle; RVTRAB- right ventricular trabecula; LVTRAB-left ventricular trabecula.

heterogeneity between neighbouring cells of sympathectomized ventricles is as obvious as in control ventricles.

Chemical sympathectomy, therefore, does not abolish any of the forms of myosin ATPase staining heterogeneity to be found in the rabbit heart.

#### Electrophysiological Results

Cardiac electrophysiological properties have been found to show regional variations (Gulch, 1980). The experiments reported here were conducted to see if electrophysiological variations were correlated with regional differences in myosin ATPase staining. Eleven correlation experiments were carried out; in nine of these, electrophysiological recordings were taken from two pieces of cardiac muscle, in the remaining two experiments recordings were taken from three pieces of muscle. Mean values and standard errors of action potential durations (APD) at 50,75 and 90% of repolarisation, of resting membrane potentials and of action potential amplitudes are listed in Table 4. The statistical significance levels of the differences between these values in each piece of muscle are listed in Table 5.

I will describe first the results of Expts.1-9 (those which involved two muscle samples each). In Expts.1,4,5,6,7,8 and 9 recordings were made in a right ventricular papillary muscle and compared with recordings from: a left ventricular papillary muscle (Expts.1,5 and 6); a left ventricular trabecula (Expts.7,8 and 9) or a right ventricular trabecula (Expt.4). When action potential durations at 50,75 and 90% of repolarisation from the two pieces of muscle in each experiment were compared they were found to be

|         | APD <sub>50</sub> | APD <sub>75</sub> | APD <sub>90</sub> | E <sub>m</sub> | APA            |
|---------|-------------------|-------------------|-------------------|----------------|----------------|
| Expt,1  | <0,001            | <0,001            | <0,001            | N,S,           | N,S,           |
| Expt,2  | N,S,              | N,S,              | N,S,              | N,S,           | N,S,           |
| Expt,3  | <0,001            | <0,001            | <0,001            | N,S,           | N,S,           |
| Expt,4  | <0,001            | <0,001            | N,S,              | N,S,           | N,S,           |
| Expt,5  | <0,001            | <0,001            | <0,001            | N,S,           | N,S,           |
| Expt,6  | <0,001            | <0,001            | <0,001            | <0,05          | N,S,           |
| Expt,7  | <0,001            | <0,001            | <0,001            | N,S,           | <0,01          |
| Expt,8  | <0,001            | <0,001            | <0,001            | N,S,           | N,S,           |
| Expt,9  | <0,001            | <0,001            | <0,01             | <0,01          | N,S,           |
| Expt,10 | <0,001            | <0,001            | <0,001            | N,S,           | N,S, (1 vs 2)  |
|         | <0,001            | <0,001            | <0,001            | N,S,           | N,S, (1 vs 3)  |
|         | N,S,              | N,S,              | N,S,              | N,S,           | N,S, (2 vs 3)  |
| Expt,11 | N,S,              | N,S,              | N,S,              | N,S,           | <0,01 (1 vs 2) |
|         | <0,01             | <0,001            | <0,05             | <0,01          | N,S, (1 vs 3)  |
|         | <0,001            | <0,001            | <0,001            | N,S,           | <0,05 (2 vs 3) |

Table 5. Significance levels calculated using Student's t-test, significance was taken as  $p < 0.05$ .

significantly different in all cases ( $p < 0.001$  in 19 of the 21 cases). At every level of repolarisation, APD is shorter in the right ventricular papillary muscle than in any other muscle with which it is compared.

In experiments 6 ( $p < 0.05$ ) and 9 ( $p < 0.01$ ) resting membrane potential was significantly different between the two muscles and in experiment 7 the action potential amplitudes from the two muscles were significantly different ( $p < 0.01$ ). No significant differences were found between these parameters in any other experiments.

In summary, action potentials are longer in left ventricular papillary muscles and trabeculae of the right and left ventricles than in right ventricular papillary muscles, however, resting membrane potentials and action potential amplitudes are not, in general, significantly different in these muscles.

In experiments 2 and 3 recordings from two right ventricular papillary muscles were compared. In experiment 2 action potential durations from the two muscles were not significantly different at any of the stages measured. In experiment 3, however, the action potential durations at 50, 75 and 90% of repolarisation are significantly different ( $p < 0.001$  in each case). In neither experiment do resting membrane potentials or action potential amplitudes differ significantly between the two muscles.

In experiment 10 a statistical comparison of the recordings made in the two right ventricular papillary muscles, shows no significant differences between any of the parameters measured. However, a comparison between left ventricular trabecula recordings

and those of the first right ventricular papillary muscle (RVPAP<sub>1</sub>) shows that action potential durations at 50,75 and 90% of repolarisation are shorter in the trabecular muscle. No significant differences were found between the resting membrane potentials or the action potential amplitudes in these two muscles.

The comparison of the two right ventricular papillary muscles in experiment 10 agrees with that of experiment 2, however, finding shorter action potentials in the left ventricular trabecula differs from experiments 7,8 and 9 where right ventricular papillary muscle action potentials were found to be shorter.

No significant differences were found between action potential durations at 50,75 and 90% of repolarisation of left ventricular papillary muscle and right ventricular trabecula in experiment 11, nor was there any significant difference between the resting membrane potentials recorded in these muscles. However, action potential amplitudes were significantly smaller ( $p < 0.01$ ) in the left ventricular papillary muscle when compared to those of the right ventricular trabecula. When the recordings from these two muscles were compared with those from a right ventricular papillary muscle it was found that, in both cases, the action potentials of the right ventricular papillary muscle were significantly longer at all measurement points.

Action potentials from the right ventricular papillary muscles were not significantly different in amplitude from those of the left ventricular papillary muscle but were significantly smaller than those of the right ventricular trabecula. Resting membrane potentials were significantly greater in the right ventricular



muscle when compared with those of the left ventricular papillary muscle but not significantly different from those of the right ventricular trabecula.

For the purposes of summarising these results I would like to group together the left ventricular papillary muscles and trabeculae of both ventricles (the high chronic stress muscles) in group 1 and put right papillary muscles in group 2. In seven experiments action potentials of group 1 muscles were significantly longer than those of group 2 muscles, in two experiments action potentials of group 1 muscles were significantly shorter than those of group 2 muscles, in two experiments action potentials of two group 2 muscles were not significantly different whereas in one experiment they were significantly different. Resting membrane potentials and action potential amplitudes were not, in general, significantly different within or between the two groups.

#### Histochemical and Electrophysiological Results

Histochemical data (alkaline myosin ATPase densitometry) and electrophysiological data (action potential durations at 50, 75 and 90% of repolarisation) for each experiment are presented in histograms on facing pages for easy comparison. The electrophysiological results are presented as follows: where recordings from two pieces of muscle are to be compared, three sets of two histograms are shown. The uppermost set shows the distribution of action potential durations at 50% of repolarisation ( $APD_{50}$ ), the middle set shows the distribution of  $APD_{75}$  for each and the lower set the distribution of  $APD_{90}$ . Only the upper histograms of each set have their axes labelled as the same labels

apply to each histogram in the set. Where three pieces of muscle were compared, three sets of three histograms are shown.

The histochemical data for both (or all three) muscles used in an experiment appear in the same histogram. Readings from different muscles are shown in black, white or, where a third muscle was included, grey. Columns which appear in one shade contain readings from one muscle only, whereas columns which have differently shaded portions contain readings from two muscles. An example of this is seen in Fig.23, the column indicated by the arrow contains three readings; two black and one white i.e. this column contains two readings from the right ventricular papillary muscle and one from the left ventricular papillary muscle used in this experiment. Fibres which have stained relatively strongly for myosin ATPase activity appear to the right of the histogram and more weakly stained fibres to the left.

Comparing, in Fig.24, the distributions of APD at 50,75 and 90% of repolarisation in the two muscles used in this experiment, shows that all three are on average shorter in the right than in the left ventricular papillary muscle although there is considerable overlap of the distributions of  $APD_{75}$  and  $APD_{90}$ . In Fig.23, where the photometric data are shown for these muscles, there is also considerable overlap but the majority of readings from the right ventricular papillary muscle (black) lie to the right of those from the left ventricular papillary muscle (white) i.e. they have stained more strongly for myosin ATPase activity than the fibres of the left ventricular papillary muscle.

Similarly in experiments 4-9 (Figs.29-40) the action potential durations are shorter in the 'group 2' muscles than in the 'group 1' muscles and the photometry histograms show the fibres of 'group 2' muscles to the right of those of 'group 1' muscles.

In experiment 2, where two right ventricular papillary muscles were compared, the distributions of action potential duration at 50, 75 and 90% of repolarisation overlap almost completely (Fig.26). The photometry histogram (Fig. 25) shows that the distributions of readings from these muscles also overlap almost completely. The fibres of these muscles, therefore, have very similar action potentials and myosin ATPase activities. Although the numbers are smaller for one of the right ventricular papillary muscles in experiment 10 (RVPAP<sub>2</sub>, n=3; Figs.41 & 42) a similar overlap of APD and photometry histograms appears to occur.

In the above experiments, therefore, it appears that longer action potentials are strongly associated with less strong myosin ATPase staining and that muscles which have action potentials of similar durations also have similar myosin ATPase staining.

The remaining experiments confirm this association of long action potentials with low myosin ATPase staining intensity. In experiment 3, where two right ventricular papillary muscles were compared, the action potential durations (as seen in Fig.28) overlap very little. However, in contrast with experiments 2 and 10 where the myosin ATPase staining intensities overlapped almost completely, Fig.27a shows little overlap of staining intensities of the fibres from these two muscle samples; and as the black readings are from the second papillary muscle (RVPAP<sub>2</sub> in Fig.28) it appears

that again long action potentials are associated with low myosin ATPase staining intensity. This is as one would expect from experiments 1 and 4-9. Figs.27b and c show the two muscles from which the recordings were taken in this experiment. Fig.27b shows the muscle from which the black readings in Fig.27a were taken: the outer layers of this muscle have stained less darkly than the underlying layers. These outer-layer fibres are those from which electrophysiological recordings would have been taken. The white readings in Fig.27a were taken from the muscle in Fig.27c, where all fibres are uniformly dark. Although the location of these pale cells is rather unexpected, this only serves to make more convincing the fact that the association of long action potentials with pale fibres is again observed.

A very similar result is seen in experiment 11 (Figs.43 & 44), where the action potentials recorded in a right ventricular papillary muscle (Fig.44) are longer than those of two 'group 1' muscles (the two 'group 1' muscles seem to have similar action potential durations in Fig.44 and similar ranges of staining intensities in Fig.43). The staining intensity measurements shown in grey in Fig.43 were taken from the right ventricular papillary muscle. Fig.43 shows that these fibres were paler than those of the other two muscles used in this experiment. Again the association of long action potentials with pale fibres is observed in unexpected circumstances.

On just one occasion in experiment 10 this association was not found. Part of experiment 10 has already been described i.e. the comparison of the two right ventricular papillary muscles. One part

of the experiment remains to be described i.e. the comparison of a left ventricular trabecula with right ventricular papillary muscle. Fig.42 shows that the action potentials of the left ventricular trabecula were shorter than those of the two right ventricular papillary muscles. However, unlike all the other experiments in which action potential durations differed, the shorter action potentials were here associated with pale fibres i.e. the readings which appear white in Fig.41 were taken from the left ventricular trabecula.

With this one unexplained exception, it appears that long action potentials in rabbit cardiac muscle are associated with low myosin ATPase activity and short action potentials with high myosin ATPase activity, demonstrated after alkaline pre-treatment.

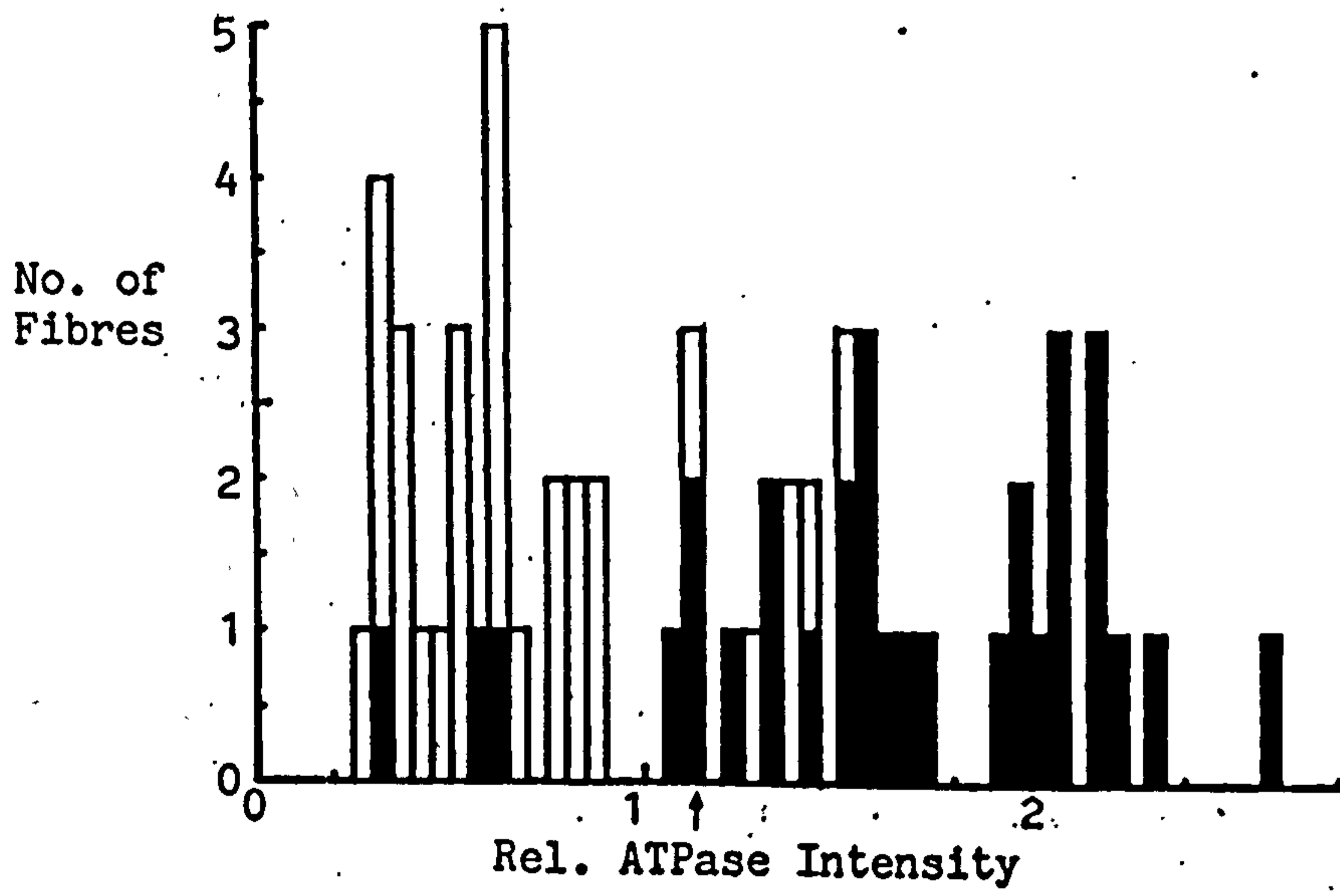


Fig.23 Histogram of photometric readings taken from right (black) and left (white) ventricular papillary muscles stained for myosin ATPase activity after alkaline pre-treatment.

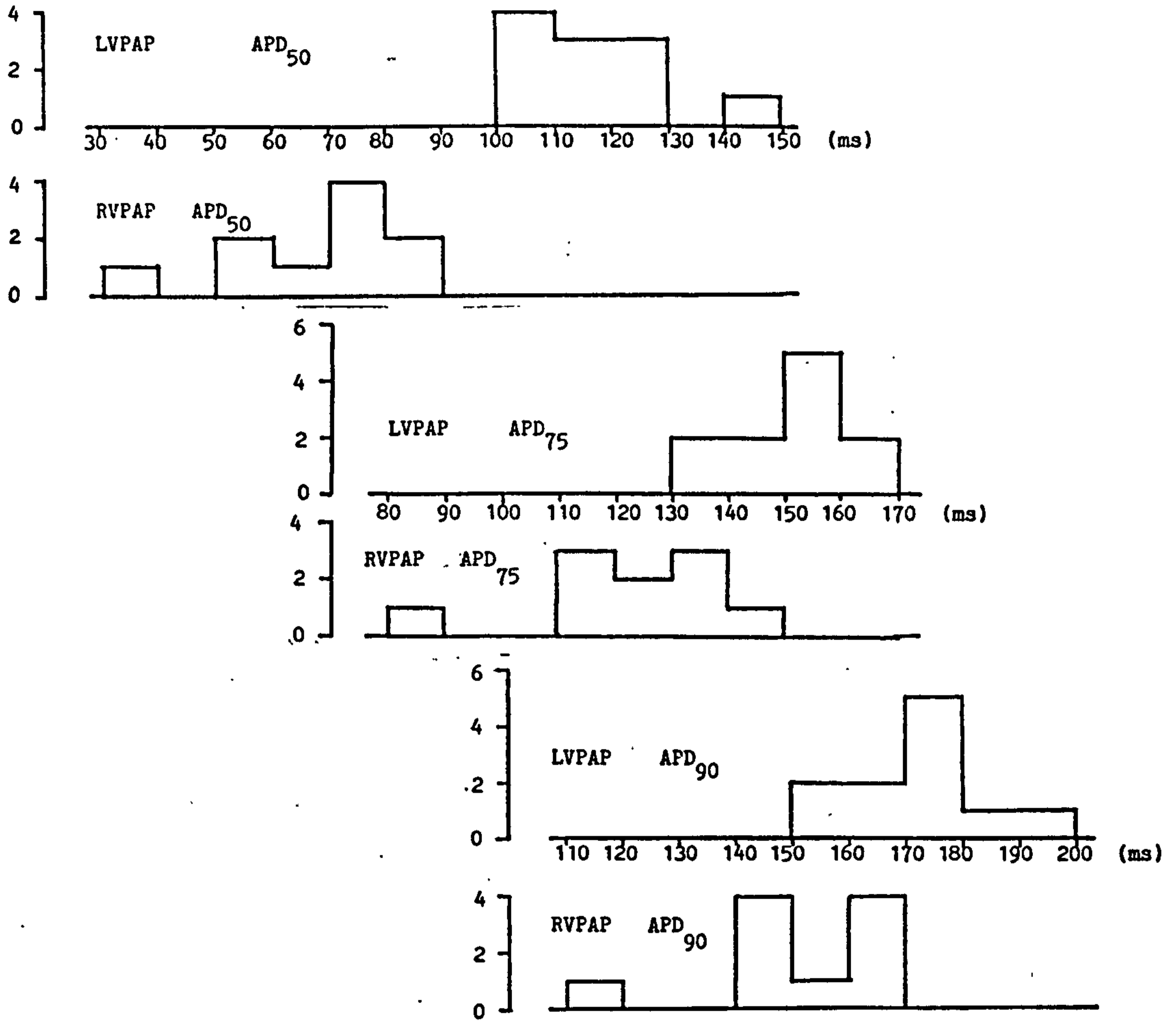


Fig.24 Histograms of action potential duration at 50% (APD<sub>50</sub>), 75% (APD<sub>75</sub>) and 90% (APD<sub>90</sub>) of repolarisation measured in the muscles featured in Fig.23.

Vertical axes are numbers of recordings.

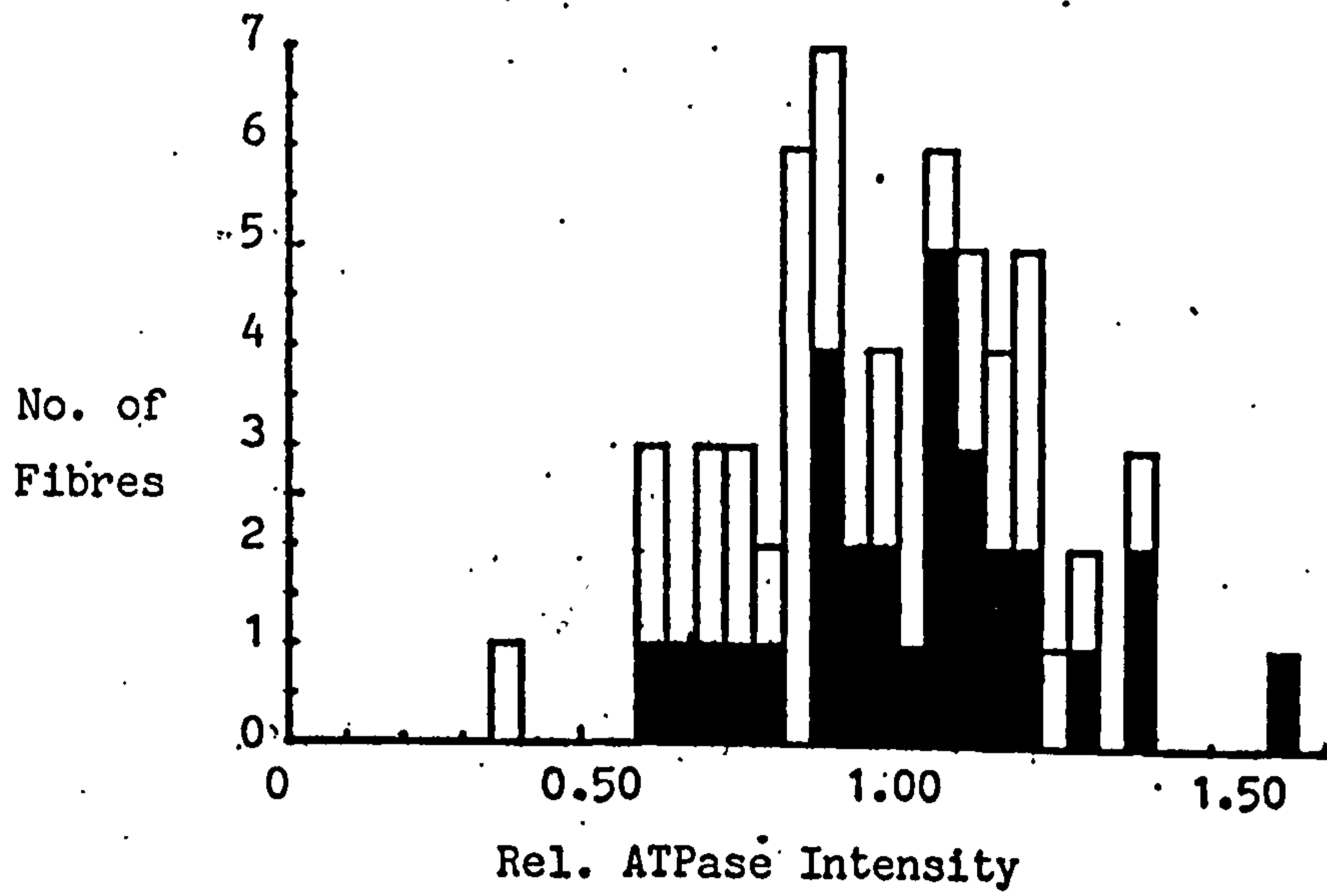


Fig.25 Histogram of photometric readings taken from two right ventricular papillary muscles stained for myosin ATPase activity after alkaline pre-treatment.



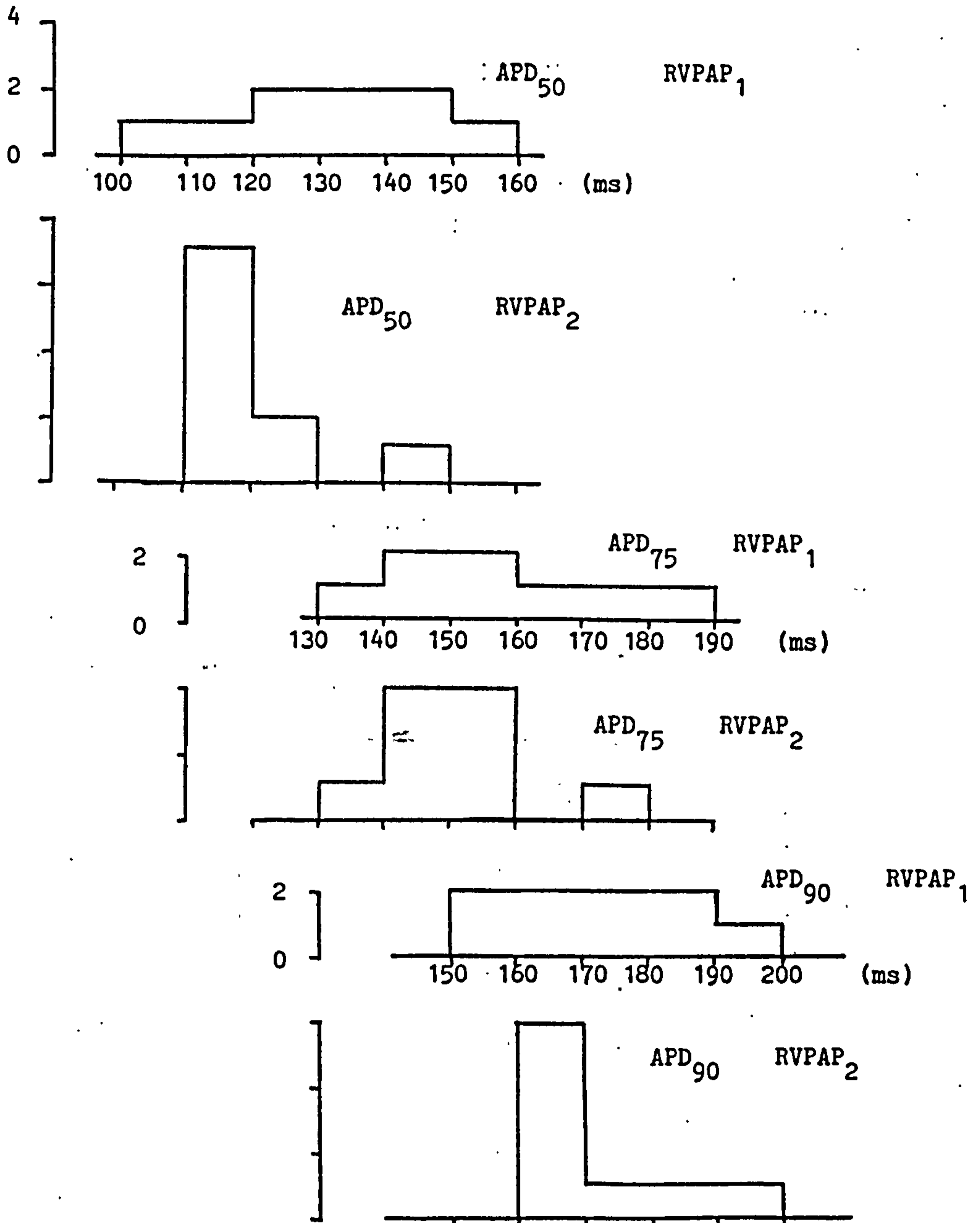


Fig.26 Histograms of action potential duration at 50%, 75% and 90% of repolarisation measured in the muscles featured in Fig.25.

Vertical axes are numbers of recordings.

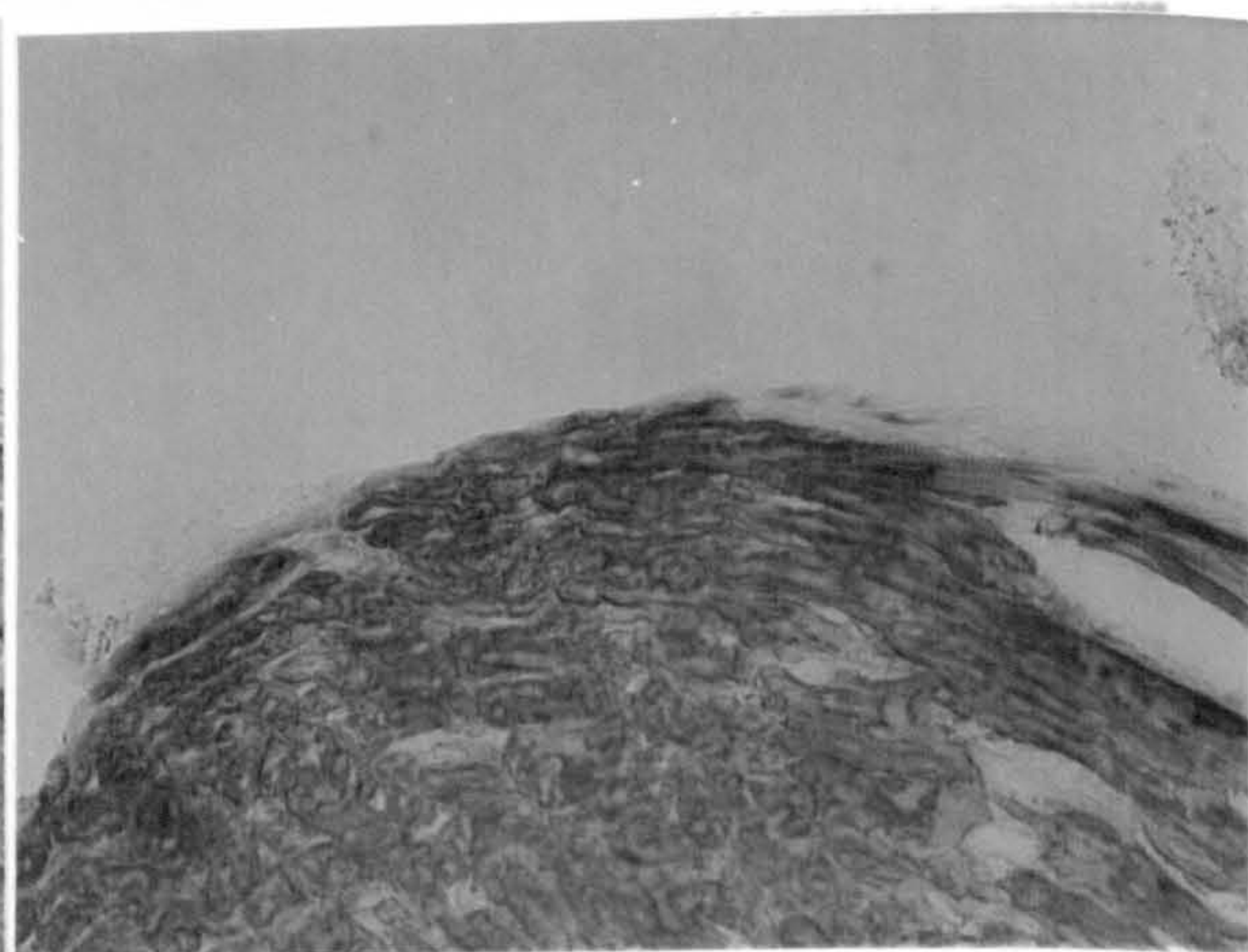
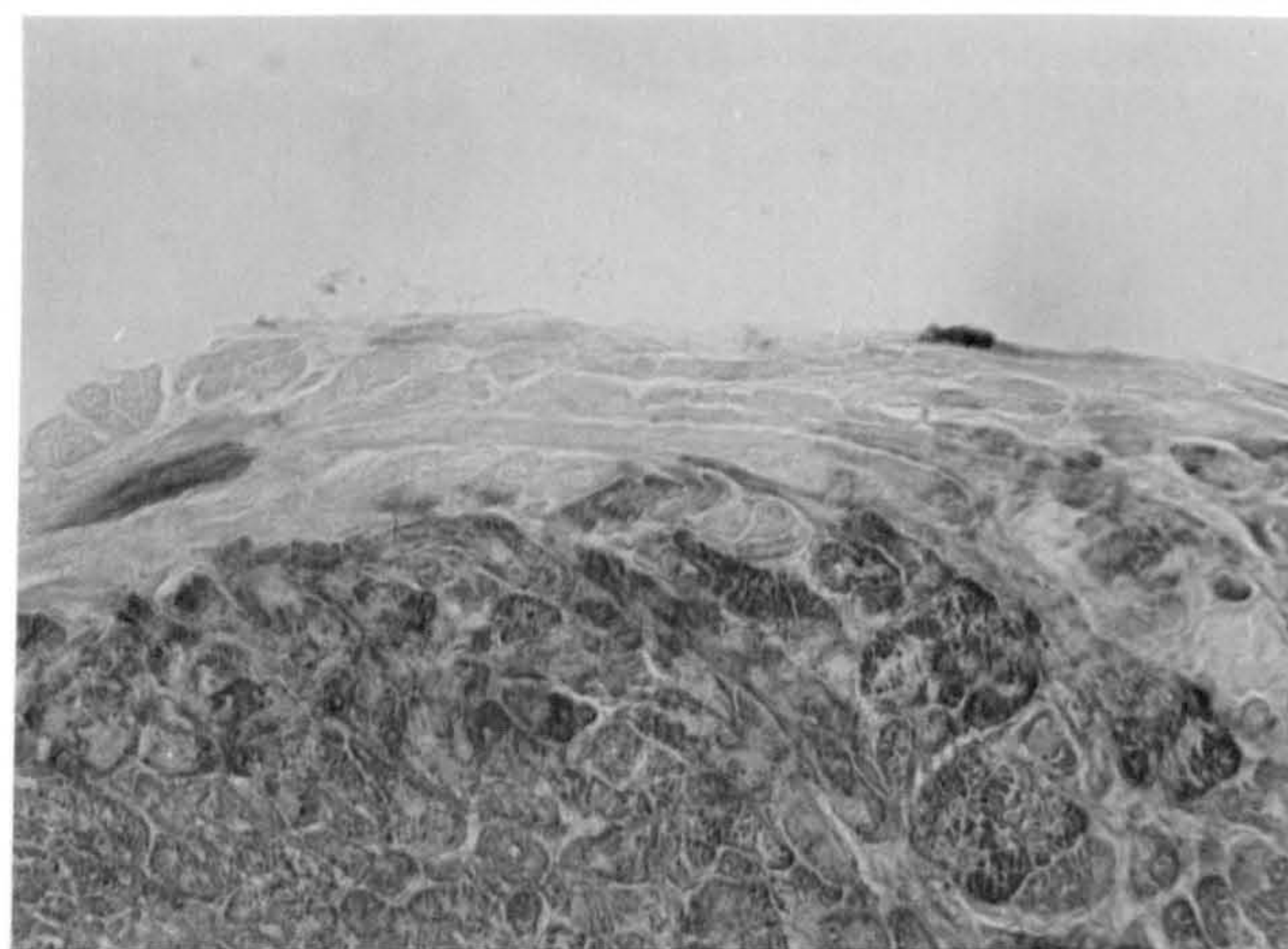
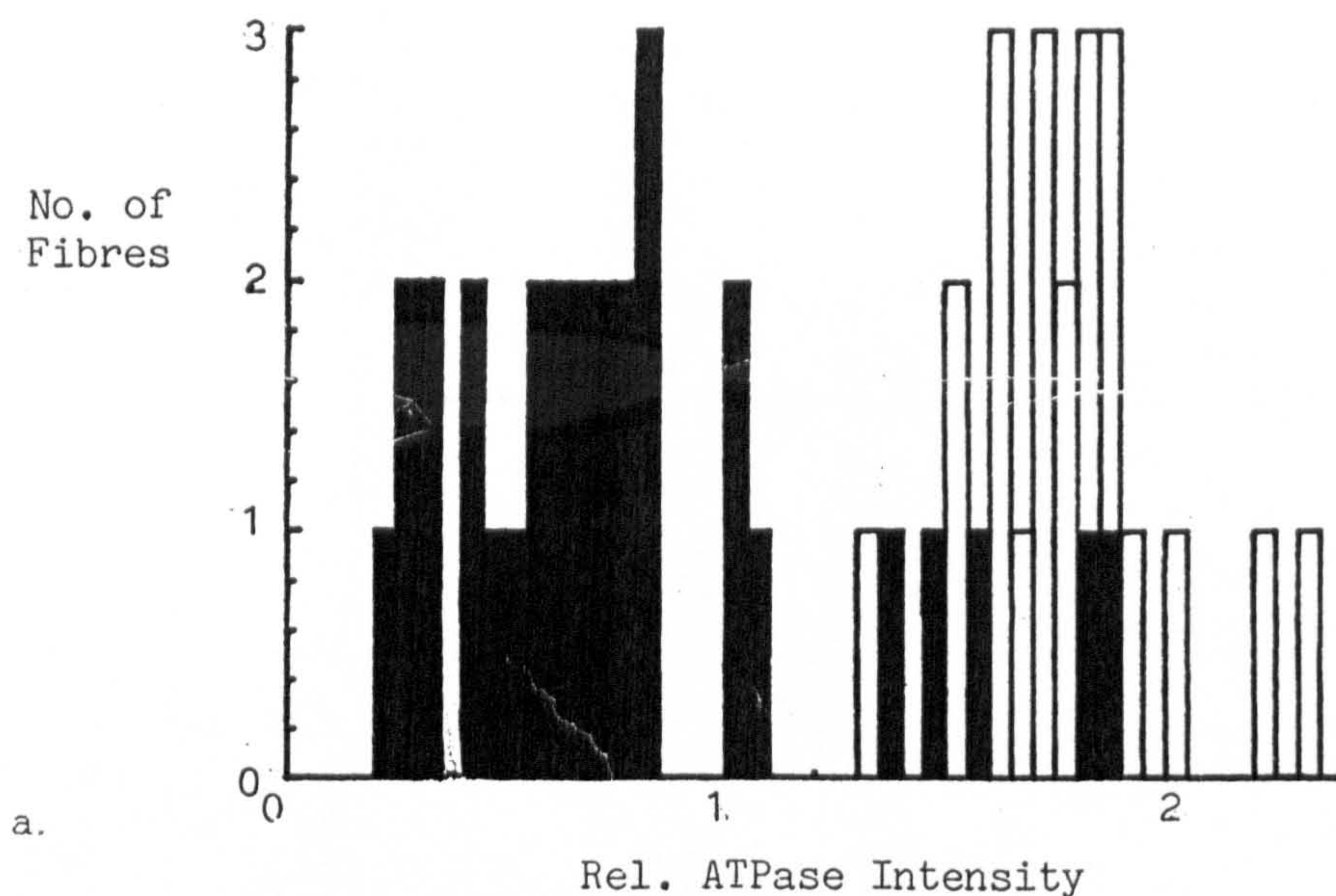


Fig.27a. Histogram of photometric readings taken from two right ventricular papillary muscles stained for myosin ATPase activity after alkaline pre-treatment. The readings shown in black correspond to those from RVPAP<sub>2</sub>, those shown in white correspond to those from RVPAP<sub>1</sub> of Fig.28.

b. Right ventricular papillary muscle (RVPAP<sub>2</sub>). The outer layer of fibres correspond to the black readings in Fig.27a.

c. Right ventricular papillary muscle (RVPAP<sub>1</sub>). The fibres in this muscle correspond to the white readings in Fig.27a.

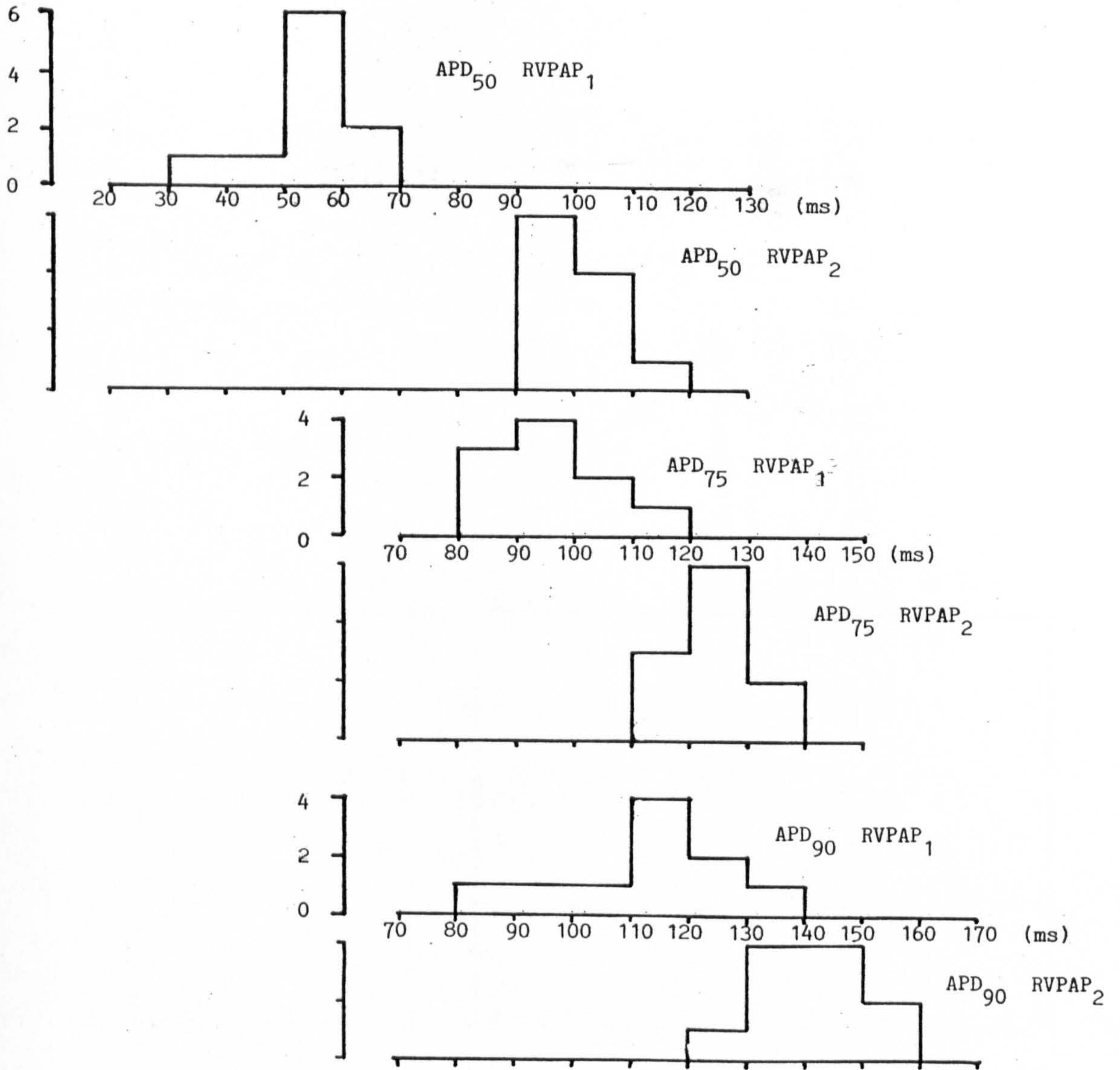


Fig.28 Histograms of action potential duration at 50%, 75% and 90% of repolarisation measured in the muscles featured in Fig.27a.

Vertical axes are numbers of recordings.

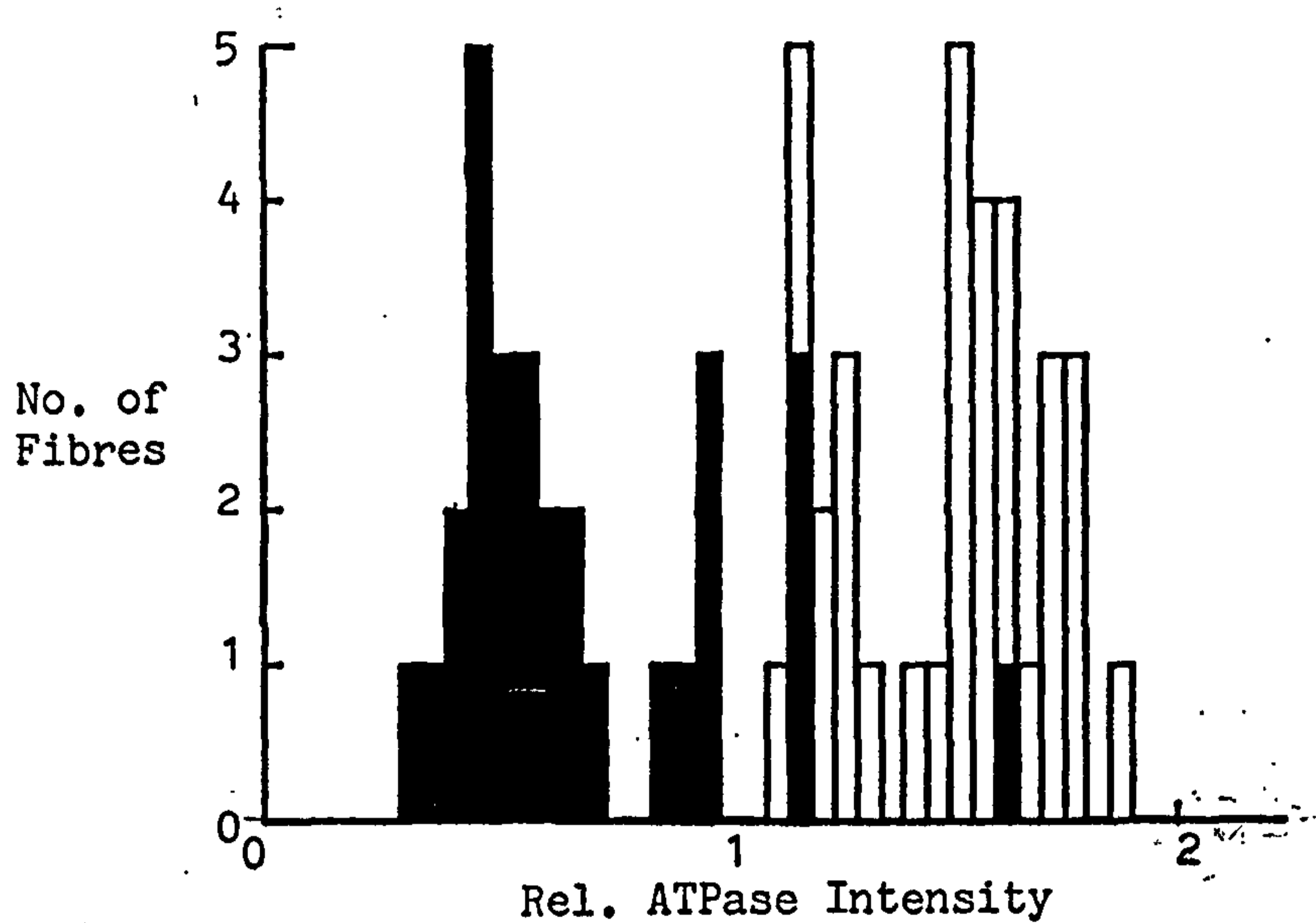


Fig.29 Histograms of photometric readings taken from a right ventricular trabecula (black) and a right ventricular papillary muscle (white) stained for myosin ATPase activity after alkaline pre-treatment.

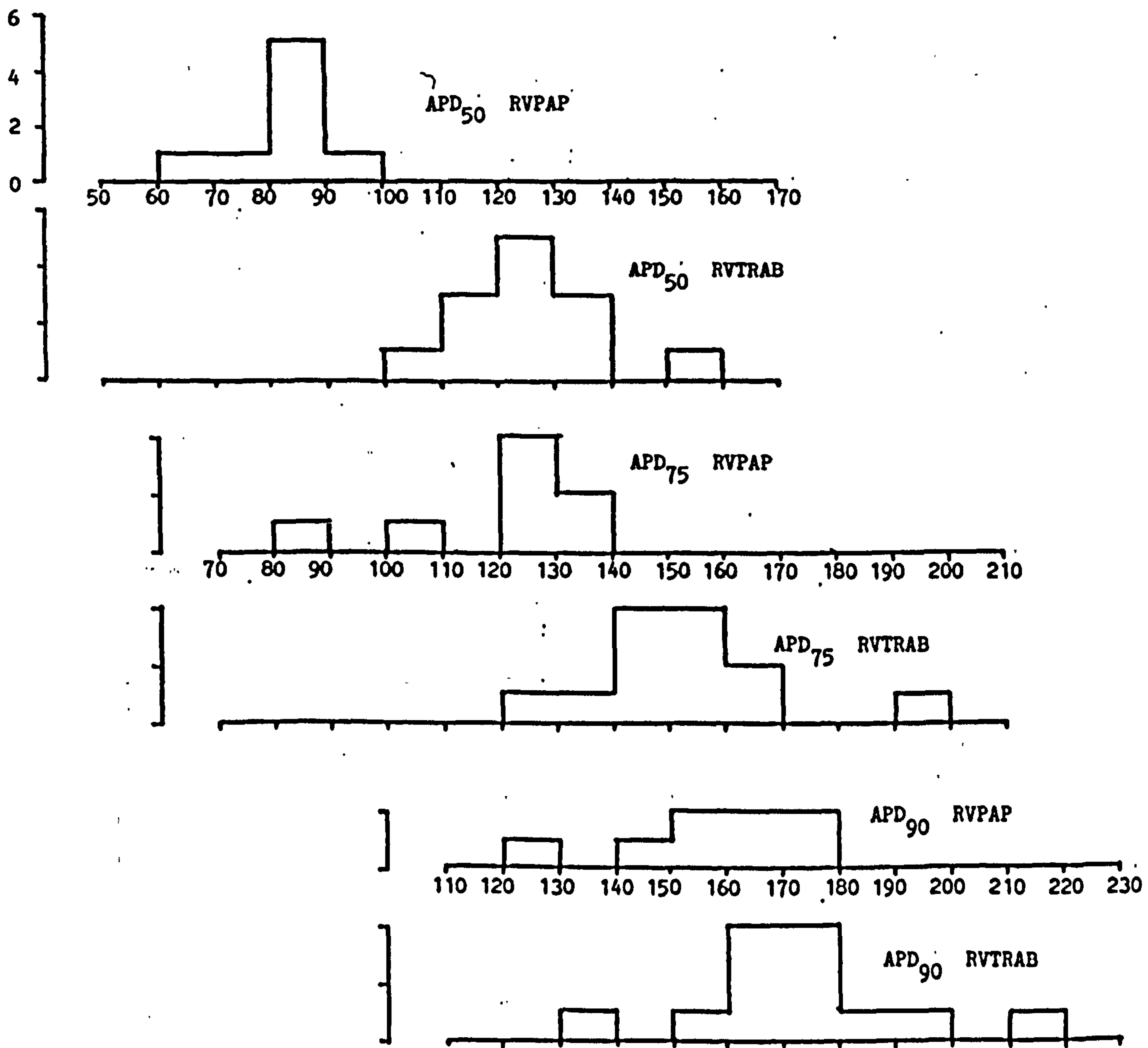


Fig.30 Histograms of action potential duration at 50%, 75% and 90% of repolarisation measured in the right ventricular papillary muscle (RVPAP) and the right ventricular trabecula (RVTRAB) featured in Fig.29.

Vertical axes are numbers of recordings.

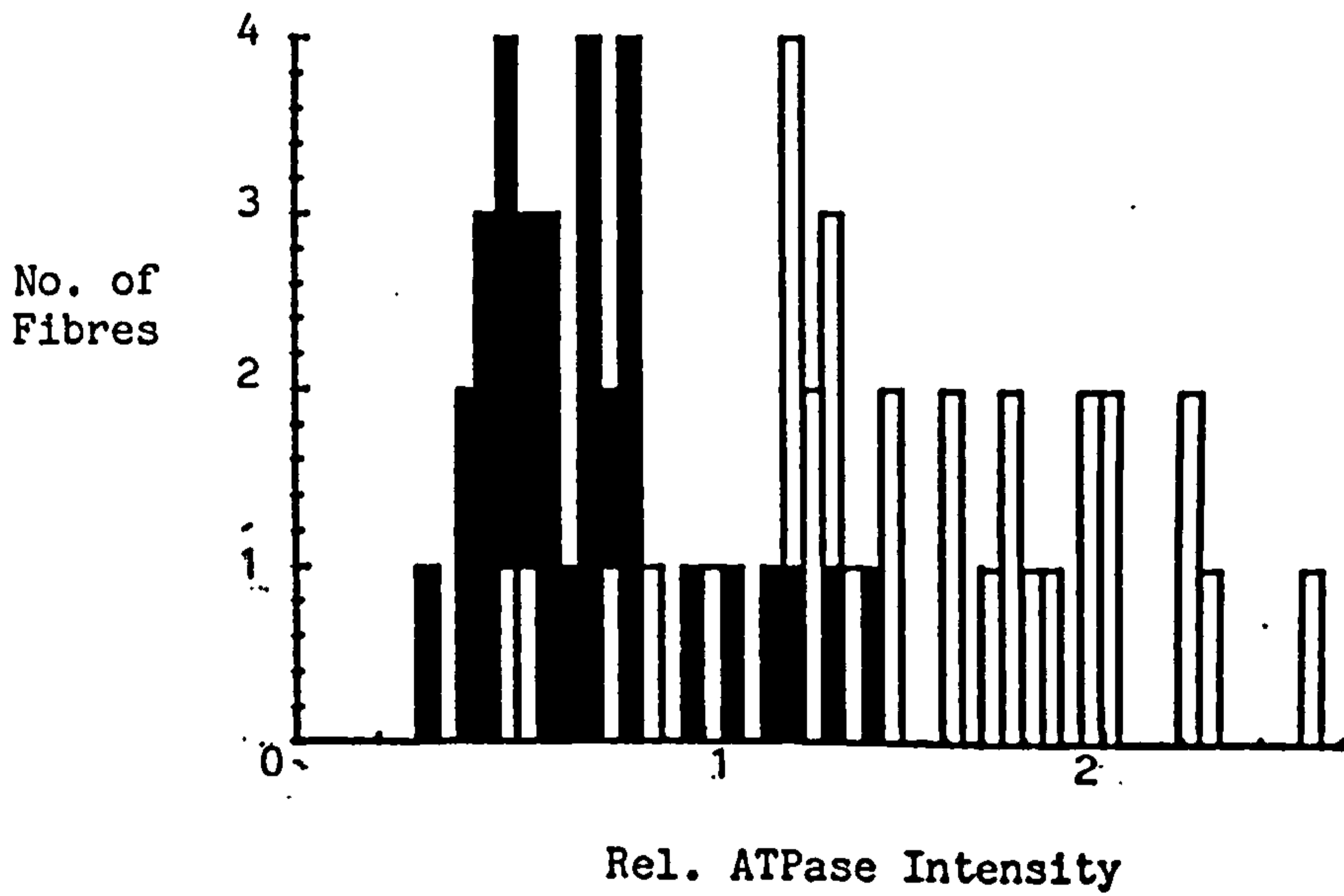


Fig.31 Histogram of photometric readings taken from a right ventricular (white) and a left ventricular (black) papillary muscle stained for myosin ATPase activity after alkaline pre-treatment.

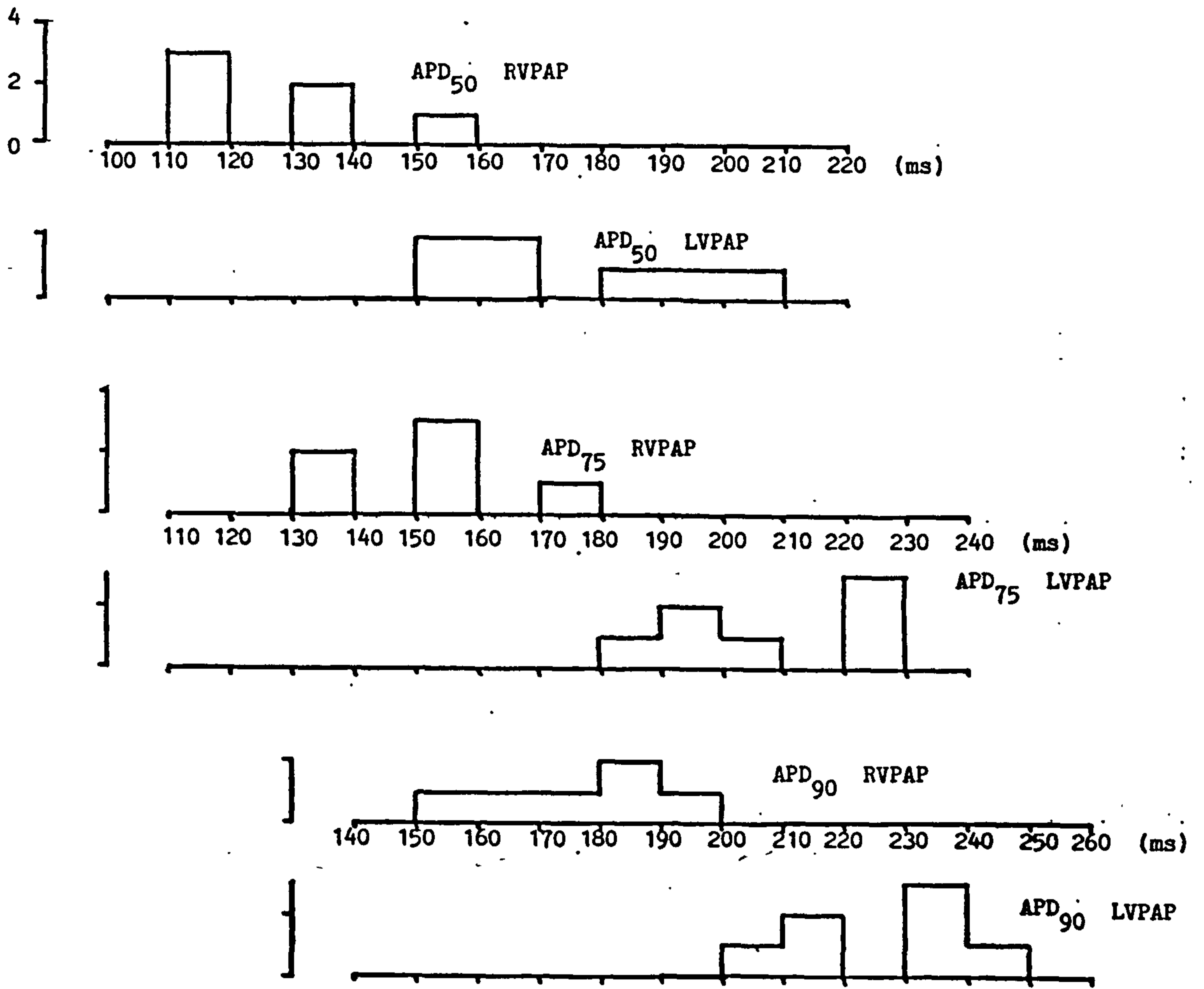


Fig.32 Histograms of action potential duration at 50%, 75% and 90% of repolarisation measured in the right ventricular (RVPAP) and left ventricular (LVPAP) papillary muscles featured in Fig. 31.

Vertical axes are numbers of recordings.

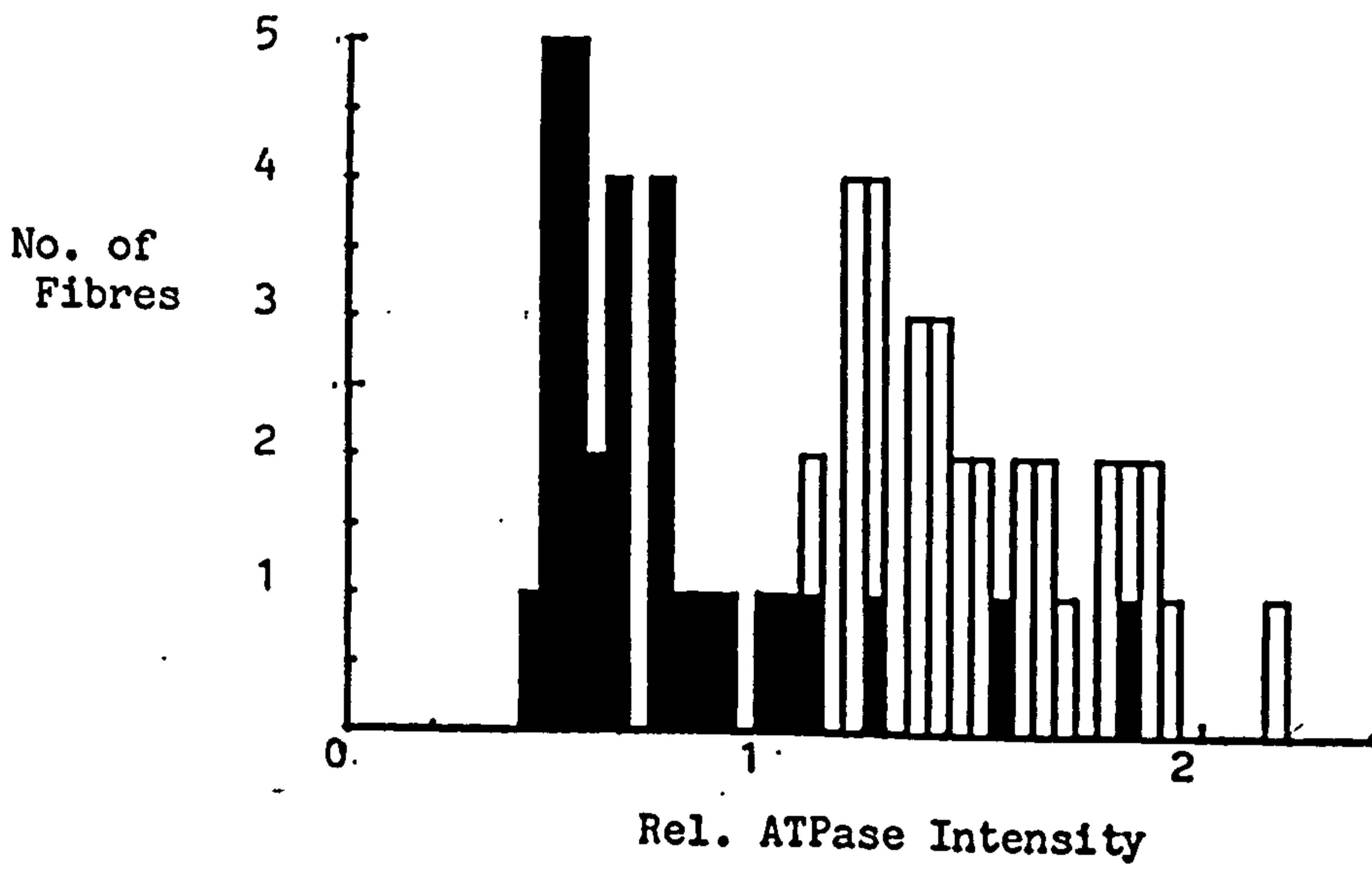


Fig.33 Histogram of photometric readings taken from a right ventricular (white) and a left ventricular (black) papillary muscle stained for myosin ATPase activity after alkaline pre-treatment.



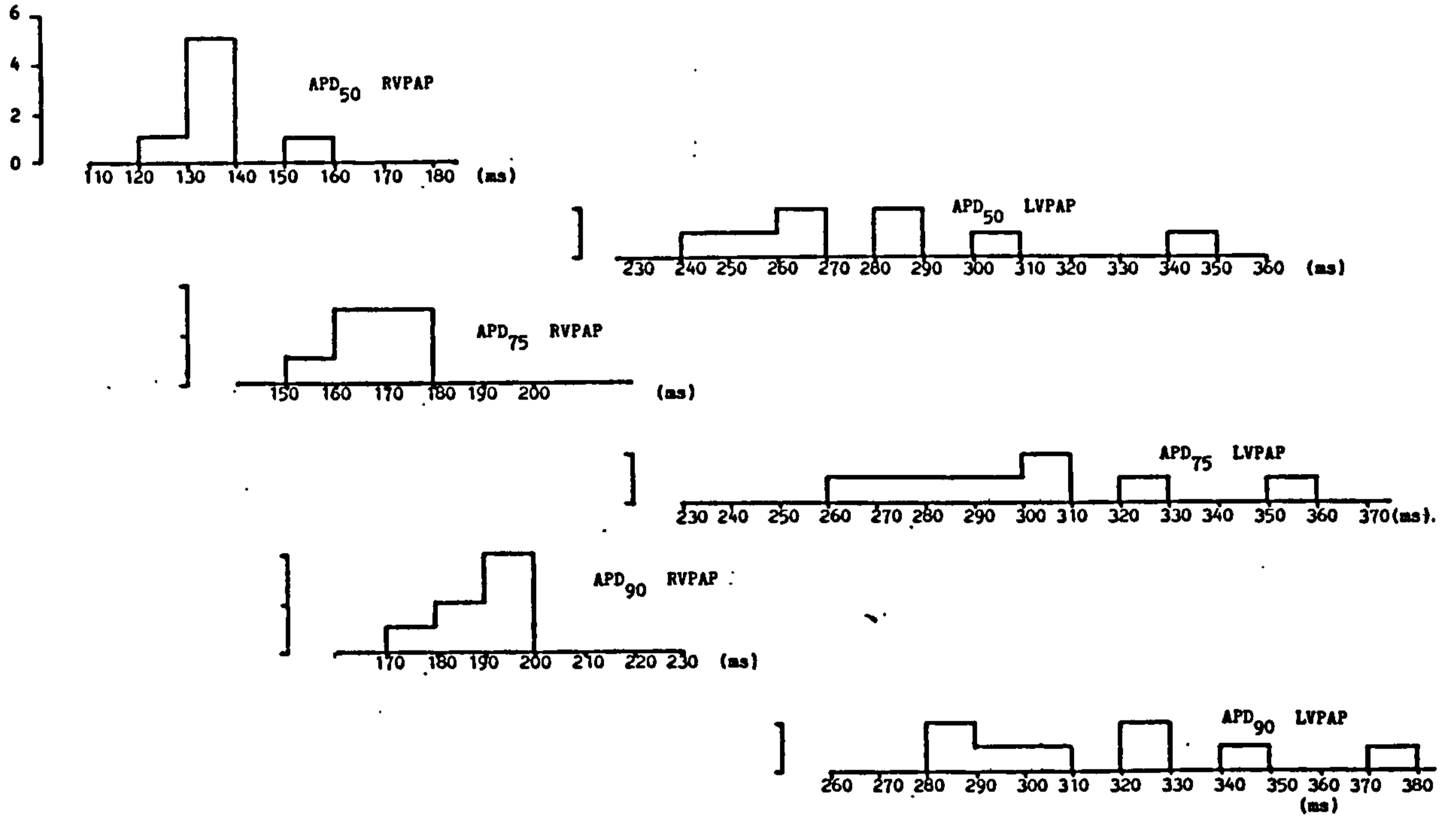


Fig.34 Histograms of action potential duration at 50%, 75% and 90% of repolarisation measured in the right ventricular (RVPAP) and left ventricular (LVPAP) papillary muscles featured in Fig.33.

Vertical axes are numbers of recordings:

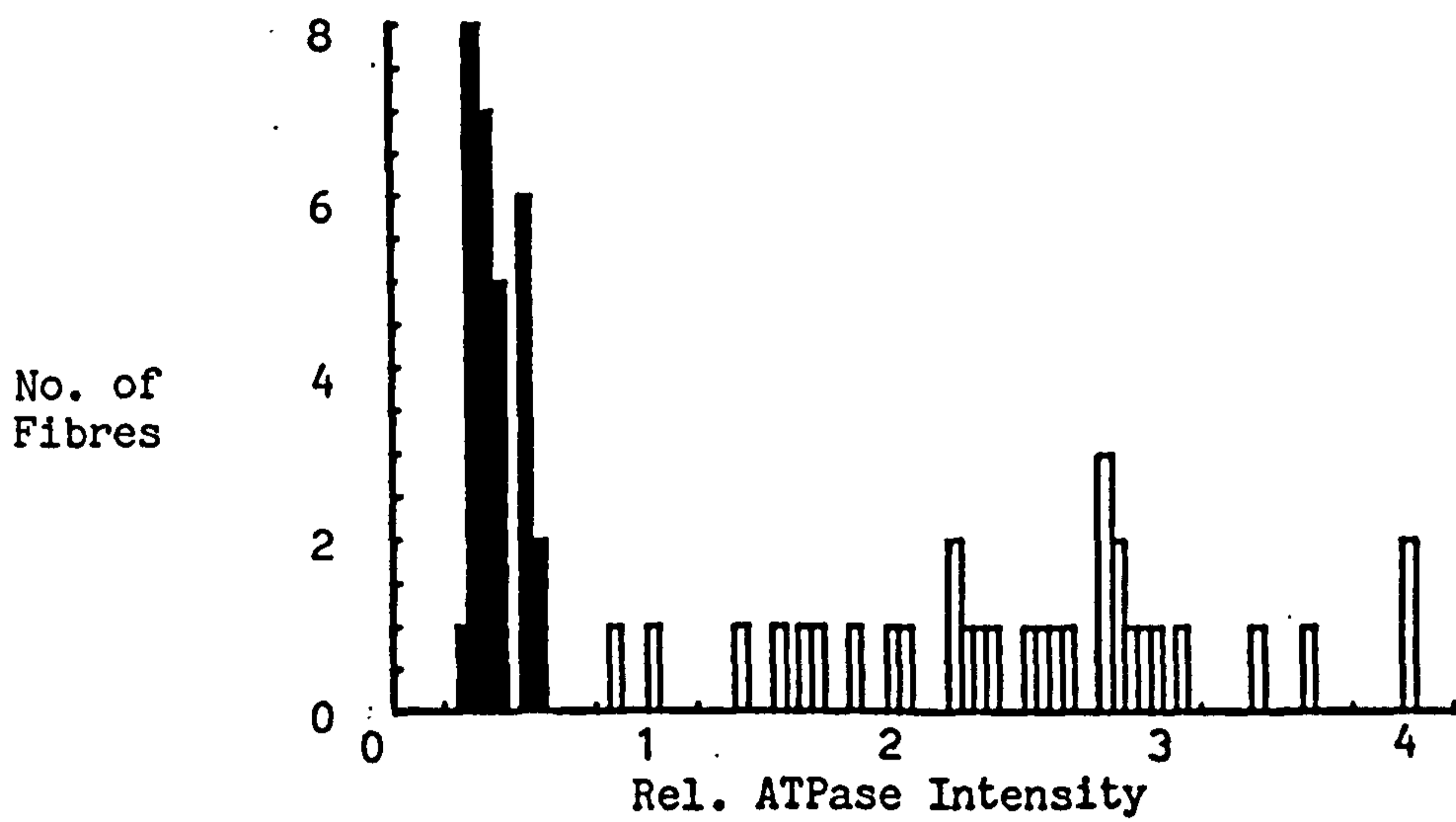


Fig.35 Histogram of photometric readings taken from a right ventricular papillary muscle (white) and a left ventricular trabecula (black) stained for myosin ATPase activity after alkaline pre-treatment.

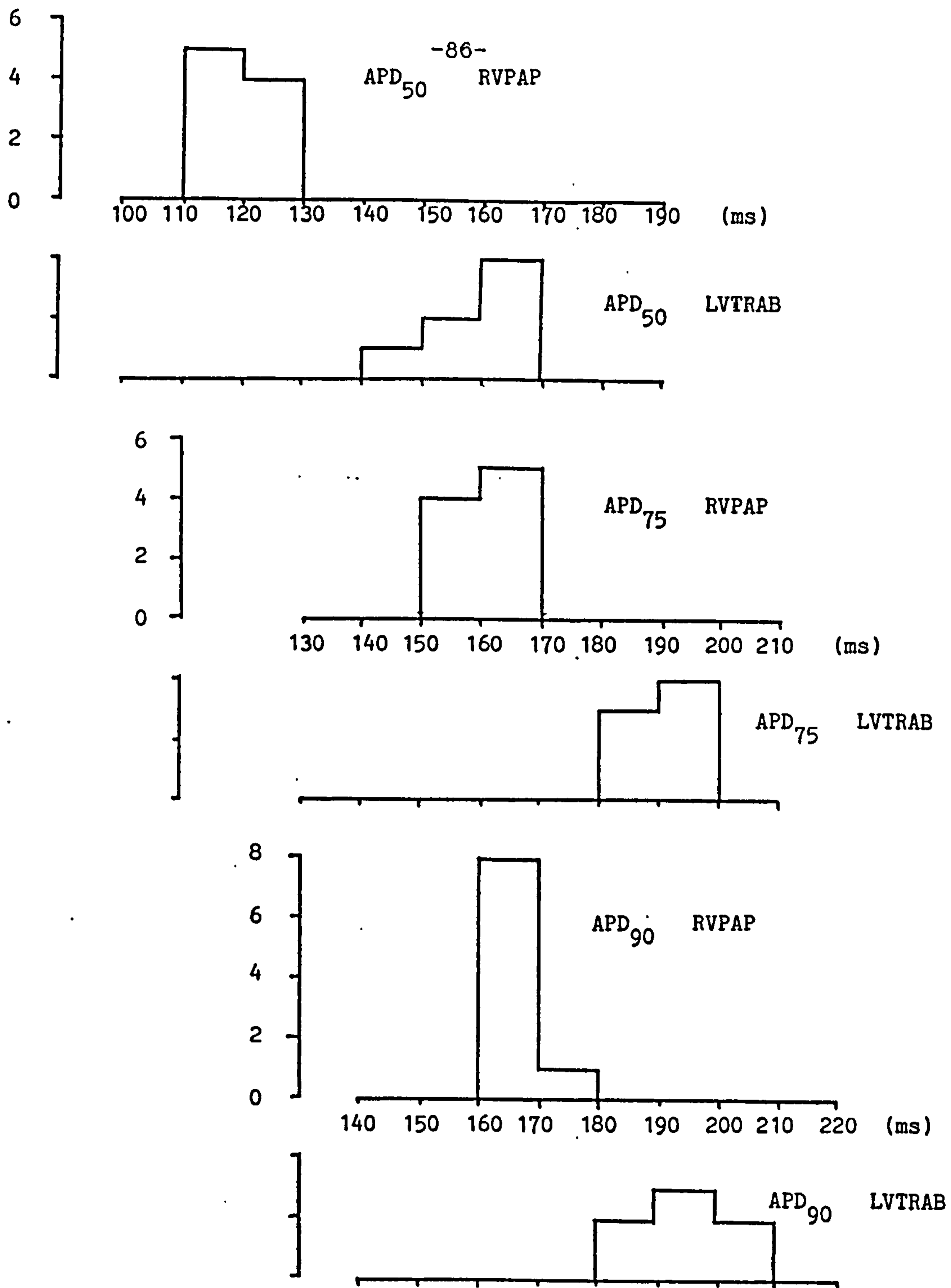


Fig.36 Histograms of action potential duration at 50%, 75% and 90% of repolarisation measured in the right ventricular papillary muscle (RVPAP) and the left ventricular trabecula (LVTRAB) featured in Fig.35.

Vertical axes are numbers of recordings.

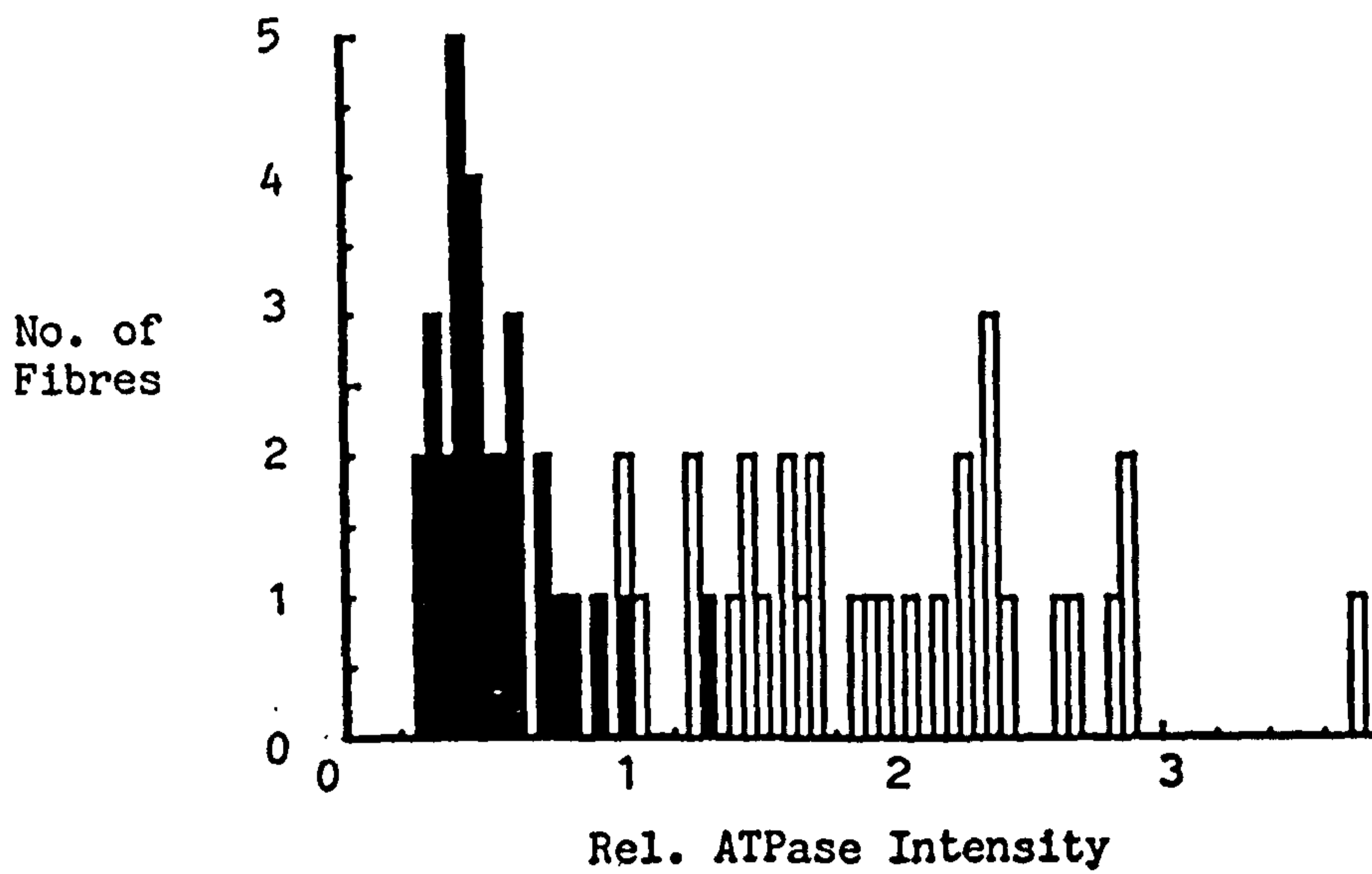


Fig.37 Histogram of photometric readings taken from a right ventricular papillary muscle (white) and a left ventricular trabecula (black) stained for myosin ATPase activity after alkaline pre-treatment.

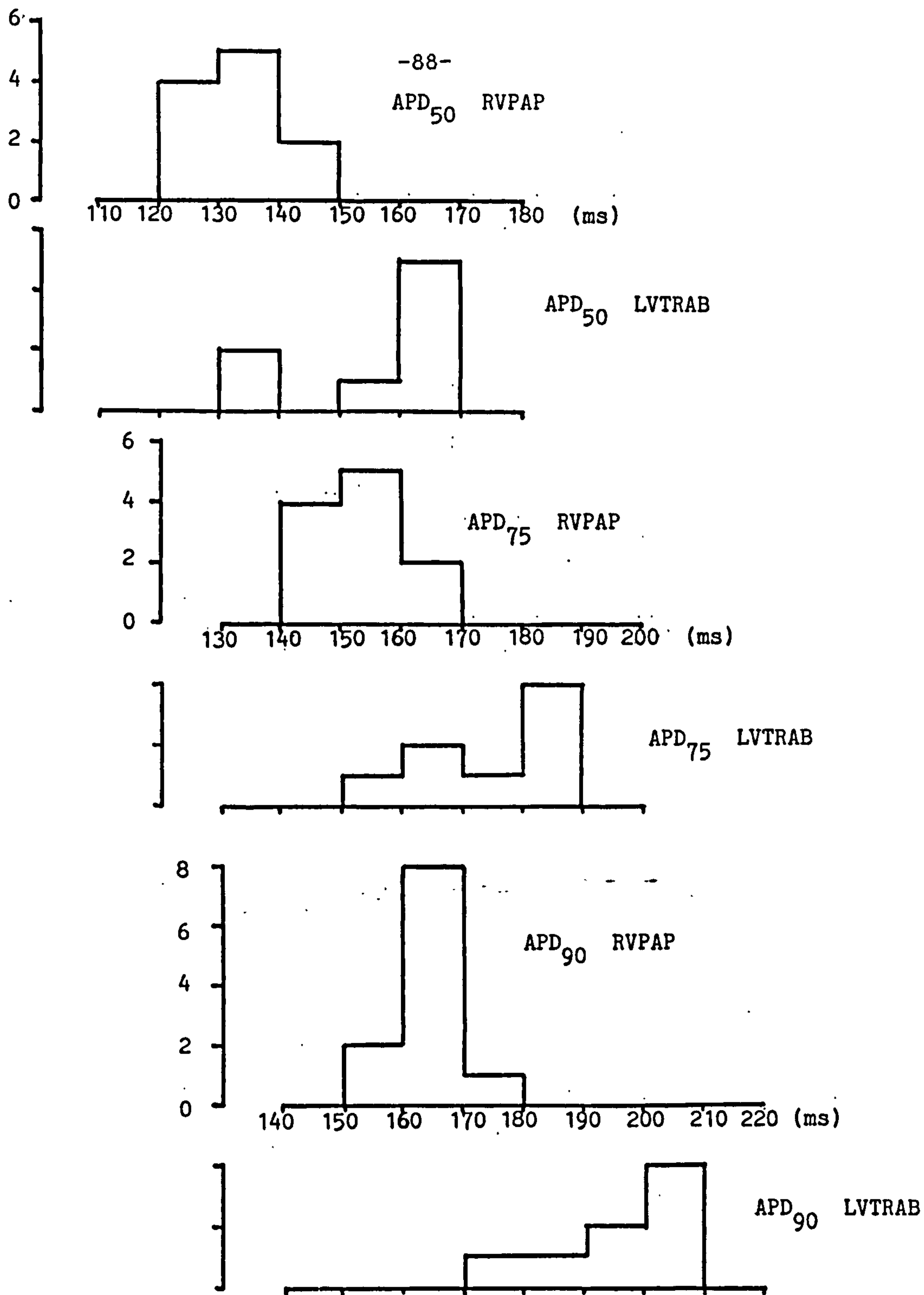


Fig.38 Histograms of action potential duration at 50%, 75% and 90% of repolarisation measured in the right ventricular papillary muscle (RVPAP) and the left ventricular trabecula (LVTRAB) featured in Fig.37.

Vertical axes are numbers of recordings.

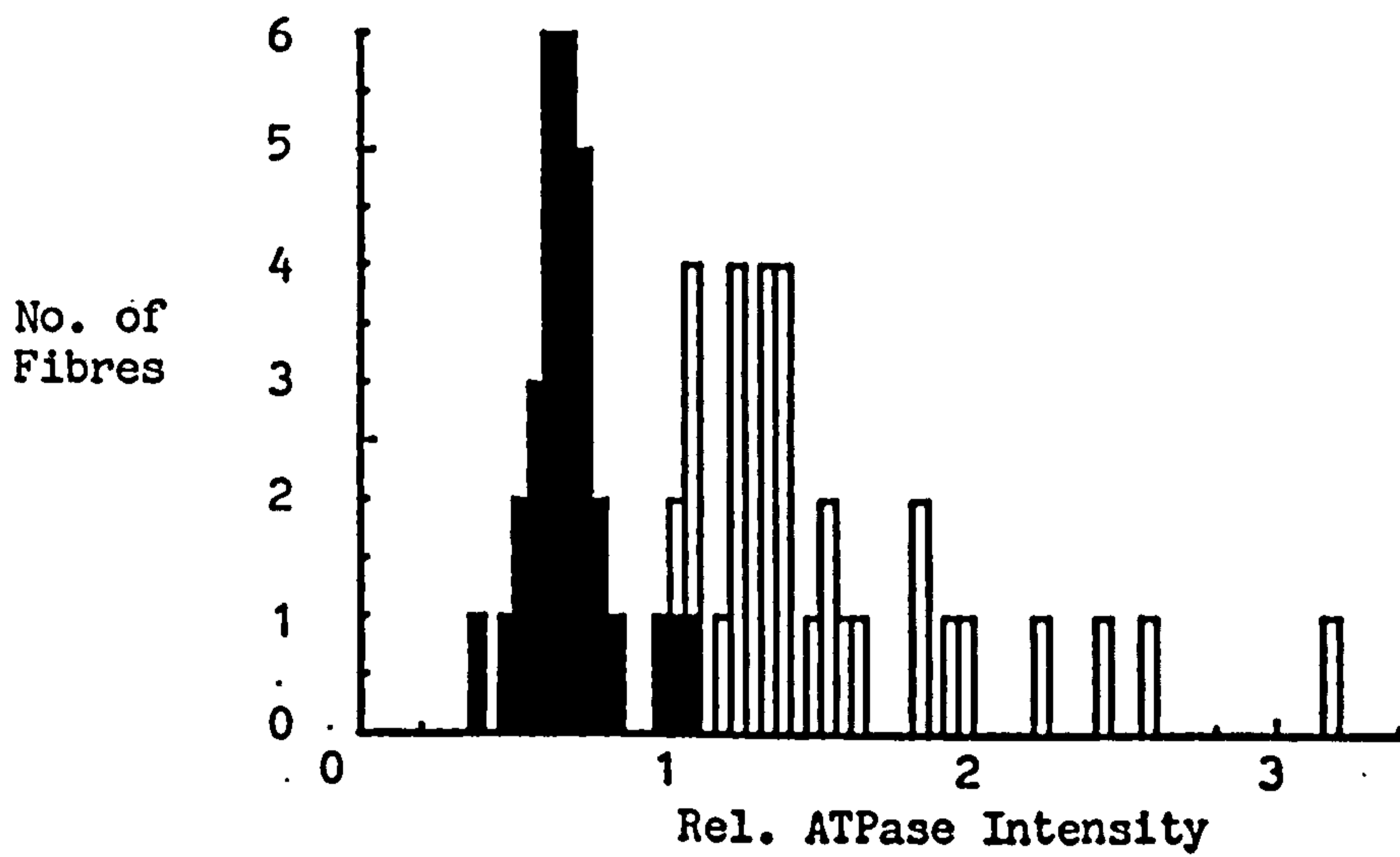


Fig.39 Histogram of photometric readings taken from a right ventricular papillary muscle (white) and a left ventricular trabecula (black) stained for myosin ATPase activity after alkaline pre-treatment.

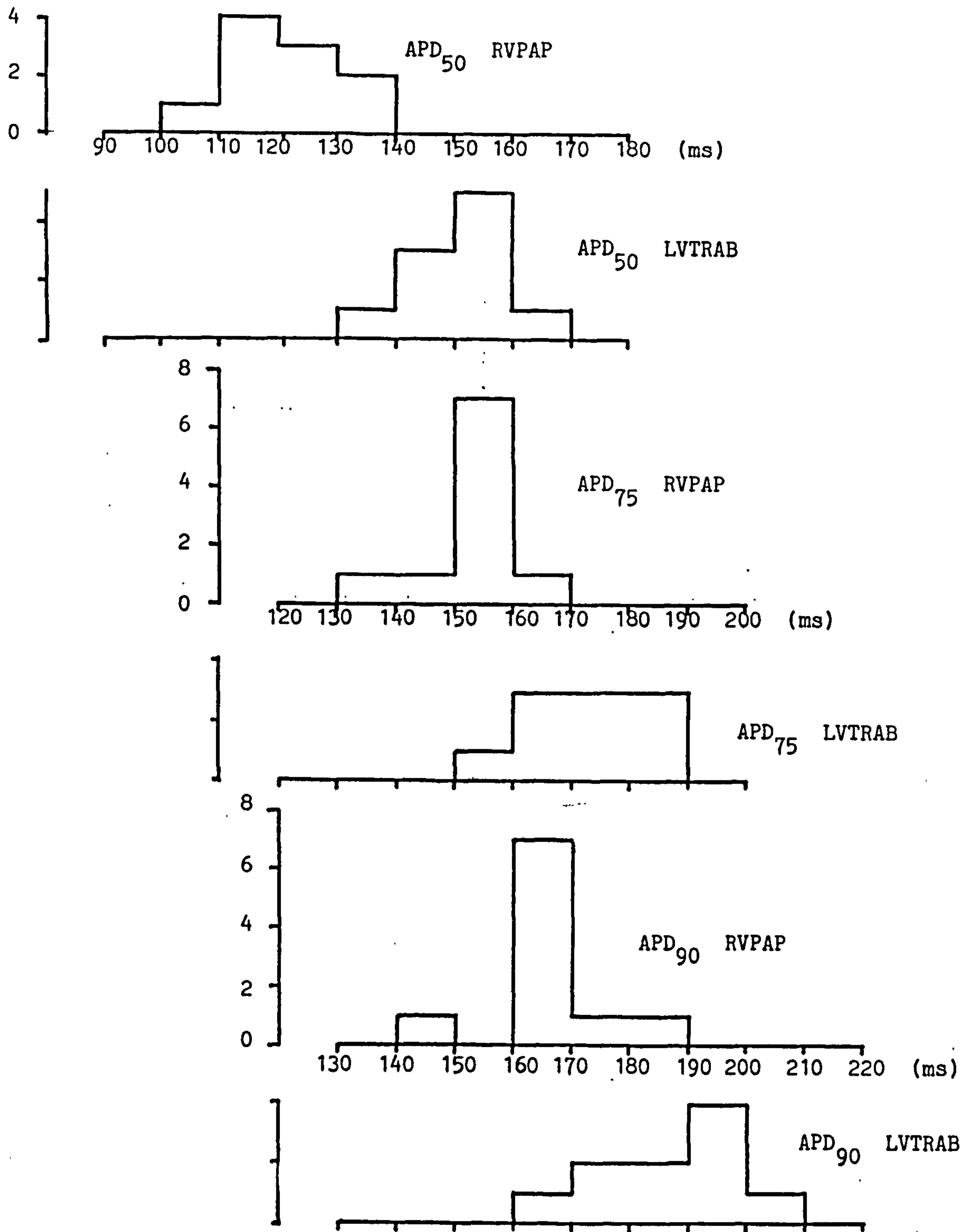


Fig.40 Histograms of action potential duration at 50%, 75% and 90% of repolarisation measured in the right ventricular papillary muscle (RVPAP) and left ventricular trabecula (LVTRAB) featured in Fig.39.

Vertical axes are numbers of recordings.

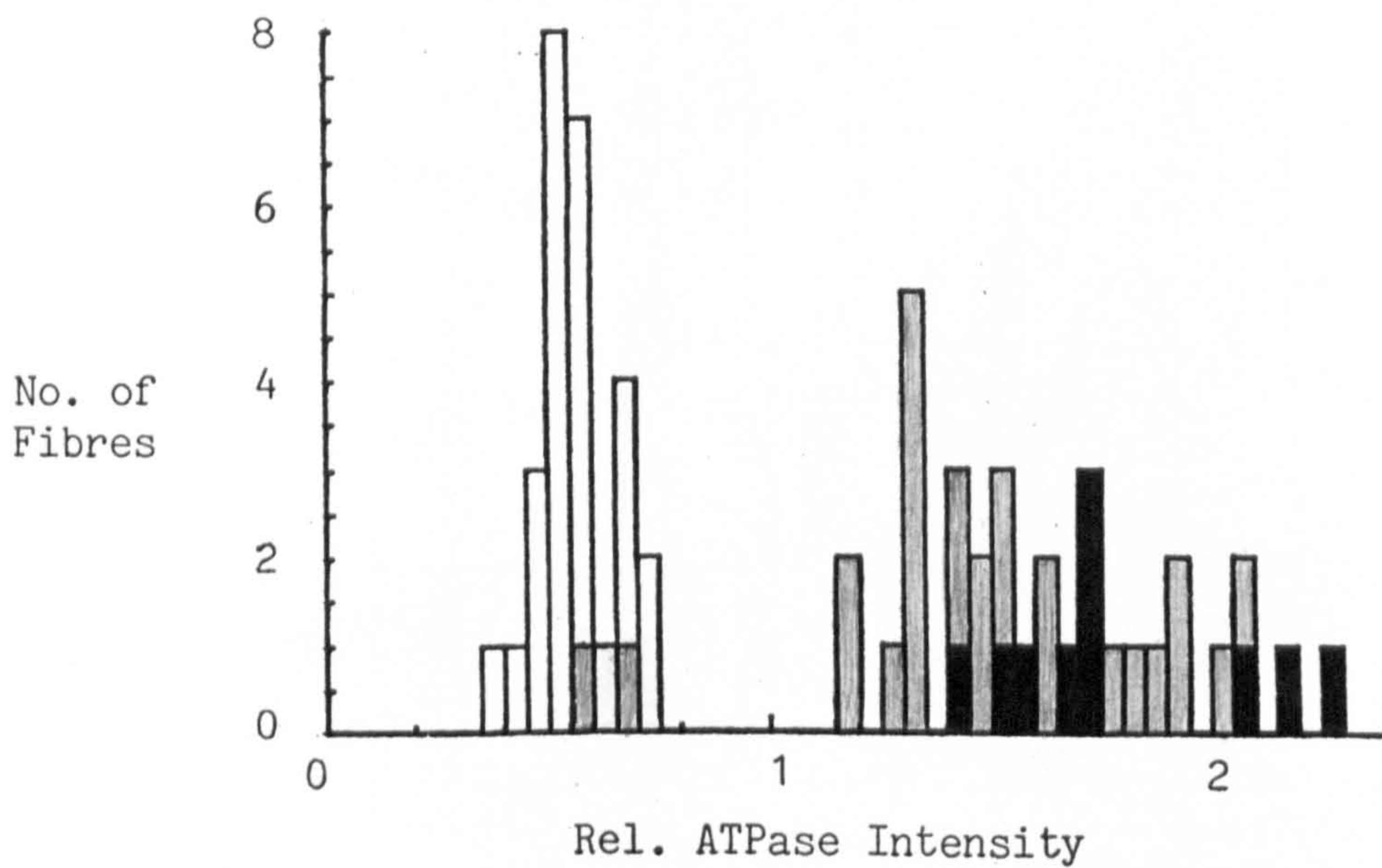


Fig.41 Histogram of photometric readings taken from two right ventricular papillary muscles (grey and black) and a left ventricular trabecula (white) stained for myosin ATPase activity after alkaline pre-treatment.

RVPAP<sub>1</sub> readings are shown in grey, RVPAP<sub>2</sub> readings in black.



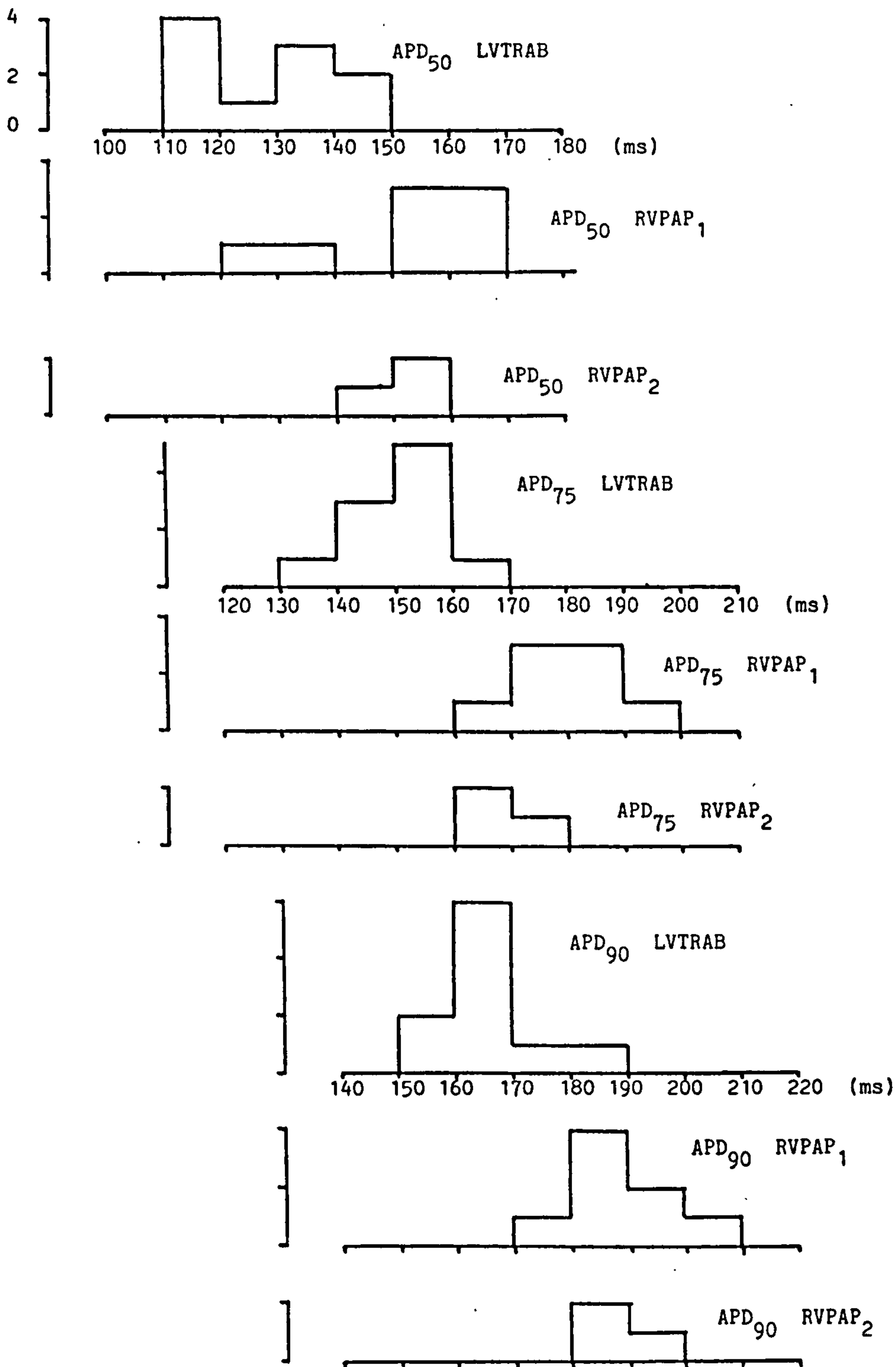


Fig.42 Histograms of action potential duration at 50%, 75% and 90% of repolarisation measured in the left ventricular trabecula (LVTRAB) and the two right ventricular papillary muscles (grey readings- RVPAP<sub>1</sub>; black readings- RVPAP<sub>2</sub>) featured in Fig.41.

Vertical axes are numbers of recordings.

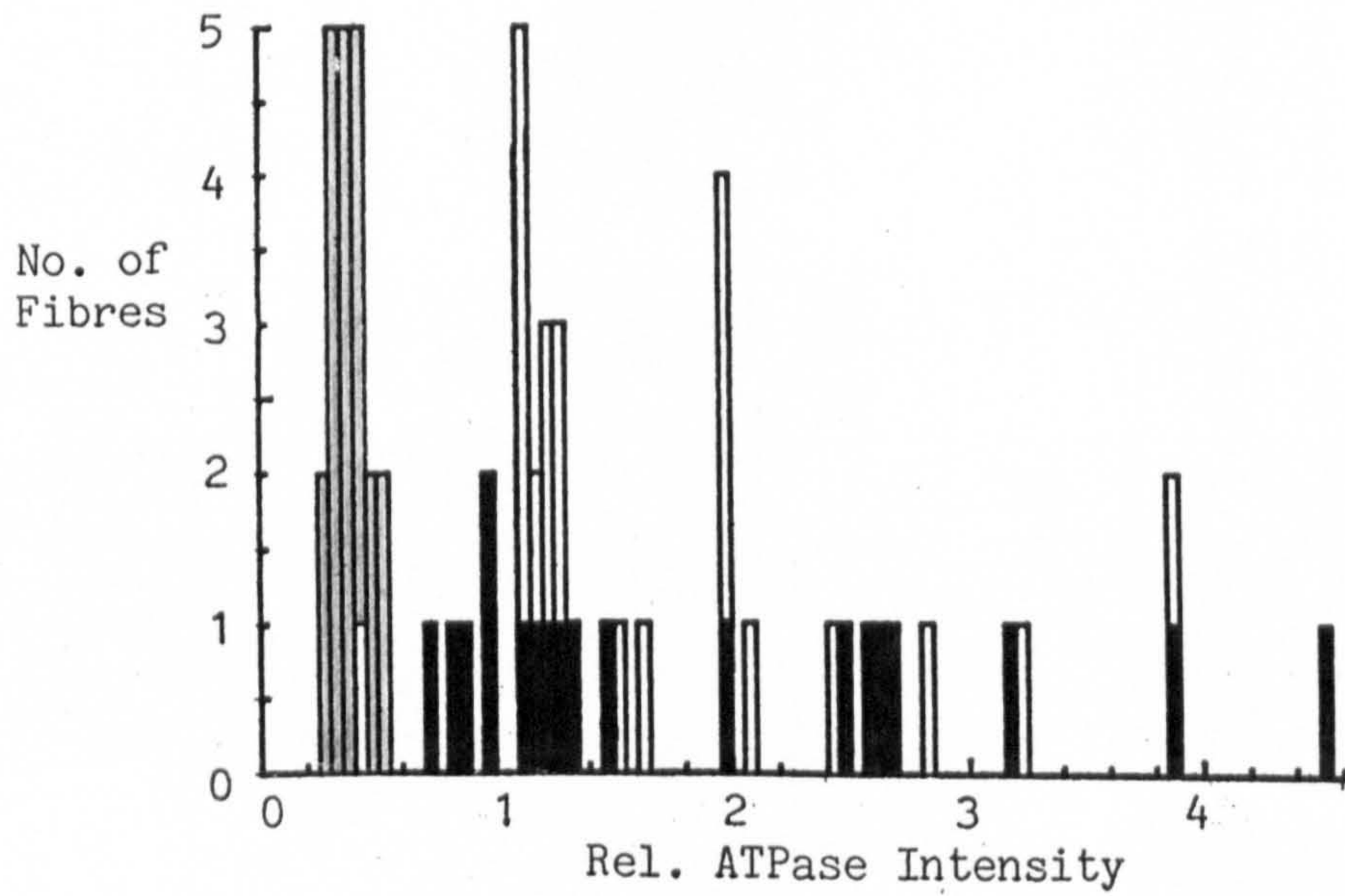


Fig.43 Histogram of photometric readings taken from a right ventricular papillary muscle (grey), a left ventricular papillary muscle (black) and a right ventricular trabecula (white) stained for myosin ATPase activity after alkaline pre-treatment.

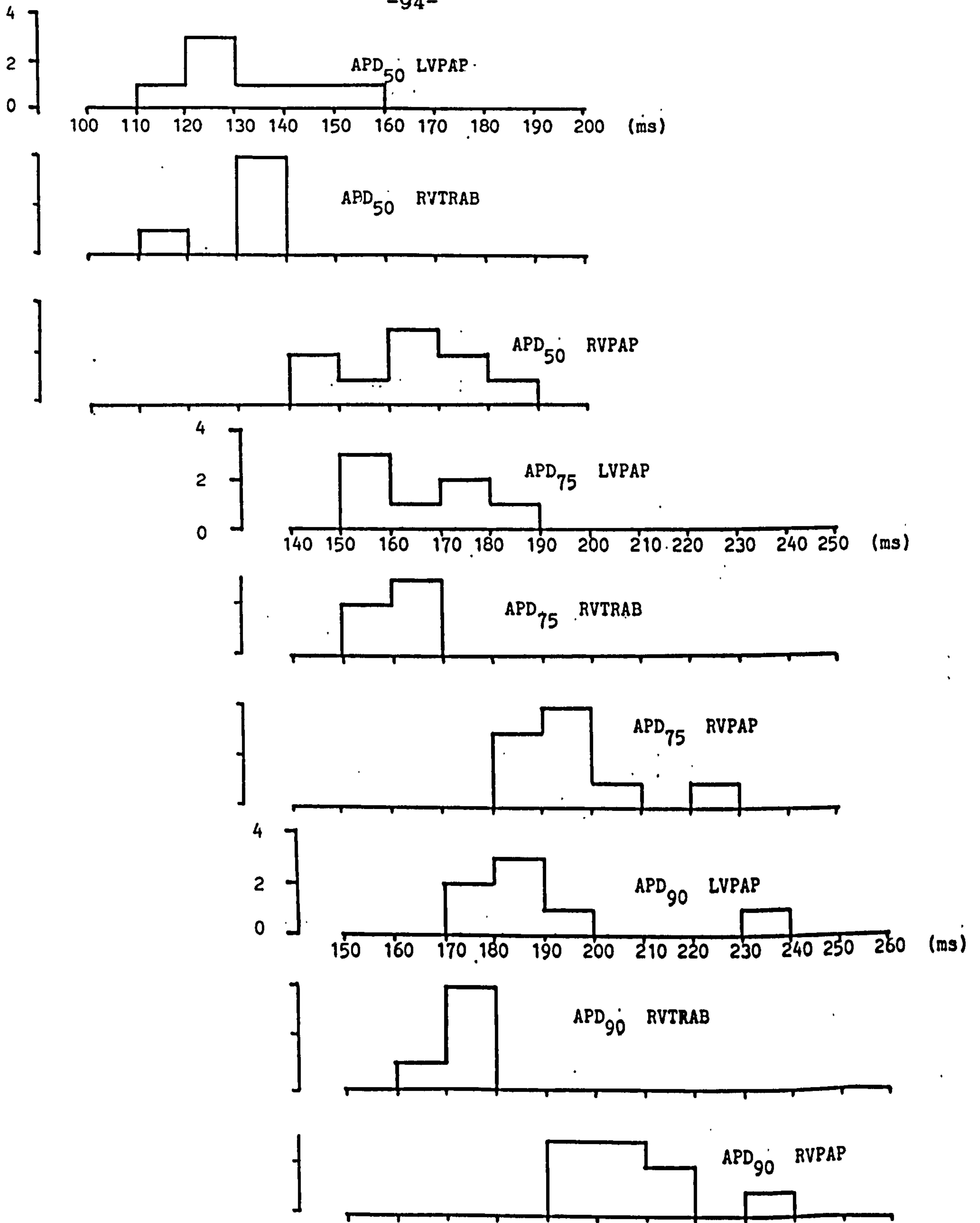


Fig.44 Histograms of action potential duration at 50%, 75% and 90% of repolarisation measured in the right ventricular papillary muscle (RVPAP), left ventricular papillary muscle (LVPAP) and right ventricular trabecula (RVTRAB) featured in Fig.43.

Vertical axes are numbers of recordings.

DISCUSSION

### Myosin ATPase

The first task undertaken in this work was the search for a species in which myosin heterogeneity in the ventricles could be demonstrated, using the myosin ATPase staining technique. Before considering the results achieved in this search it will be worthwhile discussing some aspects of the technique itself.

The myosin ATPase staining technique used in this thesis is system A of Snow et al. (1982); employing an alkaline pre-treatment before incubation in an ATP containing medium. During this incubation the ATPase of the myosin in the sectioned fibres (activated by calcium ions) hydrolyses ATP, releasing inorganic phosphate in the process. This inorganic phosphate is immediately trapped, in the form of the insoluble salt calcium phosphate ( $\text{Ca}_3(\text{PO}_4)_2$ ), within the fibre. A deposit of calcium phosphate therefore builds up in each fibre, in amount proportional to the expressed activity of the myosin ATPase. This deposit is visualised in the section in the form of cobalt sulphide (CoS) by a series of inorganic chemical reactions (see p.22). Each fibre is therefore stained in proportion to the Ca-activated, pH 9.4 ATPase activity of the myosin it contains.

The alkaline pre-treatment takes advantage of the fact that different myosin isoforms often have different sensitivities to alkaline pH. In the case of cardiac muscle it has been shown by Pope et al. (1980) that the ATPase activity of rat ventricular  $V_1$  myosin is more alkali-stable (i.e. is inhibited less by alkaline conditions) than that of  $V_2$  ventricular myosin. Relative alkaline

stability is a common feature of 'faster' myosins (Guth & Samaha, 1969; Pope et al., 1980) (V<sub>1</sub> myosin has a higher ATPase activity than V<sub>2</sub> myosin and can be considered 'faster'). The difference between the normal myosin ATPase activities is, therefore, exaggerated by the alkaline pre-treatment.

This difference in ATPase activity between the ventricular myosin isoforms means that it is possible to relate staining intensity to myosin type. Using this method, however, it is rarely possible to define absolutely the myosin type contained by cardiac fibres, in the way possible in skeletal muscle fibres. In skeletal muscle, the three main fibre types each contain predominately one myosin isoform (for review see Swynghedauw, 1986), in cardiac fibres the situation is complicated by the fact that all three ventricular myosins can be present in one fibre (Samuel et al., 1983). The most that can be inferred from a difference in staining intensity between two cardiac fibres is that different proportions of the three myosin isoforms are present in each (only when it is known that a single myosin isoform is present as e.g. by Weisberg et al., 1982 can a difference in staining intensity be interpreted with certainty in terms of myosin isoform; also see discussion of photometry histograms p.112).

The difference between skeletal and cardiac muscle in this respect probably stems from the control of gene expression. In cardiac muscle it is known that the two ventricular heavy chain (HC<sub>α</sub> and HC<sub>β</sub>) genes can be expressed simultaneously and that they are controlled in an antithetic manner (Lompre et al., 1984). In skeletal muscle, however, it appears that stable combinations of

myosins are relatively rare: combinations of different myosins are not unknown (e.g. Ib, IIc and IIab fibres) but these are thought to represent fibres in transition. There are two possible causes of this disposition towards homogeneity of stable forms: a. that gene expression is controlled by neuronal firing patterns and that these are clustered, favouring expression of single myosin isoforms (e.g. Spurway, 1980)\* or b. that the simultaneous expression of two heavy chain genes is an unstable situation and cannot be maintained (e.g. Salmons, 1980). The latter of these hypotheses is weakened by evidence suggesting that rabbit soleus contains a stable population of type Ib fibres and the former hypothesis supported by the transformation of these Ib fibres to type I fibres by an imposed low frequency stimulation regime (Salmons & Sreter, 1976). A flexible genome is also suggested by the work of Spurway (1980) which shows that sharp divisions between fibre types in individual muscles are lost when more than one muscle is compared, suggesting that slight differences exist between the fibre types of different muscles.

In summary the technique of myosin ATPase staining of cardiac muscle fibre sections, allows the comparison of myosin isoform distribution in individual fibres but it is more difficult to draw conclusions as to the absolute amounts of these isomyosins as, unlike skeletal muscle, mixtures of these isomyosins are much more common.

### Species Differences

Differences in the staining intensities of atrial and ventricular fibres after alkaline pre-treatment were found in all the species investigated (Figs.2-7). However in only three species were differences in staining intensities found between ventricular fibres i.e. ferret, guinea pig and rabbit. Why should it be that some but not all species show heterogeneity of this type in the ventricle ?

In some of the above cases, where ventricular staining heterogeneity was not seen, it has been shown that myosin heterogeneity does exist e.g. in bovine heart Sartore et al. (1981) have shown, using specific anti-myosin antibodies, that individual cells of the ventricular myocardium can contain different myosins. They found however that most fibres in bovine ventricle bind similar amounts of antibody and therefore contain the same myosin. The lack of myosin ATPase staining heterogeneity in bovine ventricle probably reflects the small number of cells which ought to stain differently as well as the lesser sensitivity of ATPase than antibody techniques.

Heterogeneity of staining intensity is, however, seen in the atria of both bovine and human hearts (Figs. 6 & 7). It is known that ventricles of large species, such as humans, do not appear to undergo changes of myosin heavy chain type, when subjected to chronic mechanical overload (Cummins, 1984). It is also known that in response to pressure overload human atria do show myosin distribution shifts (Schaub et al., 1984; Schiaffino et al., 1984). It is, therefore, appropriate that the atria, not the ventricles of



human and bovine hearts should show histochemical evidence of myosin heterogeneity, suggesting the existence of more than one atrial myosin isoform.

In the case of rat heart it is known that the three ventricular myosin isoforms are present simultaneously (e.g. Hoh et al., 1978), that they have different ATPase activities and alkaline stabilities (Pope et al., 1980) and that these differences can be demonstrated with the techniques used here (Weisberg et al., 1982). Weisberg et al. showed that ventricles of hypothyroid and young euthyroid rats, in the first of which  $V_3$  and in the second of which  $V_1$  predominate, stain differently for myosin ATPase activity after alkaline pre-treatment (hypothyroid ventricles staining less darkly than those of young euthyroid animals). They also showed that individual fibres stained differently in the ventricles of hypothyroid animals if they were examined before the change to  $V_3$  had been completed. This they explained could arise if the myosin change induced by hypothyroidism was not synchronised in all fibres. In view of these facts the only possible explanation for a lack of staining heterogeneity in normal rat ventricle must be that all fibres contain the same proportions of the three ventricular myosin isoforms.

In the cat to my knowledge it is not known how many ventricular myosin isoforms exist (no studies of the type done in the rat by Hoh et al. 1978 have been done for the cat) and the only evidence suggesting the existence of multiple myosin isoforms is a reduction in biochemically assayed myosin ATPase activity in pressure overloaded ventricles (Carey et al., 1978; a reduction in myosin

ATPase activity in response to pressure overload in rat ventricles is accomplished by an increased proportion of  $V_3$  myosin). It is not possible to say from the evidence presented here whether one or more ventricular myosin isoforms is present in the cat. It is not possible, therefore, to say whether the lack of myosin ATPase staining heterogeneity in cat ventricle is similar to that of the rat or of the bovine heart in origin. One other possibility is that multiple myosin isoforms exist in the cat ventricle but that differences cannot be demonstrated between them using histochemical ATPase staining techniques. However, the work of Carey et al. mentioned above showing that biochemical ATPase differences do exist makes this latter possibility less likely. It appears likely, therefore, that cat ventricular fibres stain homogeneously because they all have the same myosin composition.

Homogeneous ventricular staining appears to occur because either one ventricular myosin isoform predominates (as in bovine ventricle) or because multiple isoforms are distributed similarly in all fibres (as in rat). Those species in which ventricular staining heterogeneity can be demonstrated (ferret, guinea pig and rabbit) differ from those whose ventricles stain homogeneously in that multiple isoforms of myosin are distributed differently in individual fibres.

#### Heterogeneity in the rabbit

The degree of ventricular myosin heterogeneity which can be demonstrated, using myosin ATPase staining techniques, in the rabbit is much greater than in the other species investigated in this work. Not only do individual fibres stain heterogeneously with

respect to their neighbours but large regional variations in staining intensity also exist, such as the transmural differences illustrated in Fig.12. No such regional variations in staining intensity were visible in either ferret or guinea pig. Similar myosin ATPase staining heterogeneity in the rabbit has since been reported by Eisenberg et al. (1985)

What is the cause of this heterogeneity in the rabbit and in what way is the rabbit different from those species which show less or no ventricular heterogeneity ?

#### Sympathetic Endings

The role of sympathetic innervation in the production of ventricular myosin heterogeneity in the rabbit was investigated in this work. Rupp et al. (1983) have shown that sympathetic innervation in the rat heart has an influence on ventricular myosin distribution. They found that chemical sympathectomy (which selectively destroys peripheral catecholaminergic nerve fibres) leads to an increased proportion of  $V_3$  myosin in the rat ventricle. It has also been shown that some conditions leading to changed myosin distribution in the ventricle also lead to changes in the <sup>number</sup> of  $\beta$ -receptors on myocytes and probably, therefore, a change in sensitivity to catecholamines. Niizoe et al. (1984) have shown that hyperthyroidism leads to an increased number of  $\beta$ -receptors on ventricular myocytes whereas hypothyroidism leads to a decrease in the number of these receptors. These conditions, of course, lead to opposite changes in myosin distribution (hyperthyroidism increases  $V_1$ , whereas hypothyroidism increases  $V_3$ ). It has also been concluded by Riondel et al. (1986) that changes in sensitivity of rat cardiac

muscle to isoproterenol with age involve a reduction in the number of  $\beta$ -receptors present in aged rat hearts. Aging (as with hypothyroidism) is accompanied by an increase in the proportion of  $V_3$  myosin in ventricular myocytes.

From the evidence quoted here it appears that situations in which one would expect sympathetic stimulation of myocytes to be reduced (either by removal of nerve terminals or by reduction of  $\beta$ -receptor numbers), lead to an increased proportion of  $V_3$ , whereas increased stimulation of myocytes appears to increase the proportion of  $V_1$ .

The possibility seemed to exist therefore, that differences in myosin distribution in rabbit heart, at both regional and individual fibre levels, could be due to non-uniform distribution of sympathetic nerve endings. It also seemed possible that differences in sympathetic nerve ending distribution could be responsible for inter-species differences in ventricular myosin heterogeneity.

These possibilities were tested in two ways: a. comparison of cat and rabbit ventricular sympathetic innervation and comparison of sympathetic nerve terminal distribution between endocardial and epicardial regions of the rabbit ventricle and b. investigation of the effect of chemical sympathectomy on the myosin heterogeneity seen between different regions and individual fibres of the rabbit ventricle.

For the first part of this work (the comparison of sympathetic nerve ending distribution) nerve endings were visualised by formaldehyde-induced fluorescence (FIF) of catecholamines. Figs.

18a-c show that no differences in nerve ending distribution are apparent either between cat and rabbit ventricle or between rabbit epicardium and endocardium. Regional myosin differences in the rabbit and differences in myosin distribution between cat and rabbit cannot therefore, be due to different distributions of sympathetic endings.

It could be maintained, however, that distribution of nerve endings gives insufficient indication of the release of transmitter from individual endings. It is conceivable that release of transmitter could be different on a regional or individual level because of differences in firing rate or in the amount of transmitter released by each impulse travelling along a fibre.

Removal of all sympathetic endings ought to test whether differences in activity of sympathetic fibres are responsible for the myosin heterogeneity in the rabbit ventricle. This was achieved by the intravenous administration of 6-hydroxydopamine (6-OHDA), which causes a selective degeneration of sympathetic nerve endings. The absence of sympathetic endings after treatment with 6-OHDA, has been demonstrated electron-microscopically and using FIF treatment of tissue (Tranzer & Thoenen, 1968; De Champlain, 1971).

Recovery of sympathetic nerve terminals is quite rapid with this method of sympathectomy but can be prevented by further administration of 6-OHDA. In these experiments sympathectomy lasting six weeks was achieved according to a protocol which employs repeated intravenous doses of 6-OHDA to ensure that regeneration of sympathetic nerve terminals does not occur. Fig.19 shows catecholamine containing nerve fibres present in control

rabbit ventricle after the six week course of injections. No fluorescent fibres were present in the ventricles of sympathectomized rabbits.

Figs.20 & 21 show that neither regional nor individual fibre myosin heterogeneity has been abolished by long-term chemical sympathectomy. There is, however, some evidence to believe that, as reported by Rupp et al. (1983), sympathectomy leads to an increased proportion of  $V_3$  in the ventricles. The muscles photographed in Fig.20&21 were all alkali pre-treated before ATPase staining however, optimum differentiation in muscle from sympathectomized hearts was achieved at less alkaline pH's than in muscle of control animal hearts. This indicates that more alkali-labile myosin is present in the sympathectomized hearts, which in turn suggests that the ratio of  $V_1/V_3$  is reduced in these hearts. However, a rigorous test for a change in this ratio would require that muscle from control and sympathectomized animals be included in one block so that section thickness variations could be excluded (a thinner section would cause an apparent increase in alkali-lability).

The presence of myosin ATPase staining heterogeneity between different regions and between individual fibres of sympathectomized ventricles, means that differences in sympathetic nerve terminal distribution and/or transmitter release cannot be responsible for these differences of myosin distribution in normal rabbit ventricles. However, it is still possible that the sympathetic nervous system could be responsible for myosin heterogeneity in rabbit ventricles.

Although all peripheral sympathetic nerve terminals degenerate after 6-OHDA administration, the adrenal medulla is unaffected (Fronek, 1980). This means that the sympathectomized heart would still be exposed to catecholamines carried from the adrenal medulla via the blood. It is extremely unlikely that different regions (and even less so that neighbouring fibres) of the ventricles would experience different concentrations of blood-borne catecholamines. However, it is not inconceivable that different regions and different fibres might have different numbers of  $\beta$ -receptors. Niizoe et al. (1984) and Riondel et al. (1986) have shown that  $\beta$ -receptor numbers change in some conditions which also alter myosin distribution) causing different sensitivities to a given concentration of catecholamine.

For this to be the cause of myosin heterogeneity in the rabbit ventricle requires the further assumption that differences in  $\beta$ -receptor number survive the general increase in the numbers of these receptors seen in sympathectomized tissue, resulting in the super-sensitivity to catecholamines seen in sympathectomized tissue (e.g. Nadeau et al., 1971). Vascular tissue from the animals sympathectomized in these experiments was indeed found to be super-sensitive to catecholamines (A. Templeton and N.C. Spurway; personal communication).

If the above hypothesis is true then, despite uniform distribution of sympathetic terminals and uniform exposure to blood-borne catecholamines in sympathectomized animals, the sympathetic nervous system could still have different effects on different regions or fibres of the rabbit ventricle. The weight of

the evidence presented here, however, suggests that the sympathetic nervous system has no role in the production of myosin heterogeneity in the rabbit ventricle.

Workload: Metabolic Capacity

Evidence was presented earlier to the effect that cardiac myosin distribution and hence myosin ATPase activity can be altered by a change in workload (Carey et al., 1978; Klotz et al., 1981). Regional variations in workload could therefore be responsible for the heterogeneity of staining intensity in rabbit ventricles. Two indirect indexes of workload were used here to investigate this possibility: metabolic capacity and fibre cross-sectional area.

Much work has been done in skeletal muscle to show that the metabolic capacity of fibres is governed to a large extent by their workload (i.e. frequency of use x load; see Salmons, 1980 for review). It therefore seemed reasonable to use metabolic capacity of cardiac fibres as an index of their chronic workload.

Differences in workload are known to exist between atrium and ventricle and between epicardial and endocardial layers of the ventricular wall. The lower workload of the atrium is due to the lower pressure in this chamber. If the ventricular wall is considered as a series of concentric spheres then tension is greatest in the inner layers. This means that the endocardial layers of the ventricular wall are under higher tension than the epicardial layers (Katz, 1977). It has been shown that sarcomere length is greater at a given left ventricular filling pressure in endocardial fibres than in epicardial fibres (Spotnitz et al., 1966), indicating that the greater stress on endocardial fibres



stretches their sarcomeres more. A higher workrate in the endocardium is also indicated by the higher production of lactate in this region (as compared with the epicardium) under anaerobic conditions (Dunn & Griggs, 1975). In this situation all ATP must be supplied using anaerobic glycolysis, the end product of which is lactate (this higher production of lactate does not necessarily indicate a higher glycolytic capacity in this region only that more work is being done in the endocardium under these anaerobic conditions).

Two measures of metabolic capacity were used: succinate dehydrogenase staining (SDH; an oxidative capacity marker) and  $\alpha$ -glycerophosphate dehydrogenase staining ( $\alpha$ GPDH; a glycolytic capacity marker). Using these enzymes, differences in metabolic capacity were sought between areas which stained differently for myosin ATPase activity i.e. epicardium/endocardium (Fig. 13a & b) and atrium/ventricle (Fig. 13c & d). The different SDH staining intensities of the atrium and ventricle are as one would expect from the different workloads of these chambers i.e. high intensity staining in the harder working ventricles. However no difference is visible in  $\alpha$ GPDH staining intensity between atrium and the endocardial layers of the ventricle (the difference in myosin ATPase staining would be greatest between these two areas). The transmural distribution of SDH does not follow the same association with workload as is seen in the atrio-ventricular comparison (SDH is uniformly distributed across the ventricular wall), however  $\alpha$ GPDH staining appears to be lower in the harder working

endocardial layer of the ventricular wall than in the less hard working epicardial layer.

It is interesting to note that endocardial muscle is more susceptible to hypoxic injury than the epicardial layers. This is probably a consequence of the lower glycolytic capacity (indicated by lower  $\alpha$ GPDH staining intensity) and the lower  $PO_2$  (Winbury et al., 1971; Moss, 1968) in this region. The lower  $PO_2$  could itself be a consequence of the lower density of capillaries (Gerdes et al., 1979) and the higher intramyocardial pressure in the endocardial layers (Heineman & Grayson, 1985; which will tend to reduce blood flow to this region with respect to the epicardium). It has also been shown that partial coronary artery occlusion leads to a greater reduction of  $PO_2$  in the endocardium than in the epicardium, this would also make hypoxic injury more likely in the endocardial layers.

The lack of transmural variation in SDH staining is rather surprising, as the atrio-ventricular difference in both myosin ATPase and SDH staining intensities would suggest that the epicardial layer ought to have a lower SDH staining intensity. This result is, however, in agreement with the work of Rakusen & Tomanek (1986), who showed that mitochondria formed the same volume fraction of myocytes in the endocardium and epicardium. However, the higher efficiency of  $V_3$  myosin (which predominates in the endocardium) might mean that the higher workload in this region can be met by the same rate of energy supply as is sufficient in the epicardium (where the less efficient  $V_1$  myosin isoform is more plentiful). Indeed, Eisenberg & Salmons (1979) have shown that the

initial increase in mitochondrial volume fraction, in fast skeletal muscle fibres chronically stimulated at low frequency, falls to nearer (but still above) control levels once more efficient, slower myosin starts to be produced.

The correlation of  $\alpha$ GPDH and myosin ATPase staining intensities on a regional scale led me to examine the possibility that a correlation also existed between these stains at the level of individual fibres. The intensities of these two stains in individual fibres are plotted against each other in Fig. 15. The correlation coefficient for this plot ( $r=0.15$ ) appears to show that there is little relationship between these two enzymes in individual fibres. However I will return to this point after considering the relationship between myosin ATPase staining and fibre cross-sectional area.

#### Workload: Fibre Cross-Sectional Area

Fibre cross-sectional area was the other index of fibre workload used in this work. Hypertrophy is a well known response of cardiac muscle (Gulch, 1980) to increased chronic workload. It has also been shown in cardiac muscle that endocardial fibres of rat heart have larger cross-sectional areas than epicardial fibres (Gerdes et al., 1986). I have investigated whether similar differences in fibre cross-sectional area exist in the rabbit ventricle and also if fibre cross-sectional area is related to myosin ATPase staining heterogeneity at the regional and individual fibre levels.

Table 1 shows that significant transmural differences in fibre cross-sectional area do exist in the rabbit ventricle although the

differences are smaller (20% as opposed to 38%) than those seen by Gerdes et al., (1986)\* in the rat heart. The larger fibres are located in the sub-endocardial region indicating that, in individual rabbit hearts, fibres subjected to high chronic loading tend to have larger cross-sectional areas than fibres subjected to low chronic loading (at least at the regional level).

These regional differences in fibre cross-sectional area are as one would expect from the relative loads of epicardial and endocardial layers and they also seem to match the differences in myosin distribution between these two regions i.e. slow myosin ( $V_3$ ) appears to be associated with larger fibres and fast myosin ( $V_1$ ) with smaller fibres. The association of  $V_3$  myosin with larger fibres is, of course, similar to the situation in hearts hypertrophied by pressure overload, where the fibres are larger and contain more  $V_3$  than normal hearts. Workload therefore appears to affect, at the regional level, myosin isoform distribution (as was also indicated by  $\alpha$ GPDH staining). I also looked at the relationship between fibre cross-sectional area and myosin distribution in individual, neighbouring fibres to test whether differing workloads were responsible for the myosin heterogeneity between individual fibres.

Fig. 16 shows that this relationship is not a linear correlation. However, close examination, with the aid of the dividing lines, shows that the upper right quadrant contains few points relative to the lower right quadrant. The two left hand quadrants contain roughly equal numbers of points. The dividing

lines were chosen for this reason and to maximise the difference between the two right hand quadrants.

One factor which could influence the shape of the distribution is variability of cross-sectional area along the length of individual fibres. For instance, a thin portion of an otherwise large fibre, containing  $V_3$  myosin which ought to appear in the lower right quadrant, will appear in the lower left quadrant. By controlling for changes in myocyte shape, I was able to ensure that fibres appearing in the lower left quadrant were truly small fibres. Fig. 17 shows that controlling for changes in myocyte shape does not affect the shape of the distribution.

It appears, therefore, that large fibres are more likely to contain slow  $V_3$  myosin, whereas smaller fibres are not restricted to either fast or slow myosin. High workload (in so far as cross-sectional area can be taken as indicating workload; other factors could, of course, influence fibre cross-sectional area) seems to be associated with a higher proportion of  $V_3$  myosin whereas low workload appears to have no influence on myosin distribution.

Closer examination of Fig. 15 supports this last statement. The distribution of points in Fig. 15 bears some similarity to those in Figs. 16 & 17. In this case the upper left corner contains no points i.e. the distribution is somewhat triangular in shape. This indicates that fibres which are low in  $\alpha$ GPDH are also low in myosin ATPase staining (as was the case with the endocardial/epicardial comparison) whereas, fibres high in myosin ATPase staining are found over the full range of  $\alpha$ GPDH staining intensities. It seems low intensity myosin ATPase staining (and therefore  $V_3$  myosin)

tends to be associated with large cross-sectional area and low  $\alpha$ GPDH staining. High intensity myosin ATPase staining is a poor predictor of fibre size and  $\alpha$ GPDH staining intensity i.e. at the level of neighbouring fibres high workload seems to favour production of  $V_3$  myosin whereas low workload seems not to influence myosin expression greatly.

#### Photometry

Photometry was used to confirm and quantify differences in staining intensity between fibres and tissues. For example the difference between atrial and ventricular fibres in Fig.8 is shown quantified in Fig.9. The subjective view that most ventricular fibres (at the right in Fig.8, shown in black in Fig.9) are stained less darkly than atrial fibres is confirmed by Fig.9. However, Fig.9 also shows that there is a small overlap of the staining intensities of the atrial and ventricular fibres. One black reading falls within the range of the white readings i.e. one of the ventricular fibres sampled has a staining intensity which falls within the range of the atrial fibres.

It is generally thought that, in the rabbit, atrial heavy chain is identical to the ventricular  $V_1$  heavy chain ( $HC_\alpha$ ). Sinha et al. (1984) have concluded that the heavy chain produced by thyrotoxic rabbit ventricle (exclusively  $HC_\alpha$ ) is identical to that in the atrium. The fact that peptides maps, electrophoretic and immunological properties (Chizzonite et al., 1984) of these heavy chains show no differences supports the claim that they are identical. It is, therefore, possible that fibres of the atrium and ventricle staining with similar intensity contain the same  $HC_\alpha$

dimer myosin (not strictly speaking the same myosin as the light chains will be different between the atrium and ventricle, but as has been argued above these have no influence on the myosin ATPase activity and hence are unimportant here).

Chizzonite et al. (1982) have shown using monoclonal antibodies that the ventricles of rabbits of the age used in this work (about 12 weeks) contain 10-20% HC<sub>α</sub>. This figure is not inconsistent with the result presented here of only one ventricular fibre staining as intensely as atrial fibres, as 20% HC<sub>α</sub> in the ventricle does not mean 20% of the fibres of the ventricle contain only HC<sub>α</sub> (and hence only V<sub>1</sub>). It is more likely that most cells contain some HC<sub>α</sub> and that the number of cells containing only HC<sub>α</sub> will be very small. It is, therefore, not unreasonable to find that only one ventricular fibre out of fifty appears to contain only HC<sub>α</sub> as in this case. The unusual shape of the distribution of ventricular fibres in Fig. 9 (c.f. Fig. 11a-d) is probably due to the particular area sampled; as this was chosen only for its proximity to a piece of transversely sectioned atrium.

Another interesting point from Fig.8 (which might complicate the above discussion somewhat) is the fact that the range of staining intensities in the two tissues is quite comparable. This would perhaps suggests the presence of more than one myosin isoform in the rabbit atrium. It has indeed been shown, in the rat, that two atrial myosin isoforms exist, A<sub>1</sub> and A<sub>2</sub> which differ in their heavy chain structures (Hoh et al., 1978).

Photometric measurements were made in ventricular muscle of the rabbit after alkaline pre-treatment in order to discover if

distinct fibre types, similar to these found in skeletal muscle (Brooke & Kaiser, 1970; Tunell & Hart, 1977), existed; if so, they should show as peaks in the distribution of staining intensities.

The distributions of fibre-staining intensities in Fig.11(a-d) all show positive skewing, indicating that the majority of fibres stain palely for myosin ATPase activity after alkaline pre-treatment and hence contain slow myosin. Again this distribution is in agreement with the majority of ventricular myosin heavy chain being of the  $\beta$  variety (Chizzonite et al., 1982).

Fig.11 shows that although the skewed distribution is quite constant the number and size of peaks can be variable. Before discussing the number of peaks in Fig.11 it will be necessary to examine some of the factors which could influence this number.

The high pH pre-treatment used on these sections is intended to exaggerate differences in myosin ATPase activity by inhibiting slow myosins more than faster myosins. If the alkaline pre-treatment used is too severe then it is possible to inhibit completely all the myosin present so that no staining is achieved. Less extreme examples of this can still be a problem: where slightly too high pH pre-treatment is used not all myosin will be inhibited but more than one fibre type can appear to have effectively the same staining intensity and where more than one peak would be seen at a lower pH only one larger peak containing pale fibres is seen. This can be avoided by using a range of pH's on serial sections so that the 'optimal' pH for differentiation can be found.

The above illustrates how peaks can be lost from the left of the distribution by using too severe a pre-treatment, it is also



possible to lose peaks at either end of the distribution by choosing the wrong region of the ventricle for measurement. Fig.12 shows that fibre staining intensity varies within the ventricles. Clearly if one chooses to take readings from an endocardial region of the ventricular wall no dark fibres will be found, as only pale fibres are found in the endocardial layers of the rabbit ventricle. Similarly, few pale fibres will be found in an epicardial region. It is necessary, therefore, to choose a region such as that presented in Fig.10 where a wide range of fibre staining intensities, from very dark to very pale, can be seen. A region such as this is more likely to contain all existing fibre types.

Unless these problems are guarded against they will tend to reduce the number of peaks in a photometric analysis. However there is one other problem, to do with the technique of the photometry itself, which can add spurious peaks to a distribution. The problem lies in the orientation of fibres and the effect of this on distribution error.

Distribution error is caused by unstained gaps in fibres which allow light to pass through. Obviously if the proportion of fibres taken up by unstained gaps is variable this will in itself be a problem (there is no reason to suppose this is the case here as SDH staining shows little variation between fibres; mitochondria being the major component of cardiac fibres unstained by myosin ATPase reactions as performed here). However distribution error becomes more troublesome with increasing stain intensity (Spurway, 1986).

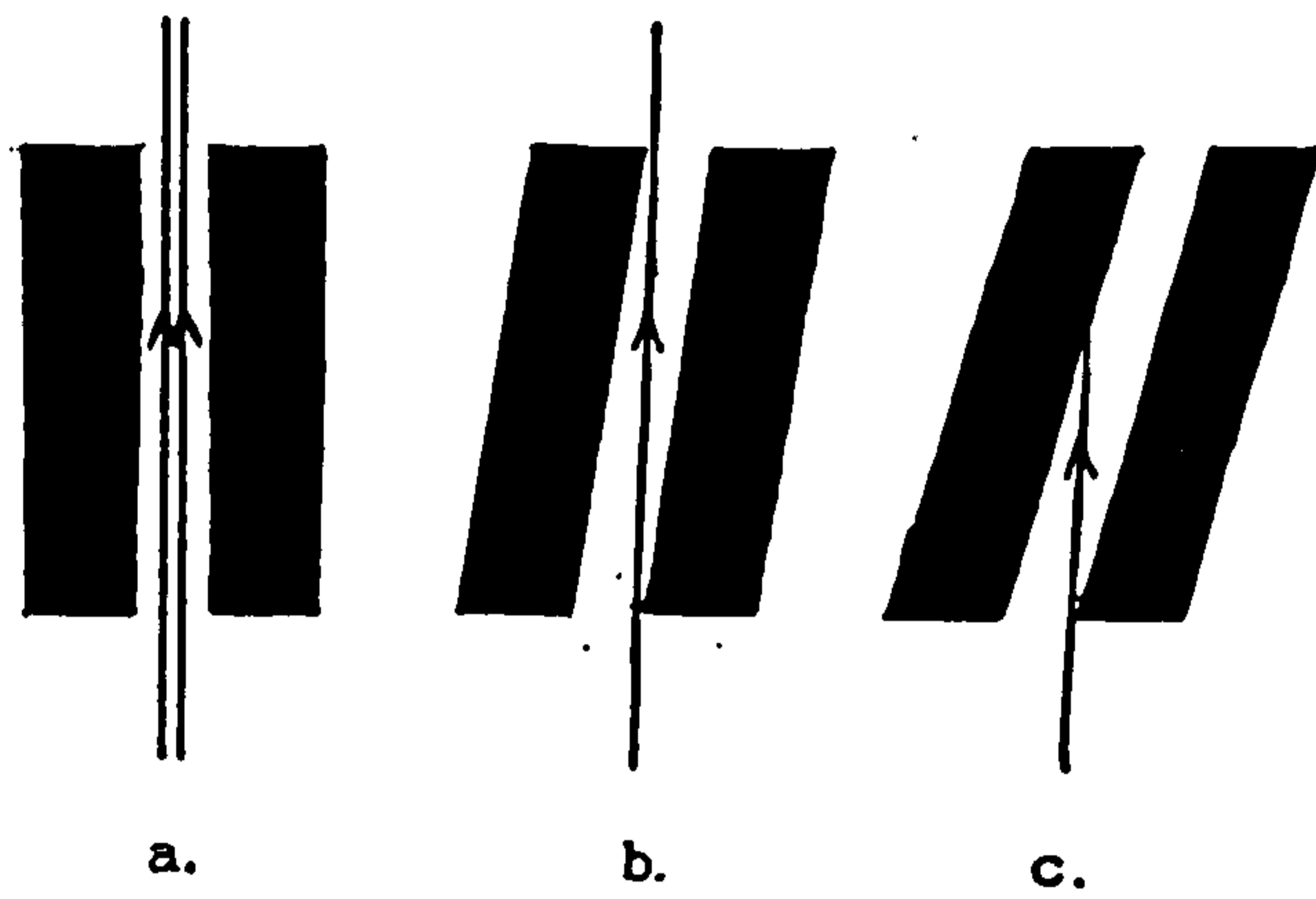


Fig.45 The effect of fibre orientation on distribution error. As fibre tilt increases so the amount of light able to pass between the stained myofibrils is reduced.

If the proportion of fibres taken up by unstained gaps remains constant, then the amount of light allowed to pass through these gaps will be the same in all fibres regardless of staining intensity. However the stained portions of dark fibres will allow less light to pass than those of pale fibres. The photometer measures the sum of the light passing through both stained and unstained regions of fibres. If the amount of light passing through unstained regions is very high in comparison with that passing through stained regions, then it will dominate the absorbance value for the fibre . This is more of a problem as staining intensity increases, as the transmittance of intensely stained portions is dominated more by the transmittance of unstained portions.

Distribution error tends to reduce the contrast between dark and pale fibres as seen by the photometer by reducing the importance of the stained portions in the absorbance value, however it should not alter the number of peaks to be seen. Distribution error is further affected, however, by the orientation of fibres with respect to the light incident on them. The sections used in this work were 10 $\mu$ m thick and one can think of the myofibrils contained them as columns of stain deposit. When these columns are arranged parallel to the incident light as in Fig.45a, light can pass between them. If, however, the myofibrils are tilted (because the fibre containing them has been cut obliquely) the effective gap will be reduced; allowing less light through (Fig.45b). A further tilting of the myofibrils will close the gap completely (Fig.45c). Distribution error is, therefore, reduced and so measured density increased in obliquely sectioned fibres.

If these differences in orientation are random then this effect will lead to a broadening of a peak or distribution as obliquely sectioned fibres will appear darker to the photometer than transversely sectioned fibres. Spurious peaks can be generated in a distribution if differences in orientation are not random. Cardiac muscle is arranged in layers which do not all necessarily have the same orientation. If photometric readings are taken from two adjacent layers (a & b), which contain the same fibre types but in which the fibres are differently oriented (transversely in a., obliquely in b.), then fibres in layer b. will have a higher absorbance than their fibre type counterparts in layer a. If the difference in fibre orientation between the layers is sufficiently large, the fibres of layer b. will form a 'subsidiary' peak to the right of each of the peaks formed by fibres of layer a.

The only way to reduce the chance of subsidiary peaks is to ensure that gaps between myofibrils are visible in all fibres measured. However, this is of limited use as only the extreme case of Fig.45c where no gaps will be seen is prevented, the situations illustrated in Figs.45a & b (and all those in between) will pass this selection.

Bearing in mind that subsidiary peaks are possible, we can return to Fig.11. It is possible in each of the histograms of Fig.11 to find five peaks; some with and some without subsidiary peaks (the lack of a subsidiary peak in some cases would indicate that not all muscle layers contain the same proportions of fibre types). This is, however, only a tentative identification of the peaks present and it is quite possible to come to a different

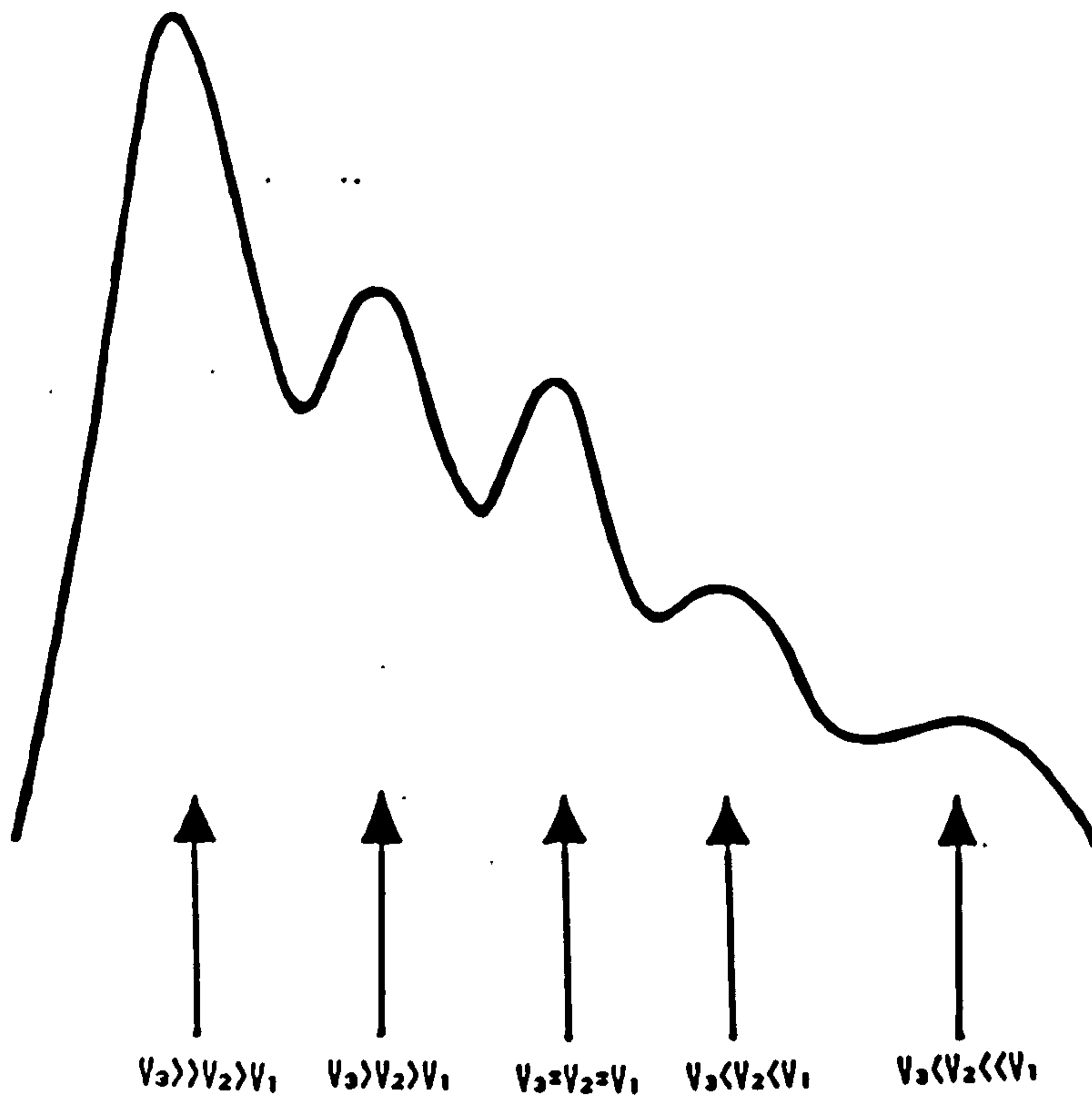


Fig.46 Possible interpretation of peaks in rabbit ventricular fibre staining intensity distributions.

conclusion about their number. Two other interpretations of the histograms in Fig.11 are: that subsidiary peaks do not exist and that each peak of the distribution represents a fibre type or; that no significant peaks are present and the distribution contains all possible staining intensities in proportions determined solely by the skew of the population.

Having decided that it is possible (but by no means obligatory) to see five peaks in Figs.11a-d it is possible to hypothesise on how these five peaks, representing different combinations of ventricular myosins, could arise from three myosin isoforms. Fig.46 shows one (of many) possible combinations which would fit this number of peaks. The extreme left peak would, in this scheme, contain readings from fibres containing only  $V_3$  myosin (this is not unreasonable as Chizzonite et al., 1982 have shown that  $HC_3$  forms between 80-90% of the ventricular myosin heavy chains in rabbits of this age, many fibres could therefore contain only  $V_3$  myosin). The peak at the extreme right of the distribution would consist of fibres containing only  $V_1$  myosin (the possibility of a very few ventricular fibres containing only  $V_1$  myosin has already been discussed in relation to Fig.9). The middle peak in Fig.46 has been positioned halfway between the two extreme peaks (representing  $V_1$  and  $V_3$  only containing fibres) and would consist of fibres containing equal proportions of the three ventricular myosin isoforms. The remaining two peaks consist of fibres where  $V_3$  myosin (left) and  $V_1$  myosin (right) predominate. The positions of these last three peaks, with respect to the two extreme peaks (which are

taken as the limits here), can vary and this will obviously affect the combination of myosin isoforms assigned to them.

All of the combinations of myosin isoforms used in Fig.46 have been shown to exist in the rat ventricle (Rupp, 1985). The whole ventricle figures for myosin distribution found by Rupp can be related directly to individual fibres because, in the rat ventricle, all fibres appear to contain the same proportions of the ventricular myosin isoforms.

### Electrophysiology

The electrophysiological study presented here was prompted by work on skeletal muscle showing that the decisive factor in myosin expression is the pattern of muscle fibre activation. Chronic low frequency stimulation has been shown to 'convert' a fast skeletal muscle to a slow skeletal muscle (eg. Salmons & Sreter, 1976). These authors showed that changes of fibre type in cross-innervation experiments are also due to changes in activity pattern by demonstrating that slow to fast conversion, after cross-innervation of a fast muscle nerve to a slow muscle, could be prevented by low frequency chronic stimulation, this removed any effect of the nerve itself.

The equivalent relationship in cardiac muscle to that described above in skeletal muscle would have to involve changes in action potential configuration rather than pattern of activity, as all cardiac fibres are activated on every beat (or ought to be). It is now known that electrophysiological properties of cardiac muscle fibres vary depending on the location in the heart. Differences in action potential configuration have been shown to exist between: atrium and ventricle (Hume & Uehara, 1985); epicardium and endocardium (Gulch, 1980; Sekiya et al., 1983); base and apex of the ventricles (Sekiya et al., 1983) and between right and left ventricular papillary muscles (Gulch, 1980).

The above action potential heterogeneity appears to follow a pattern which has been noted before in this thesis with respect to myosin distribution i.e. differences in regional workload appear to



be associated with different action potential configurations as well as different myosin distributions. Differences in workload between atrium and ventricle and between epicardium and endocardium, and the causes of these differences have already been discussed (p.106). It appears that regions subject to a relatively high workload (i.e. ventricle relative to atrium, endocardium relative to epicardium) have longer action potentials than regions subject to relatively lower workloads. Indeed, Gulch (1980) specifically investigated action potential durations in regions of the heart under different chronic loading. He reasoned from the greater loading and longer action potentials of hypertrophied hearts that regions of high chronic loading in the normal heart ought to have longer action potentials than regions of lower chronic loading. In the rabbit, of course, these regional differences in chronic loading are also associated with varying proportions of  $V_3$  myosin.

The muscles used in this study were also chosen with probable chronic loading in mind (although orientation of fibres in the muscles was as important a factor in the choices). It was assumed that right ventricular papillary muscles had been under lower chronic loading in life than left ventricular papillary muscles and right and left ventricular trabeculae (the muscles within the high chronic loading group were not ranked amongst themselves in order of loading).

The results collected from these muscles are similar to those of Sekiya et al. and of Gulch i.e. longer action potential durations in muscles from areas of high chronic loading with no consistent

significant differences between either resting membrane potentials or action potential amplitudes. However, there were some interesting exceptions to this which will be discussed later.

The rate of stimulation used in these experiments (1 Hz) is probably rather lower than the normal range of rabbit heart rates. As action potential duration is a function of stimulation rate (Carmeliet, 1977; Kleber et al., 1978) then it is possible that at normal rates of stimulation the differences in action potential duration found here could be changed. The results as presented, however, indicate a fundamental electrophysiological difference between the regions investigated, and it appears unlikely that this will be lost at higher rates of stimulation.

The circumstantial evidence that regions of the ventricle exhibiting long action potentials also contain higher proportions of  $V_{\alpha}$  myosin than regions with shorter action potentials (e.g. endocardium/epicardium) is supported by the results presented here. In all but one case, when action potentials were found to be different between two muscles the longer action potentials were associated with less intense myosin ATPase staining (and therefore a higher proportion of  $V_{\alpha}$  myosin). Where action potentials were found not to differ then myosin ATPase staining was also very similar.

On two occasions this association of long action potentials with higher proportion of  $V_{\alpha}$  myosin was particularly noticeable. In experiments 2,3 and 10 the action potentials and myosin ATPase activities of two right ventricular papillary muscles were compared. These experiments were done in order to test whether muscles

working under similar loads had similar action potential durations and myosin ATPase activities. In experiments 2 and 10 this was the case. However, in experiment 3 the action potential durations recorded from the two muscles were significantly different. When the myosin ATPase staining intensities of the muscles in this experiment were compared it was found that the muscle from which the longer action potentials were recorded was stained less strongly. The lower staining intensity was, however, only seen in those fibres from which electrophysiological recordings would have taken (i.e. the outer layers); the remaining fibres in this muscle were stained with the same intensity as those of the muscle with the shorter action potentials (see Figs. 27a & b).

The other occasion on which the association of longer action potentials with a higher proportion of  $V_3$  myosin was particularly noticeable was experiment 11. In this experiment the action potentials of a right ventricular papillary muscle were found to be longer than those of two muscles with high chronic loading (left ventricular papillary muscle and right ventricular trabecula). This was the only occasion on which action potentials of a 'low chronic loading' muscle were found to be longer than those of 'high chronic loading' muscles. When the recording sites of these muscles were compared for myosin ATPase staining, the lowest staining intensity was found in that of the right ventricular papillary muscle. Again the only part of this muscle to stain palely was the region from which recordings were taken (see Fig. 43b).

On one occasion (experiment 10) the association of long action potentials with a higher proportion of  $V_3$  myosin was not found. In

this case the action potentials of a left ventricular trabecula were found to be shorter than those of two right ventricular papillary muscles. However, the myosin ATPase staining of this muscle was lower than that of the two papillary muscles. So although the myosin distribution followed the pattern of a higher proportion of  $V_3$  myosin in muscles assumed to have experienced high chronic loading, the action potential durations did not (myosin ATPase staining intensities and the action potential durations respectively of the two right ventricular papillary muscles were similar). There is no obvious reason for the lack of correlation between action potential duration and myosin ATPase staining in this case, however it is possible that the left ventricular trabecula was damaged (by over-stretching for example) during dissection, or suffered some hypoxia not experienced by the right ventricular papillary muscles. Neither of these sources of injury would affect the myosin ATPase activity of the muscle but they both would affect the action potential duration.

The differences in action potential duration found here could be due to hypoxia of the inner layers of the muscle. Hypoxia of cardiac muscle leads to release of potassium ions from fibres (Katz, 1977). It has been argued that, if this release of potassium were different in the different muscles used, this could cause differential shortening of action potentials (Sekiya et al., 1983). There are several arguments against this possibility: resting membrane potential is not significantly different between muscles (indicating that potassium release is not different between muscles); left ventricular papillary muscles i.e. larger muscles,

in which hypoxia of the inner layers should be worse, have longer action potentials than right ventricular muscles; muscles of similar size (e.g. trabeculae and right ventricular papillary muscles) have different action potential durations; there were no time dependent variations of action potential duration (hypoxia should become worse with each stimulation) and finally the order in which muscles were used was changed so that time dependent deterioration of muscles could not cause a consistent difference in action potential duration. It seems likely, in the light of the above considerations and the fact that the results reported here largely agree with the observations of Gulch (1980), that living state differences in action potential duration do exist between the muscles studied here.

Both sets of data (photometric and electrophysiological) have been displayed in histograms to show the range of values in each case and to show whether overlap of data from each muscle occurs. It is not unreasonable that there should be some overlap of photometric data, as only rarely do pieces of rabbit cardiac muscle stain homogeneously. If the correlation between action potential duration and myosin type exists at the level of individual fibres it is to be expected that overlap of populations when it appears in one histogram, should appear in both. This is, indeed, normally the case. However, it is not possible to place too much emphasis on this point as one cannot be absolutely sure that photometric readings were taken from the exact recording site and it is most certainly not possible to identify individual fibres in terms of both staining intensity and action potential duration with the

techniques used here (see chapter on isolated myocytes for a method which would allow this).

The weight of the evidence from these experiments strongly suggests that there is a correlation on a regional scale between action potential duration and myosin type in the rabbit ventricle. It remains to be seen whether such a correlation exists in individual fibres. It should be noted, however, that a correlation between action potential duration and myosin type is not found in all species. Watanabe et al. (1983) have shown that heterogeneity exists between action potentials of individual fibres and between isolated myocytes of the rat ventricle, and Gulch (1980) has shown regional variations of action potential duration in rat and cat ventricles. The lack of heterogeneity of myosin distribution in these species is evidence that a correlation between action potential duration and myosin type does not exist in all species. It is, however, worth noting that, although no correlation exists in these species within individual hearts, hypertrophied rat hearts do have longer action potentials than normal hearts ( ; Gulch et al., 1979; Aronson & Nordin, 1984) and of course contain more  $V_{\alpha}$  myosin.

Capasso et al. (1983) have put forward two possible explanations of why parallel changes in action potential duration, myosin and S.R. function should occur. One of these is that slower myosin and S.R. need a longer period of activation in order to allow normal tension to be produced. The other is that as a result of the slower release and uptake of calcium by the S.R., inactivation of calcium current and activation of potassium conductance are slower (both of

which will tend to lengthen the action potential). Whether or not the action potential duration is lengthened by changes in the S.R. alone it is clearly an advantage to the heart in maintaining normal tension production to have it lengthened. There is no reason to suppose that the same does not apply to the regional variations found here in the rabbit heart. It would be interesting to know if differences in myosin type and action potential duration in the rabbit heart were accompanied by differences in S.R. activity.

If it is assumed for the time being that changes in action potential duration are not merely a consequence of the slower uptake and release of calcium by the sarcoplasmic reticulum then what ionic mechanisms could be responsible? Hume and Uehara (1985) concluded from a patch and voltage clamp study that action potential duration in atrial and ventricular myocytes was determined by the balance between calcium current decay and the activation of time-dependent outward current. They found that the major difference between atrial and ventricular myocytes, which could cause the difference in action potential shape, was the different properties of resting potassium channels.

Regional differences within the ventricle in action potential durations have not been investigated in this manner and there is conflicting evidence for the respective roles of calcium and potassium fluxes in the production of chronic differences in ventricular action potential durations. Gulch (1980, citing Gulch 1979) concluded that a delayed inactivation of calcium current was responsible for the lengthened action potential in hypertrophied muscle. The only previous work which has looked at regional APD

differences within one heart concluded from the effect of elevating potassium levels that potassium conductance differs between regions (Sekiya et al., 1983). These authors did not however, look for possible differences in calcium conductance. More work of the type done by Hume & Uehara would be needed in order to say which of the two conductances is responsible for regional differences in action potential duration.

It has been shown by Khatib & Lab (1984) that the mode of contraction has an influence on the duration of the action potential. They showed that action potentials recorded from isovolumically contracting rabbit ventricle were shorter than those recorded when ejection of blood was allowed to take place.

Isovolumic contraction represents the ultimate pressure overload (i.e. resistance to flow is infinite) and therefore represents an increased loading. It has already been pointed out that in the overloaded heart the ventricular action potential is longer than in the normal heart. This result of Khatib & Lab may not appear to agree with this, however, it should be pointed out that the result of Khatib & Lab represents the response to an acute increase in loading; the results in hypertrophied hearts are the response to a chronic increase in loading.

The result of Khatib & Lab is mentioned here to show that changed ventricular loading conditions have an immediate effect on action potential duration. Differences in action potential duration between regions of the ventricles and between overloaded and normal hearts should, however, be thought of as being adaptations to different loading. It may also be important that the response to



chronic overload reverses the initial change seen by Khatib & Lab. It is possible, because of the immediate response of the action potential configuration to changed loading conditions, to suggest a role for action potential configurational changes as a trigger for other adaptations.

Skeletal muscle gives a clue as to how a change in action potential duration might act as a trigger for adaptation to changed loading conditions. Obviously in skeletal muscle, one of the effects of a change in activation pattern is to change the number of times cytoplasmic calcium concentration is increased. As a result of this, cytoplasmic calcium concentration, averaged over a period of time, would be changed. It is therefore possible that cytoplasmic calcium could be involved, as a secondary messenger, in the adaptive process to changed loading.

In cardiac muscle it is also possible to suggest such a role for cytoplasmic calcium. The change of action potential configuration seen by Khatib & Lab involved a shortening of the action potential and a lowering of the plateau phase. This means that the calcium entering the cytoplasm across the sarcolemma during the action potential would be reduced in two ways: shortened time for calcium entry and smaller current due to transmembrane flux of calcium ions (suggested by the lower plateau of the action potential).

If such a role is proposed for cytoplasmic calcium then there is the problem that the adaptations of APD and S.R. would change cytoplasmic calcium concentration beyond the control value. This should not be possible unless one of the adaptations triggered is to move the set point at which calcium concentration triggers (and

also terminates) the adaptive changes. This means that whatever factors were responsible for setting cytoplasmic calcium concentration would, as part of the adaptation process, be changed so that a new, higher 'control' calcium concentration could be maintained. A fall in cytoplasmic calcium concentration could in this case trigger an adaptive change which would tend to increase the calcium concentration (slower sarcoplasmic reticulum and a longer action potential means that cytoplasmic calcium concentration is elevated for longer) beyond the original 'control' level.

The above is put forward as a possible alternative to the hypothesis of Swynghedauw et al. (1982) according to which a fall in efficiency triggers the change in myosin distribution which follows pressure overload. These two hypotheses can be combined such that the decrease in efficiency (which would lower ATP levels) could itself cause the reduction in action potential duration.

#### Summary and Conclusions

In all of the seven species investigated in this thesis, atrio-ventricular myosin ATPase staining intensity differences were demonstrated. In three of the species (ferret, guinea pig and rabbit) myosin ATPase staining intensity heterogeneity was also found within the ventricle. (Atrial heterogeneity was also found in bovine and human hearts). In the rabbit this ventricular heterogeneity was seen both between regions and between neighbouring fibres of the ventricle. Using microphotometric techniques to quantify differences in fibre staining intensities, an attempt was made to identify specific fibre types in the rabbit

ventricle. It was concluded that five fibre types exist in the rabbit ventricle although difficulties with fibre orientation allow only a tentative estimate.

Two possible causes of this heterogeneity were investigated for this thesis: workload and sympathetic innervation. Two indirect measures of workload were used: metabolic capacity and fibre cross-sectional area. It was concluded from the positive correlation of workload with high content of  $V_3$  myosin that workload is a major determinant of myosin isoform expression at the regional level in the rabbit ventricle. However, workload appears to be less involved in determining myosin type at the neighbouring fibre level. Although harder working fibres appear to contain more  $V_3$  myosin, the less hard working fibres are not restricted to either a high or a low content of  $V_3$  myosin.

Two approaches to investigating the involvement of the sympathetic nervous system in the maintenance of myosin heterogeneity in the rabbit ventricle were used: examination of nerve terminal distribution and chemical sympathectomy. It was concluded that the sympathetic nervous system was not involved for two reasons. These were that: there appeared to be no differences in nerve terminal distribution between different regions of the rabbit ventricle nor between rabbit and cat ventricles (cat ventricle shows no myosin ATPase staining heterogeneity) and because both regional and neighbouring fibre heterogeneity persisted after long term sympathectomy.

From the electrophysiological study, it was concluded that cardiac muscle under high chronic loading has, in general, longer

action potentials than muscle under low chronic loading. It was also concluded that longer action potentials are associated with a higher content of  $V_{\infty}$  myosin. Included is a discussion of how changes in action potential duration could be responsible for setting in motion the adaptive responses to changed loading conditions.

SUPPLEMENTARY CHAPTER 1

RAT ATRIO-VENTRICULAR DIFFERENCES

## Introduction

Differences between atrial and ventricular myosins in large mammals have been demonstrated using a variety of methods e.g. in bovine hearts using peptide maps of partially digested myosin heavy chains (Flink et al., 1978), and using specific antibodies (Sartore et al., 1981). In human hearts, differences have been shown in terms of immunological properties (Schiaffino et al., 1984; Yazaki et al., 1984) and in terms of myosin ATPase activities (Thornell & Forsgren, 1982; see also this thesis p.39). Differences between atrial and ventricular myosin ATPase activities have also been shown in the pig by Grandier-Vazeille et al. (1983).

In these species (human, bovine, pig) the great majority of ventricular myosin is of the  $V_3$  type, although Schiaffino et al. (1984) have shown that human ventricular fibres contain a proportion of  $V_1$  myosin.  $V_3$  myosin heavy chain is considered to be different from atrial myosin heavy chain in all mammalian species, whereas the matter of  $V_1$  myosin is debated in several instances.

In the rabbit ventricle all three ventricular myosins are expressed i.e.  $V_1$ ,  $V_2$  and  $V_3$  (Lompre et al., 1981). In this species atrial and  $V_1$  myosin heavy chains are considered to be identical in terms of ATPase activities (Banerjee, 1983) and when compared immunologically and electrophoretically (Chizzonite et al., 1984). Also Sinha et al. (1984) and Clark et al., (1984) have found no differences between the  $\alpha$ -mRNAs of rabbit atrial myosin heavy chain and ventricular myosin heavy chain.

The position in the rat is less clear. Dalla-Libera et al. (1983) have found differences between the peptide maps of partially digested atrial heavy chain ( $HC_{\alpha}$ ) and ventricular heavy chain ( $HC_{\alpha'}$ ). However, Schaub et al. (1982) and Kunz et al. (1986) have reported evidence, from peptide maps and histochemical ATPase activities, which indicates that rat atrial and ventricular myosin heavy chains are in fact identical. Further conflicting evidence is supplied by Yazaki et al. (1979) who find a large difference between the biochemically-assayed ATPase activities of rat atrial and ventricular myosins and Syrový (1985) who finds a small difference.

The work presented in this chapter was originally done as part of the search for a species displaying suitable ventricular myosin heterogeneity and is reported, in less extensive form, earlier in this thesis. In this chapter there is presented histochemical myosin ATPase and immunohistochemical evidence suggesting that the  $HC_{\alpha}$  of the rat atrium and the  $HC_{\alpha'}$  of the rat ventricle are not identical proteins.

Methods

Myosin ATPase

Myosin ATPase activity was investigated in fresh, frozen sections (the preparation of which has already been described p.20) after either alkaline or acid pre-treatment. The methods followed were those of Snow et al. (1982) (Method A). Alkaline pre-treatment of sections has already been described (p.21), the procedure for acid pre-treatment is as follows. Sections were placed in the following solution for 5 mins:

0.2 M Na acetate

pH adjusted, with a precision of  $\pm 0.025$  units, to a value within the range 4.0 - 4.8 using acetic acid.

Sections were then washed several times in alkaline pre-treatment solution at pH 9.45 to remove all Na acetate. Acid pre-treated sections were incubated in the ATP-containing solution (see p.22) for 60 mins. Visualisation of phosphate deposits was as described for alkaline pre-treated sections (p.22). After this sections were dehydrated, cleared and mounted.



### Immunohistochemical Staining

Fresh, frozen sections to be stained immunohistochemically were incubated overnight in specific guinea-pig anti-rabbit IIA myosin antibody diluted in phosphate-buffered saline (PBS; 0.145M NaCl in 0.01M phosphate buffer, pH 7.4) containing 1% bovine serum albumin (BSA). This was done by placing the sections on a moist filter paper in a Petri dish. A drop of the above antibody solution was then placed on each coverslip so that all parts of the sections were immersed. The dish was then covered. The moist atmosphere produced inside the dish by the moist filter paper ensured that the antibody solution did not evaporate during the overnight incubation. Next morning unbound antibody was washed off the sections by three five-minute washes in PBS containing 0.025% Tween 20. Sections were then incubated as above for 2-3 hours in the second antibody: peroxidase labelled goat anti-rabbit IgG (Miles; affinity-purified) i.e. antibodies raised in a goat against rabbit IgG. This second antibody binds to the first antibody (several second antibody molecules per first antibody molecule improving the sensitivity of the method). Unbound second antibody was removed after the incubation by three five-minute washes in PBS-Tween 20. Bound second antibody was visualised (in a fume cupboard, wearing gloves) by a brief incubation of sections in PBS containing about 50µg/ml diaminobenzidine (DAB) and 0.03% H<sub>2</sub>O<sub>2</sub>. The antibody-bound peroxidase produces a coloured, insoluble polymer of DAB which indicates where the second (and hence the first) antibody has located. After this sections were then dehydrated, cleared and mounted.

Control incubations were carried out with DAB/H<sub>2</sub>O<sub>2</sub> solution, applied direct to sections and to sections treated with the second antibody only. These controls ensured that any heterogeneity of staining was not attributable to endogenous tissue peroxidases or to the second antibody having bound to some component of the section other than the first antibody.

The antibodies used here were kindly supplied by Dr. A.M. Rowlerson.

The specificity testing of the antibody was done by Dr. P. A. Scapolo of Istituto di Anatomia degli Animali Domestici con Istologia ed Embriologia, Bologna, Italy using the immunoblot technique (Towbin et al., 1979). I will describe only briefly the technique used.

Myosin is separated into its component parts by SDS gel electrophoresis, which are stained by Coomassie Blue in Fig.47a. The peptides on the gel are transferred to nitro-cellulose paper by the application of an electric current which draws them from the gel onto the paper. Once the peptides are on the nitrocellulose paper, antibody is applied and it can then be seen to which components of myosin the antibody binds. This process was done for three types of skeletal myosin: cat temporalis (type IIm myosin; lane 1); cat soleus (type I myosin; lane 2) and guinea pig (type IIA myosin; lane 3). The results of this antibody-specificity testing will be presented here -it would be inappropriate to present them in the results section as they form no part of the work done for this thesis, but are important to the discussion of the results presented in this chapter.

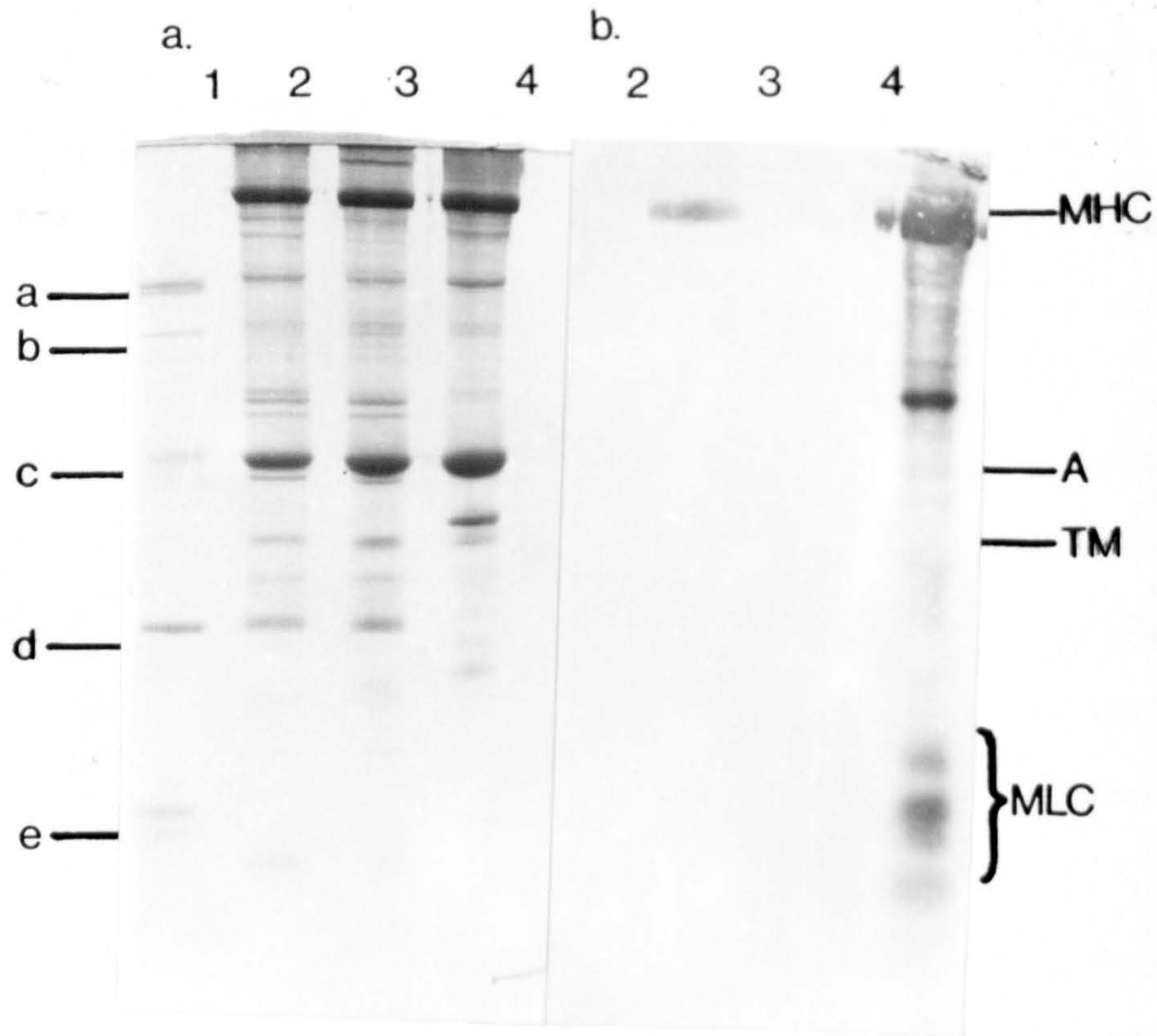


Fig.47 a. Coomassie Blue stained reference gel: lane 1 molecular weight markers (a) glycogen phosphorylase (92500 Da) (b) bovine serum albumin (66200 Da) (c) ovalbumin (45000 Da) (d) carbonic anhydrase (31000 Da) (e) lysozyme (14000 Da); lane 2 cat temporalis (type IIM) myofibrillar preparation; lane 3 cat soleus (type I) myofibrillar preparation; lane 4 guinea pig masseter (type II) myofibrillar preparation. Abbreviations: MHC - myosin heavy chain; A - actin; TM - tropomyosin; MLC - myosin light chain.

b. Immunoblot of duplicate of a. reacted with antibody, details of method in Rowleron et al. (1985). Lanes 2, 3, & 4 as in a.

Fig.47b. shows that the antibody has no affinity for type I myosin myofibrillar proteins, a small reaction in the type IIm lane only with the myosin heavy chain (IIM myosin is a type of fast myosin found in certain oxidative fibres) and a strong reaction with myosin subunits in the type IIA lane. Within the type IIA myofibrillar lane the antibody reaction is confined to the myosin heavy and light chains and one other band of intermediate mobility.

#### Photometry

The photometric techniques used in this thesis have been described before (Methods, p.24).

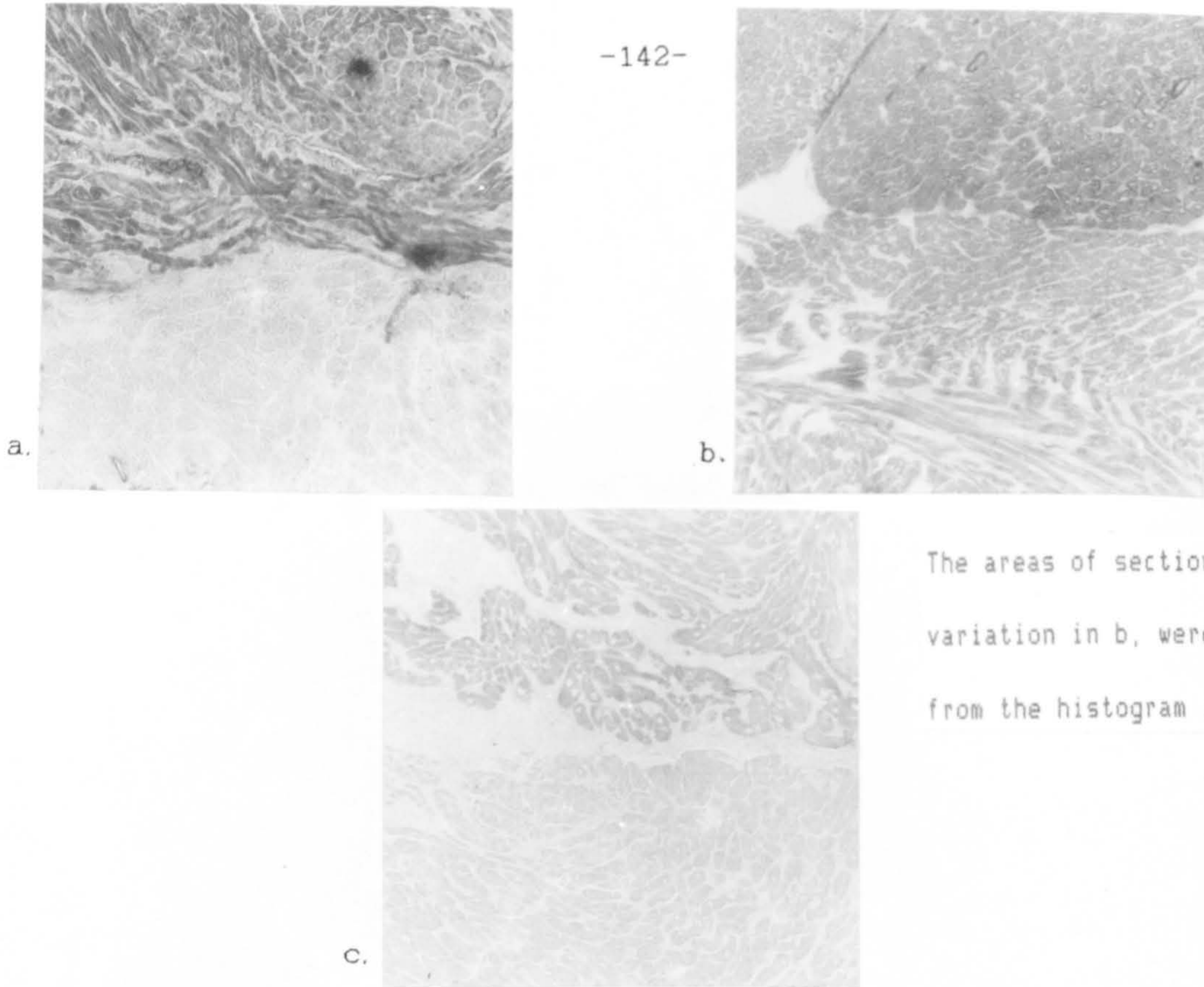


Fig.48 Myosin ATPase and immunohistochemical staining of composite blocks of atrium (top) and ventricle (bottom) of 250g rat.

a. mATPase, alkali pre-treated (pH 10.6) x100.

b. mATPase, acid pre-treated (pH 4.25) x100.

c. immunoperoxidase stain, first antibody anti-IIA myosin x100.

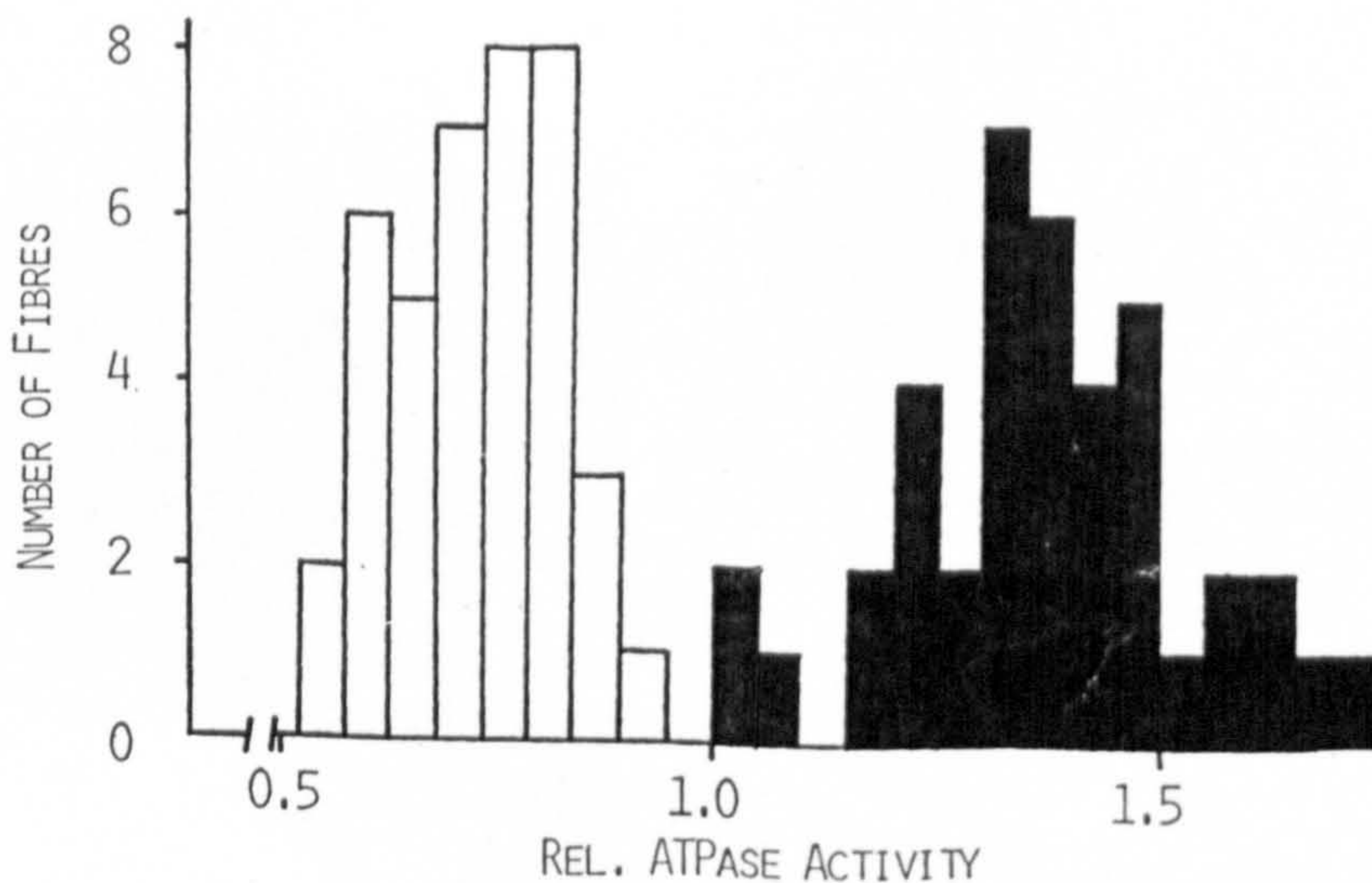


Fig.49 Histogram of staining intensity of fibres from the acid pre-treated section shown in Fig. 48b. Atrial fibres are shaded black, ventricular fibres white.

Results

Fig.48 shows examples of: a. an alkaline pre-treated myosin ATPase stained section (pH 10.6); b. an acid pre-treated myosin ATPase stained section (pH 4.25) and c. an immunohistochemically stained section. In each, atrial muscle appears at the top, ventricular muscle at the bottom. This tissue was taken from a 250g rat. After alkaline pre-treatment atrial muscle stains darker than ventricular muscle for myosin ATPase activity. After acid pre-treatment atrial muscle again stains more darkly than ventricular muscle for myosin ATPase activity. The atrial muscle in Fig.47c is also stained darker than the ventricular muscle indicating that more antibody has bound to the atrial myosin than has to the ventricular myosin.

Fig.49 confirms the difference in staining intensity between atrium and ventricle in the acid pre-treated section of Fig.48b. Photometric readings taken from atrial fibres are shaded black, readings from ventricular fibres appear white. Atrial fibres have higher relative ATPase staining than ventricular fibres and there is no overlap of the readings from the two muscles.

Fig.50 shows reactions of tissues taken from a 420g rat : a. an alkaline pre-treated myosin ATPase stained section (pH 10.55); b. an acid pre-treated myosin ATPase stained section (pH 4.4) and c. an immunohistochemically stained section. As with the smaller rat, after alkaline pre-treatment the atrial muscle stains more darkly than does the ventricular muscle for myosin ATPase activity, whereas after acid pre-treatment the ventricle in this instance

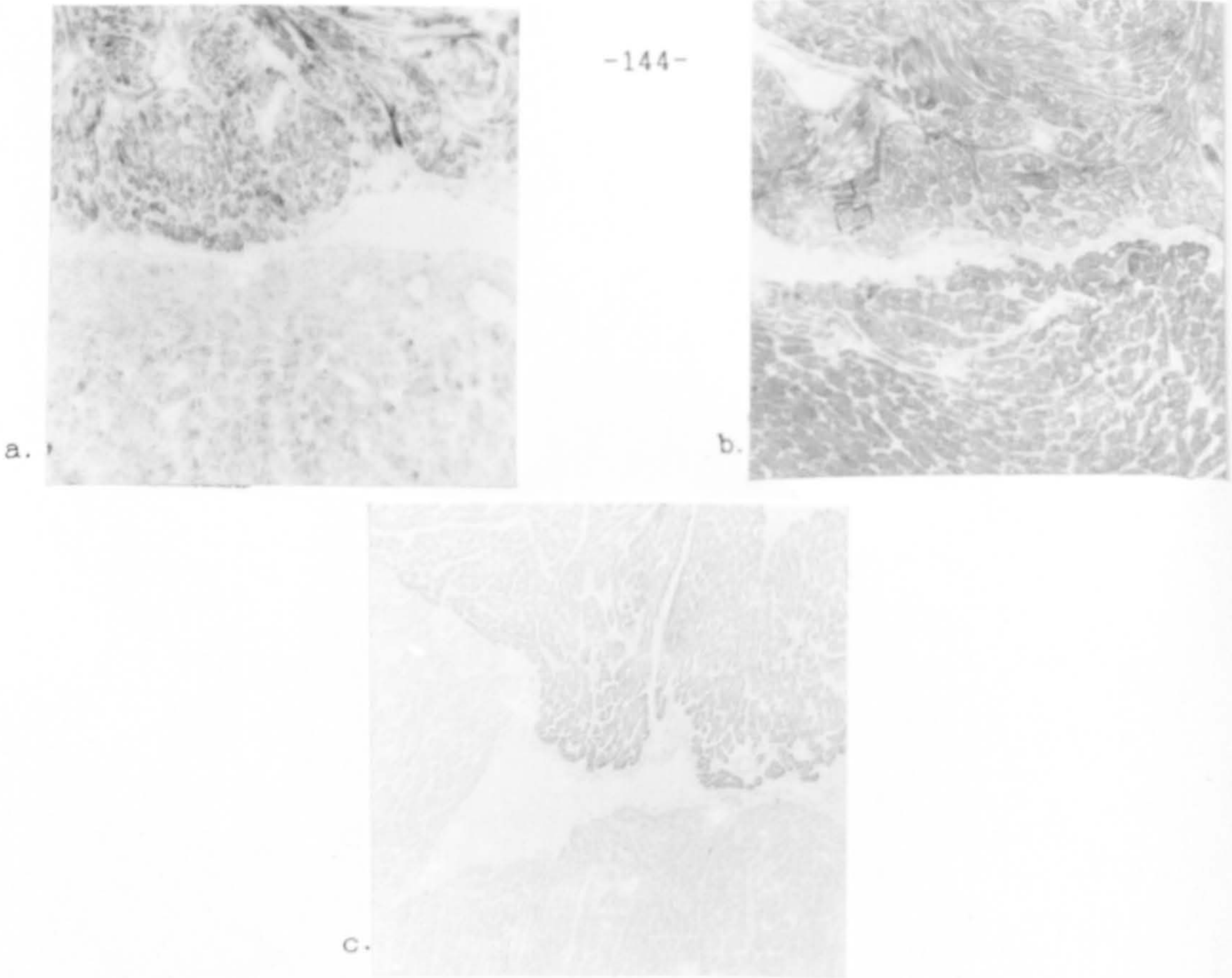


Fig.50 Myosin ATPase and immunohistochemical staining of composite blocks of atrium (top) and ventricle (bottom) of 420g rat.

- a. mATPase, alkali pre-treated (pH 10.55) x100.
- b. mATPase, acid pre-treated (pH 4.4) x100.
- c. immunoperoxidase stain, first antibody anti-IIA myosin x100.

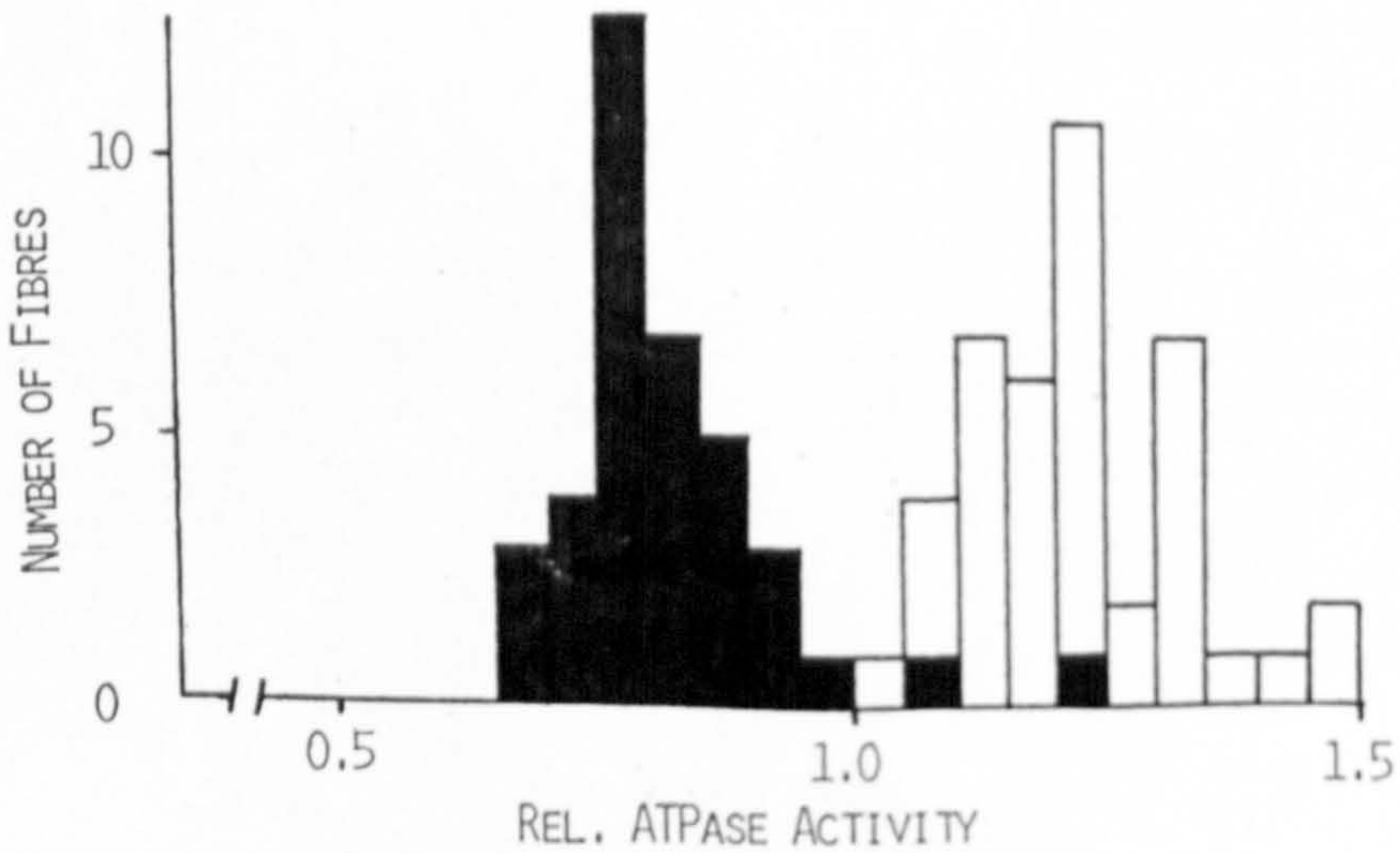


Fig.51 Histogram of staining intensity of fibres from the acid pre-treated section shown in Fig.50b. Atrial fibres are shaded black, ventricular fibres white.

stains more darkly than the atrium. As with the smaller rat the atrium binds more antibody than the ventricle.

Again the difference between atrium and ventricle after acid pre-treatment is shown quantified in Fig.51 . As with Fig.49 atrial fibres are shaded black, ventricular fibres are shown in white. In this case there is a slight overlap of the two populations with two atrial fibres falling within the staining intensity range of the ventricular fibres, however the two populations are still quite apparent and separate.

Fig. 51 (contd) The apparent lack of differentiation in Fig. 50b (which disagrees with the photometric data) can be explained by the fact that the photograph was taken under white light whereas the photometric readings were made using a blue filter to enhance myosin ATPase staining contrast.



Discussion

The myosin ATPase results presented here do not agree with those of Kunz et al. (1986), who found no differences between the histochemical ATPase activities of rat atrial and ventricular muscles. In order to obtain the differences in staining intensity shown here it was necessary to pre-treat serial sections at different pH's (both acid and alkaline), at 0.05 pH unit intervals. If the intervals used were any larger than this it was possible to miss the optimal pH for differentiation. If this happened then no difference in staining intensity between atrium and ventricle could be seen. The pre-treatment pH is probably so crucial for differentiation because of the very considerable similarities between the two myosins and may explain the lack of differentiation reported by Kunz et al.

Hoh et al. (1978) have shown that rat atrial and ventricular myosins contain different light chain species. Can differences in light chain complement be responsible for the differences in staining intensity shown here between atrium and ventricle?

Sivaramakrishnan & Burke (1982) have shown that the ATPase activity of subfragment 1 of skeletal myosin is not affected by the removal of the light chains. This indicates that the light chains have only a small role (if any) in the enzyme activity of the myosin molecule. It is also possible to draw the same conclusion from the work of Wagner (1981) which showed that the myosin ATPase activity of myosin molecule 'hybrids' (i.e. heavy chain of one myosin type combined with the light chains of another myosin type)

was determined by the heavy chain species involved and was largely independent of the light chain species. It appears, therefore, at least in vitro (the light chains may have a regulatory role in vivo; Chantler, 1981), that differences in light chain species cannot be responsible for differences in physiological myosin ATPase activity between atrium and ventricle. The differences in pH lability could nevertheless be due to the light chains, though this would require them to assume a role at extremes of pH which they do not fulfil under normal circumstances. It thus seems most compatible with the previous literature to suggest that differences in ATPase activity and pH stability reflect differences in myosin heavy chain species.

It has been shown that the rat ventricle normally contains three myosin isoforms which have different  $\text{Ca}^{++}$ -activated ATPase activities (Hoh et al., 1978) and pH stabilities (Pope et al., 1980). It could be that the differences in ATPase activity between atrium and ventricle reported here are due to the presence in the ventricle of the lower activity myosin isoforms  $V_2$  and  $V_3$ . This could certainly explain the difference after alkaline pre-treatment as  $V_2$  and  $V_3$  myosin (which are more alkali-labile than  $V_1$  myosin; Pope et al., 1980) would tend to reduce the ATPase activity of the ventricle relative to a 100%  $V_1$  ventricle (Weisberg et al., 1982). In order to test whether the different staining of atrial and ventricular muscle after acid pre-treatment could also be explained by the presence of  $V_3$  myosin in the ventricle, the atrial and ventricular myosin ATPase activities were compared in muscle from a larger rat.

It is well known that 'slower' myosins are generally more acid stable than 'faster' myosins (e.g. Brooke & Kaiser, 1970). It seems unlikely therefore that the presence of  $V_3$  myosin (which ought to retain more of its ATPase activity than  $V_1$ , and hence tend to produce a darker staining when found in higher quantities) could lead to a paler ventricle. However if this were the case then the difference ought to be more apparent in larger rats which have more  $V_3$  myosin in their ventricles (Klotz et al., 1981; Swynghedauw et al., 1982), as was the case with the alkaline pre-treated sections (comparing Fig.48a & Fig.50a). However, as Fig.50b shows the ventricle stains more darkly in this case, indicating that  $V_3$  myosin is indeed more acid stable than  $V_1$  myosin. It is therefore, not possible to explain the paler staining of the ventricle than the atrium in Fig.48b in terms of the presence of  $V_3$  myosin in the ventricle.

It is possible, however, that the different pattern of staining in Fig.50b could be due, at least in part, to a change in the myosin composition of the atrium. Hoh et al. (1978) have shown that rat atrium contains two myosin isoforms; therefore potential for a change in myosin distribution in the rat atrium does exist. However, Hoh et al. also showed that the two atrial myosin isoforms, unlike the three ventricular isoforms, had identical  $Ca^{++}$ -activated ATPase activities. It seems likely, that even if an age-dependent change in atrial myosin distribution does take place, it will not affect the ATPase activity of the atrial muscle and hence is unlikely to affect the relative staining intensities of atrial and ventricular fibres. There remains the possibility of

course that the two atrial isoforms have different pH labilities, although there is to my knowledge no information on this point.

It seems that the differences in myosin ATPase staining intensities shown here between rat atrium and ventricle cannot be explained in terms of light chains nor by the presence of multiple myosin isoforms in either muscle. This being so, it must be concluded that HC<sub>α</sub> of the rat atrium and HC<sub>α</sub> of the rat ventricle are distinct proteins.

This conclusion is supported by the immunohistochemical evidence presented here. The difference in staining intensity seen between the atrium and ventricle in Fig.48c & Fig.50c indicates that different myosin heavy chain species are present in the atria and ventricles of the rat (the immunoblot specificity test shows the antibody is directed mainly against the myosin heavy chain). It is possible that the difference seen here is due to different distribution between atrium and ventricle of the protein in the 'intermediate' band of Fig.47b. It seems likely, however, that this band represents a myosin heavy chain degradation product (to which one would expect the antibody to bind) since its molecular weight (≈60000 Da) corresponds to neither vimentin (Granger & Lazarides, 1980) nor desmin (Lazarides & Hubbard, 1978; which both have molecular weights of about 52000 Da) nor to any of the myofibrillar proteins described by Starr & Offer (1971).

Taken together the histochemical and immunohistochemical evidence presented here strongly suggests that the heavy chains of rat atrial and ventricular myosins are not identical proteins.

SUPPLEMENTARY CHAPTER 2

REPTILE ATRIO-VENTRICULAR DIFFERENCES

### Introduction

In recent years a great deal of work has been done on cardiac isoforms of myosin. Following the work of Sartore et al. (1978) which showed that atrial and ventricular fibres of the chick heart contained immunologically distinct myosin isoforms, other studies have found differences between atrial and ventricular myosins of many other species. Most of this subsequent work has been done on mammals. For example, Hoh et al. (1978) have shown differences in the electrophoretic mobilities of rat atrial and ventricular myosins and in the rabbit differences have been shown between the myosins contained by atrial and ventricular fibres using ATPase activities (e.g. see Results in this thesis), immunological properties and peptide maps (Dalla-Libera & Sartore, 1981). In larger mammals such as man and beef, differences have also been shown between atrial and ventricular myosins using these same methods (Thornell & Forsgren, 1982 and this thesis; Schiaffino et al., 1984; Flink et al., 1978).

It has, therefore, been established that differences exist between atrial and ventricular myosins in mammals. It appears, however, that no studies comparing atrial and ventricular myosins have been done in reptilian species. It seemed appropriate therefore, when a crocodile (*Caiman crocodylius*) and a terrapin (*Chrysemys scripta elegans*) became available to examine the histochemical myosin ATPase staining in these hearts for indications of heterogeneity. In order to do this acid and alkaline pre-treatments were used. These were used because it is quite

general that 'fast' myosins (atrial myosin in mammals is generally 'faster' than ventricular myosin i.e. has a higher ATPase activity; e.g. Yazaki et al., 1977) tend to be alkali-stable relative to 'slow' myosins (Pope et al., 1980) and 'slow' myosins acid-stable relative to 'fast' myosins (Brooke & Kaiser, 1970).

I also examined metabolic capacity of the atria and ventricle of each of these animals to see if, as seems to be the case in mammals, any differences in myosin distribution between atrium and ventricle can be attributed to differences in workload.

Methods

One adult terrapin (*Chrysemys scripta elegans*) and one crocodile (*Caiman crocodylius*) of about 2 months in age were used in this study. The hearts were excised and composite frozen blocks of atrium and ventricle made as described above (p.20). Fresh, frozen sections were prepared for staining also as described (p.20).

Staining Methods

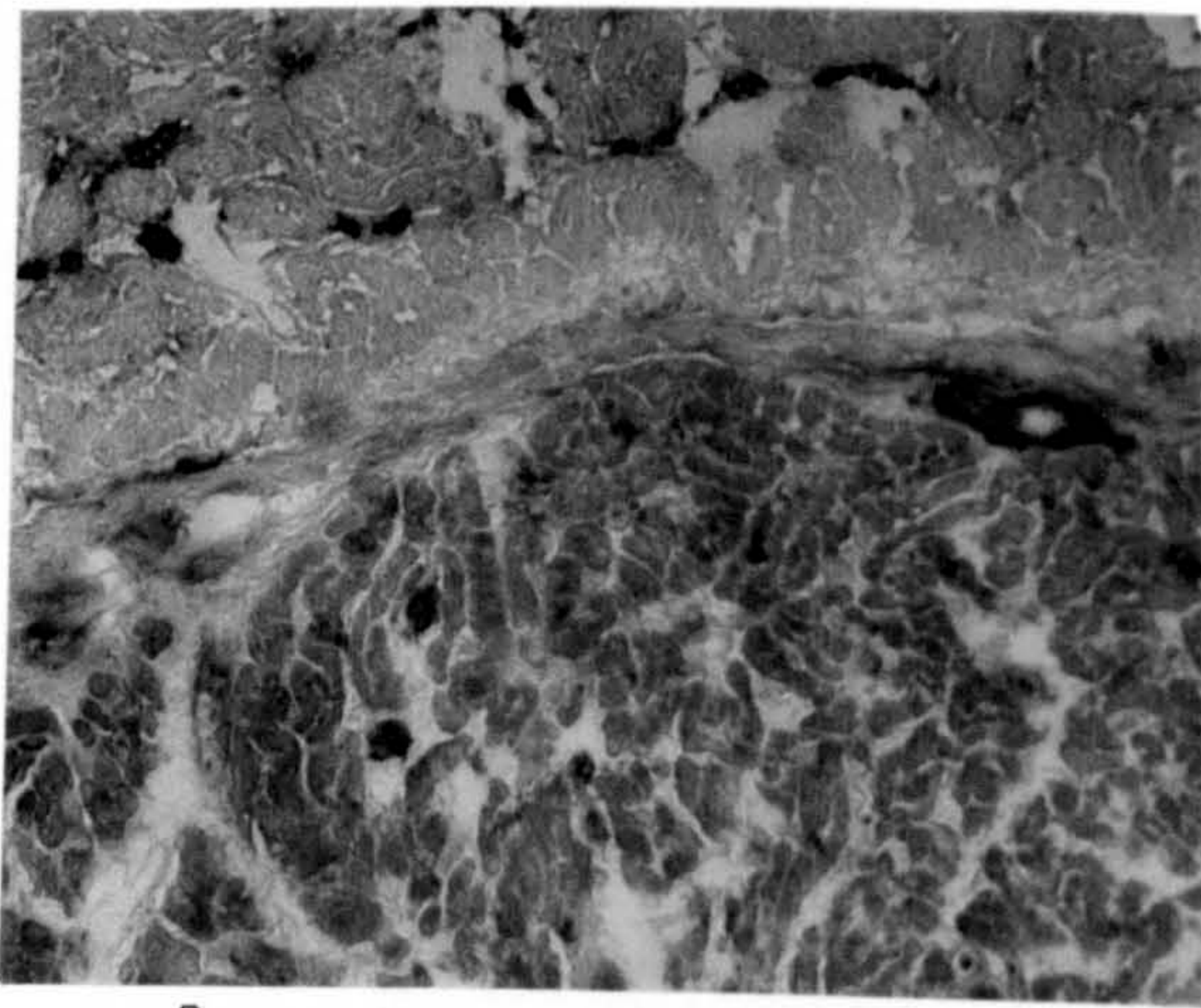
All staining procedures used in this study have been described earlier (p.21-24), with one exception: that for NADH-tetrazolium reductase. Sections were incubated for about 15 min in the solution listed below:

0.9ml NBT buffer + 0.1ml dist. water

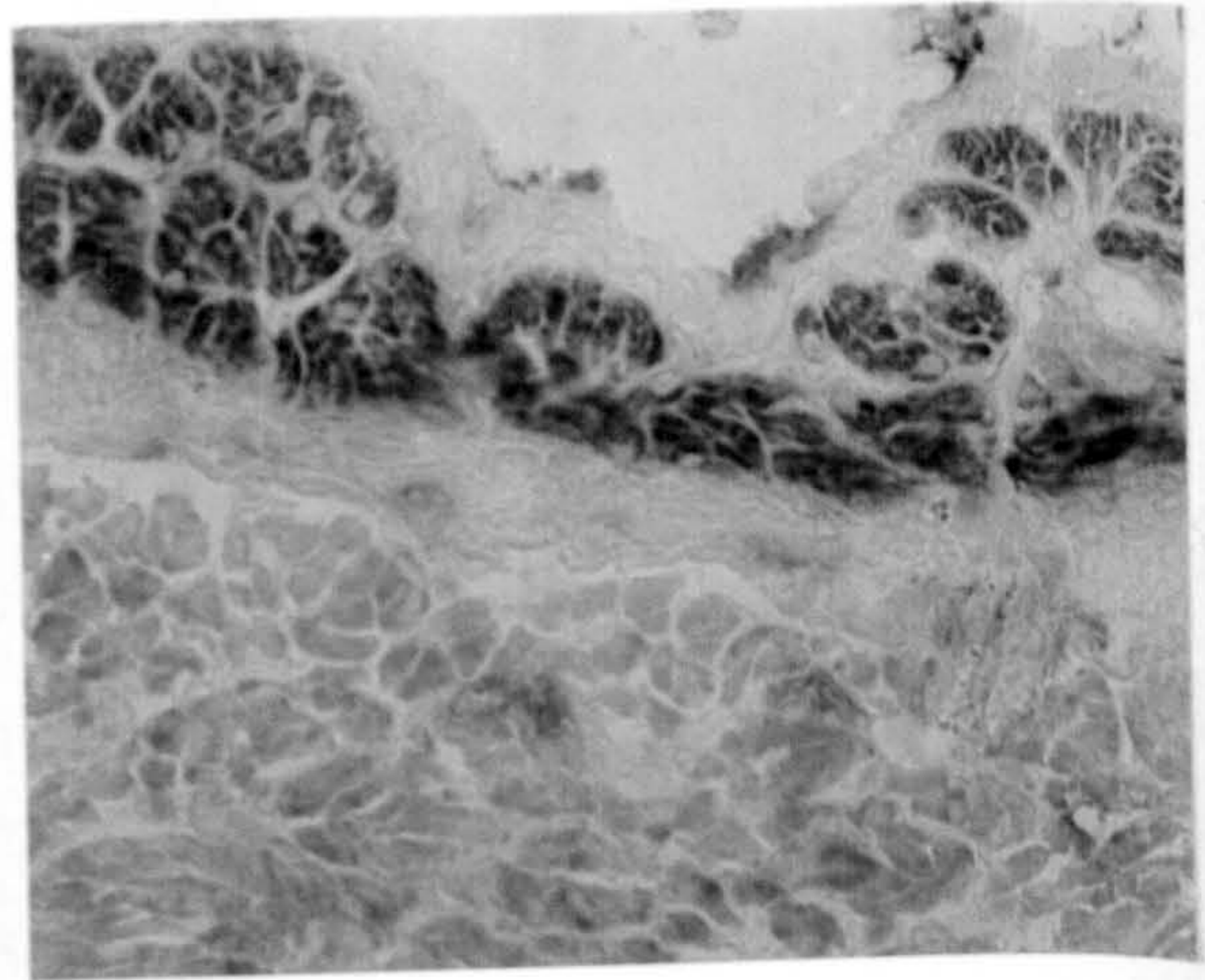
+ 2mg NADH

For composition of NBT buffer see Methods (p.23). The conditions under which incubation of sections for this stain were done were identical to those used for SDH and  $\alpha$ GPDH.





a.



b.

Fig.52 Myosin ATPase staining of composite block of terrapin atrium (top) and ventricle (bottom)

a. mATPase, acid pre-treated (pH 4.6) x160.

b. mATPase, alkali pre-treated (pH 10.2) x400

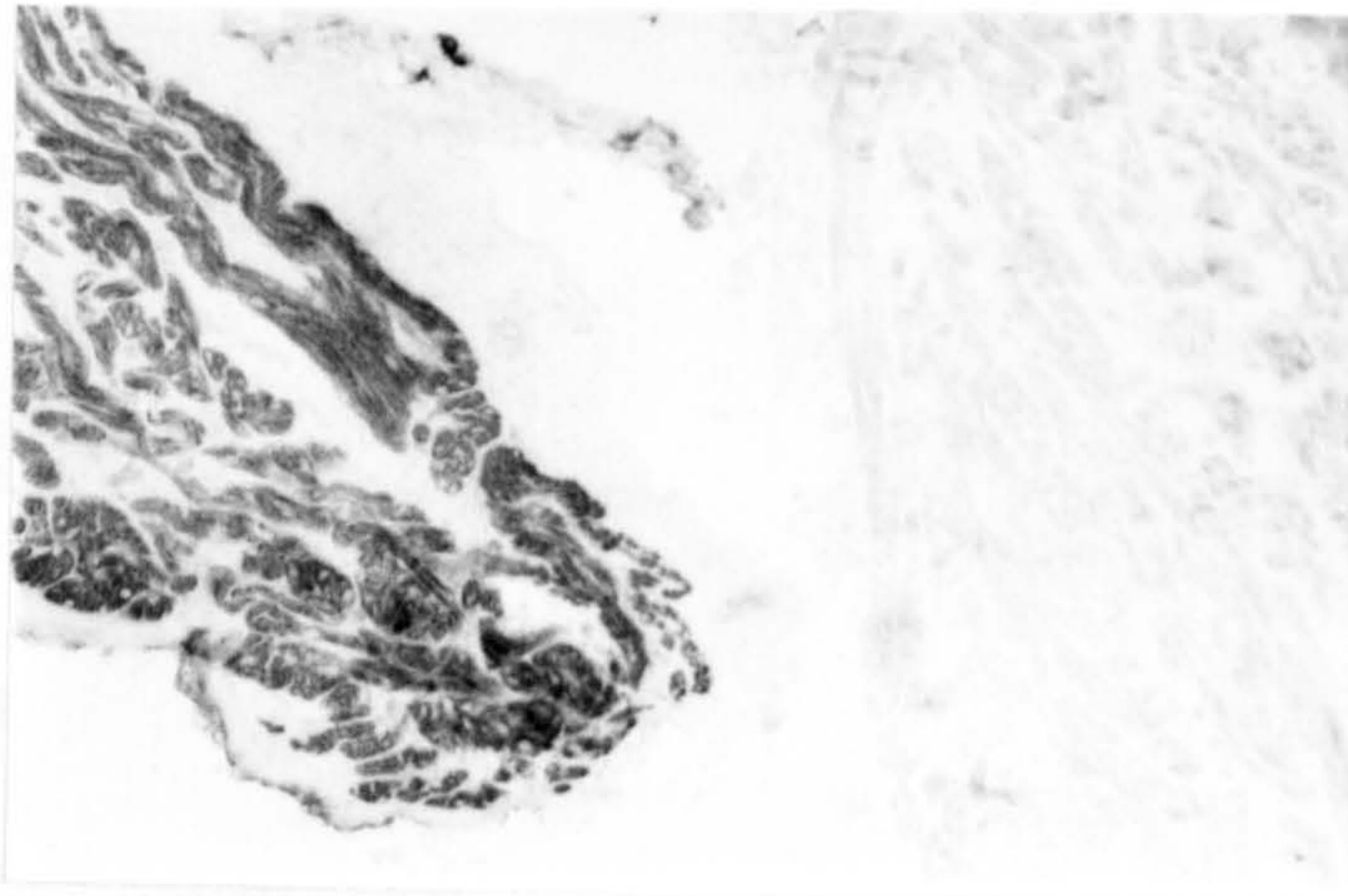


Fig.53 Myosin ATPase staining of composite block of crocodile atrium (left) and ventricle (right) after alkaline pre-treatment (pH 10.2) x160.

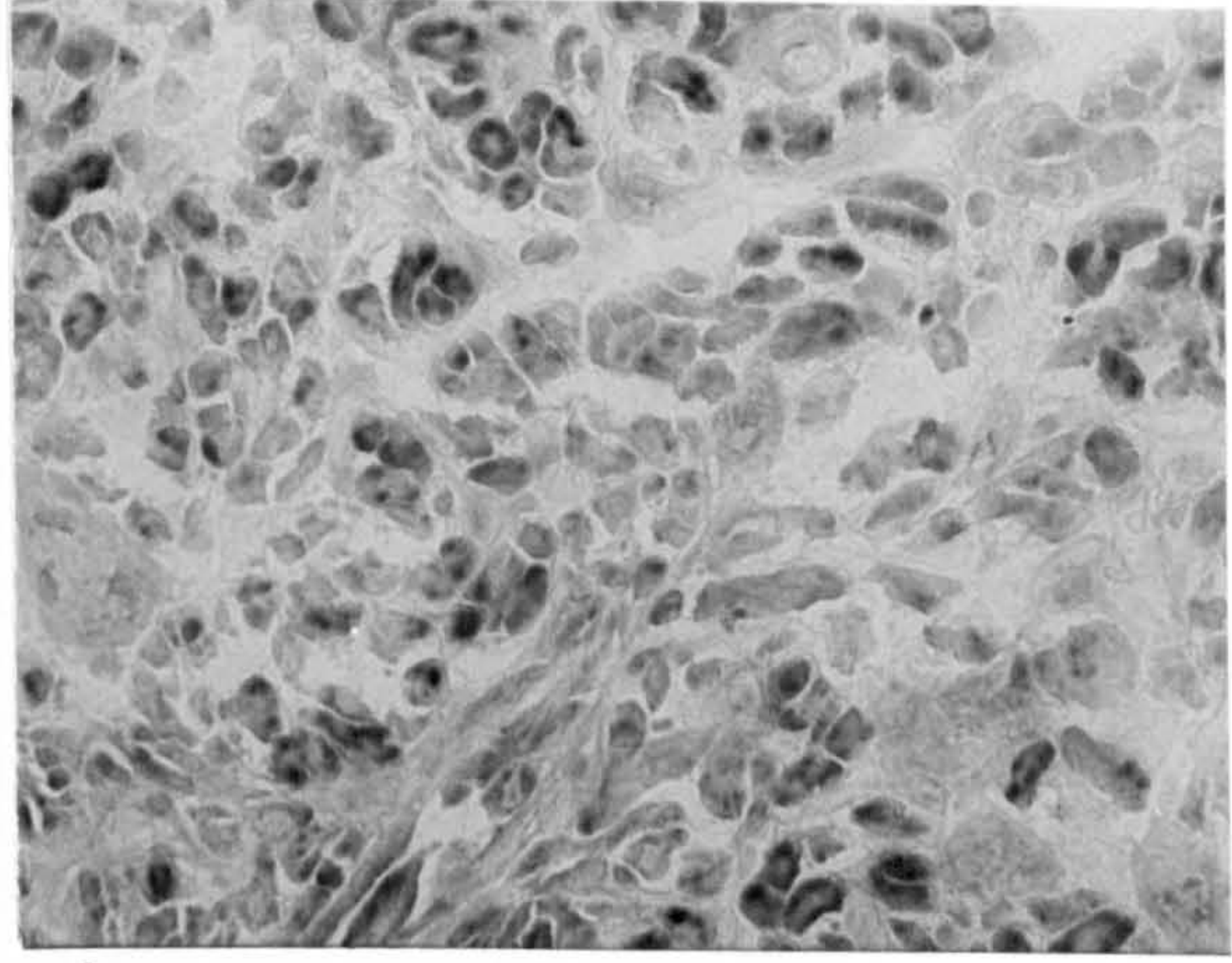
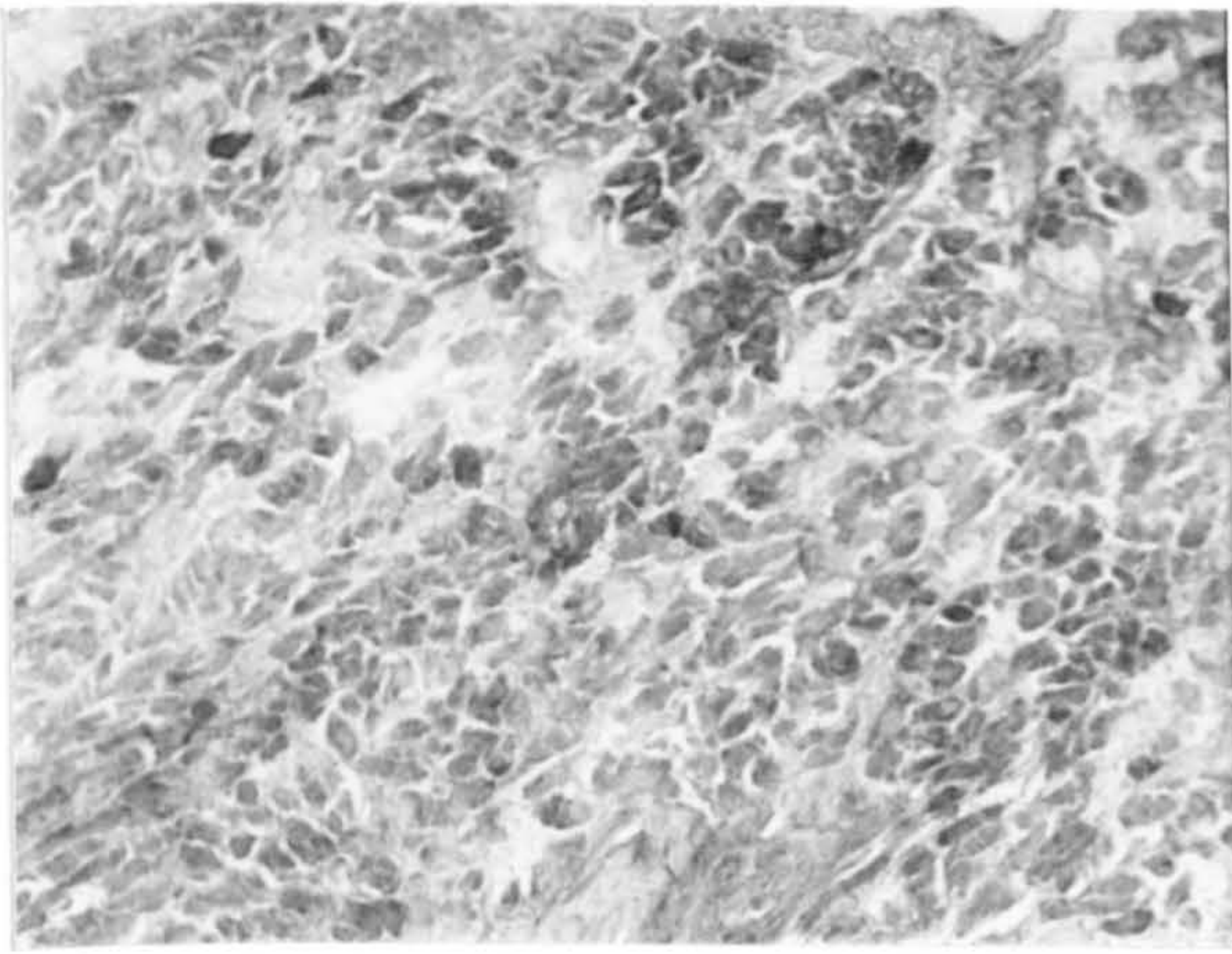
Results

Figs.52a & b show terrapin atrial (top) and ventricular (bottom) muscle stained for myosin ATPase activity after acid and alkaline pre-treatment respectively. After acid pre-treatment (pH 4.6) the ventricular muscle stains more darkly than the atrial muscle whereas after alkaline pre-treatment (pH 10.2) the atrial muscle stains more darkly than the ventricular muscle.

Fig.53 shows crocodile atrial (left) and ventricular (right) muscle pre-treated in an alkaline medium (pH 10.2) before staining for myosin ATPase activity. In this case, as with the terrapin, the atrial muscle stains more darkly than the ventricular muscle; however it was not possible to demonstrate differences in myosin ATPase staining intensity after acid between the crocodile atrium and ventricle after acid pre-treatment..

The myosin heterogeneity found in this work was not restricted to the atrio-ventricular differences presented above; shown in Figs.54a & b are regions of terrapin ventricle stained for myosin ATPase activity after (a) acid pre-treatment (pH 4.6) and (b) alkaline pre-treatment (pH 10.1). After both acid and alkaline pre-treatments individual fibres stained with different intensities. No such ventricular heterogeneity was found in the crocodile ventricle.

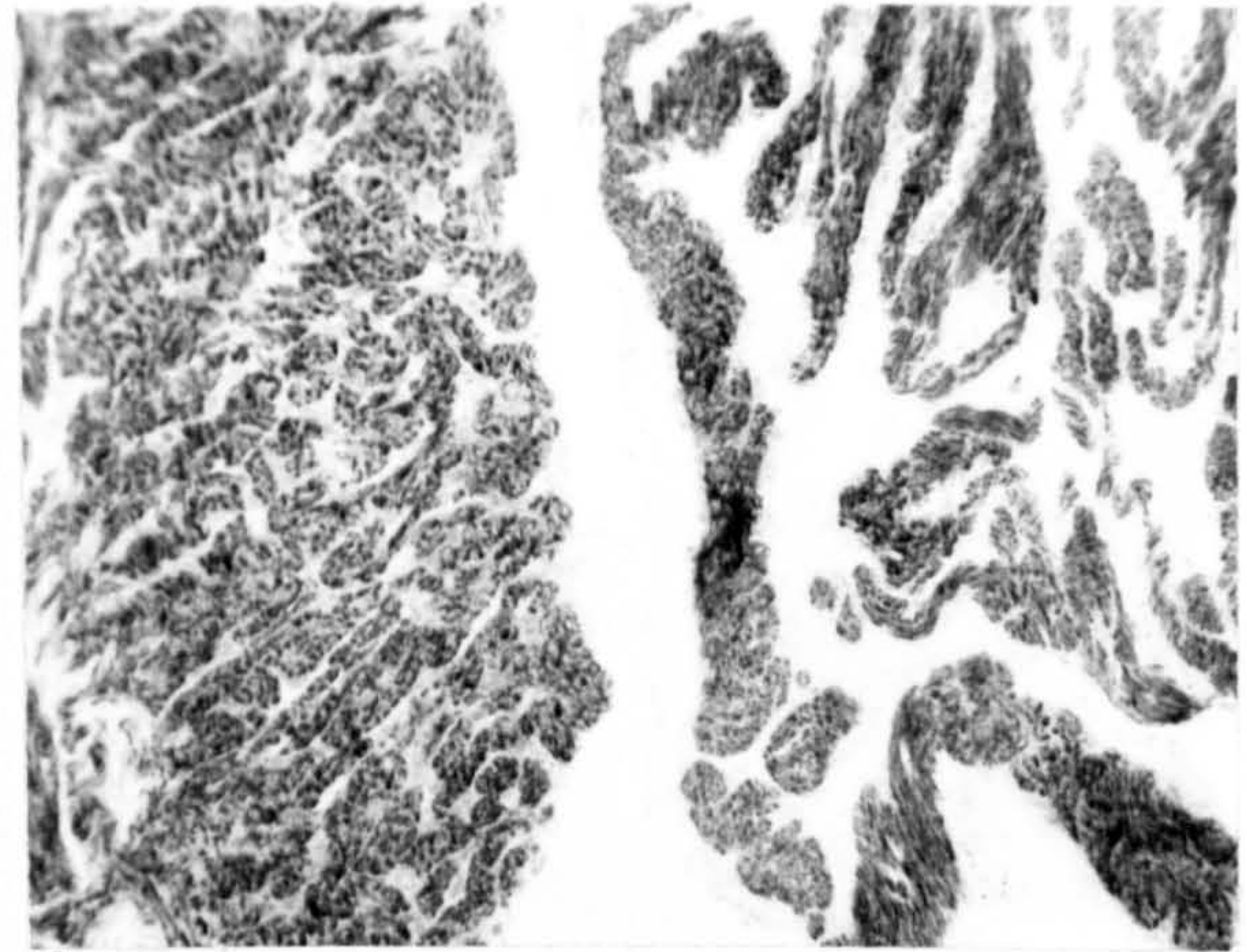
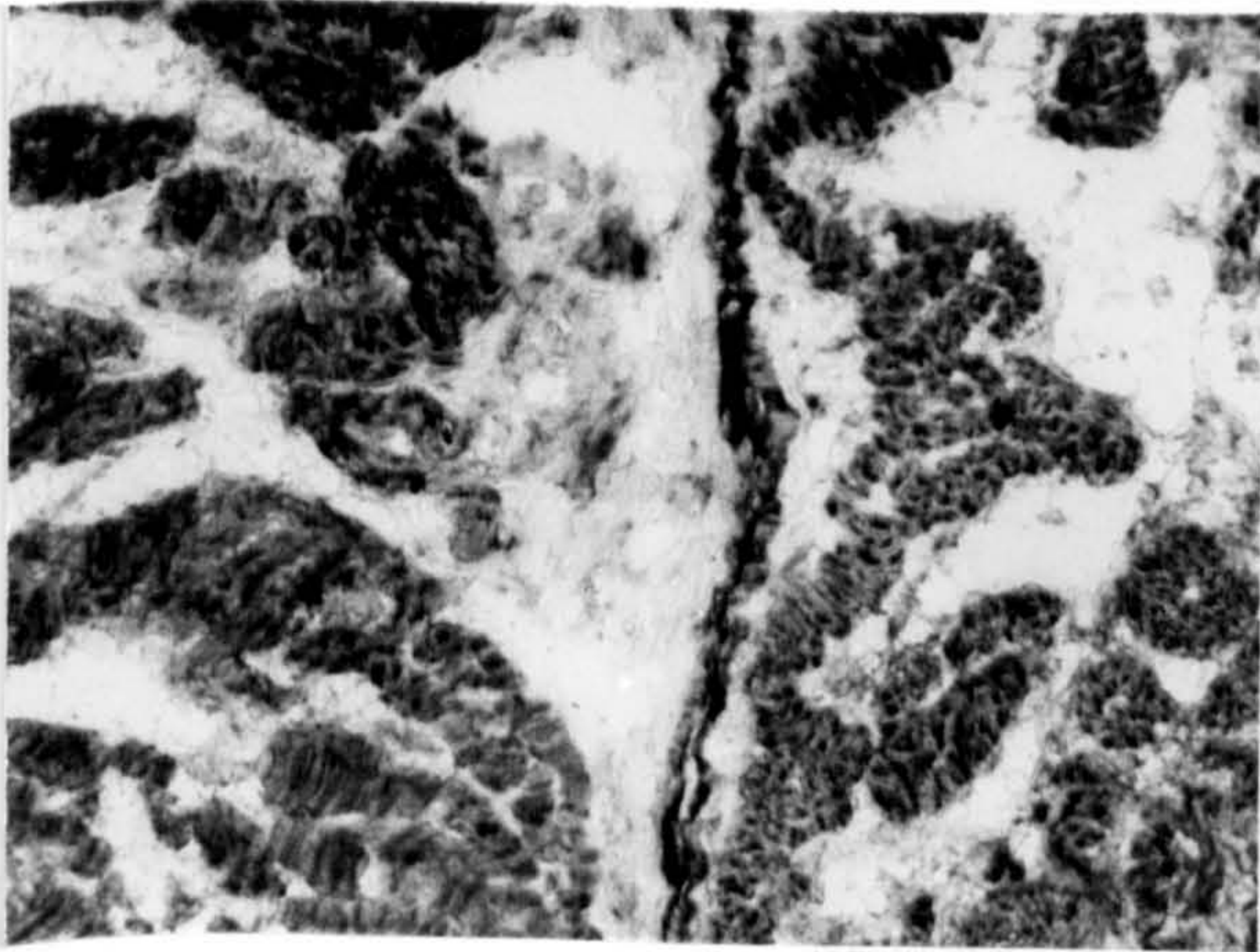
It seems that, as in the mammalian heart, two kinds of cardiac myosin heterogeneity can be demonstrated in the reptilian heart i.e. between atrial and ventricular myosins (in both terrapin and crocodile) and within the ventricle between the myosin contained by neighbouring fibres (in the terrapin only).



a. Fig.54 Myosin ATPase staining of terrapin ventricle after:

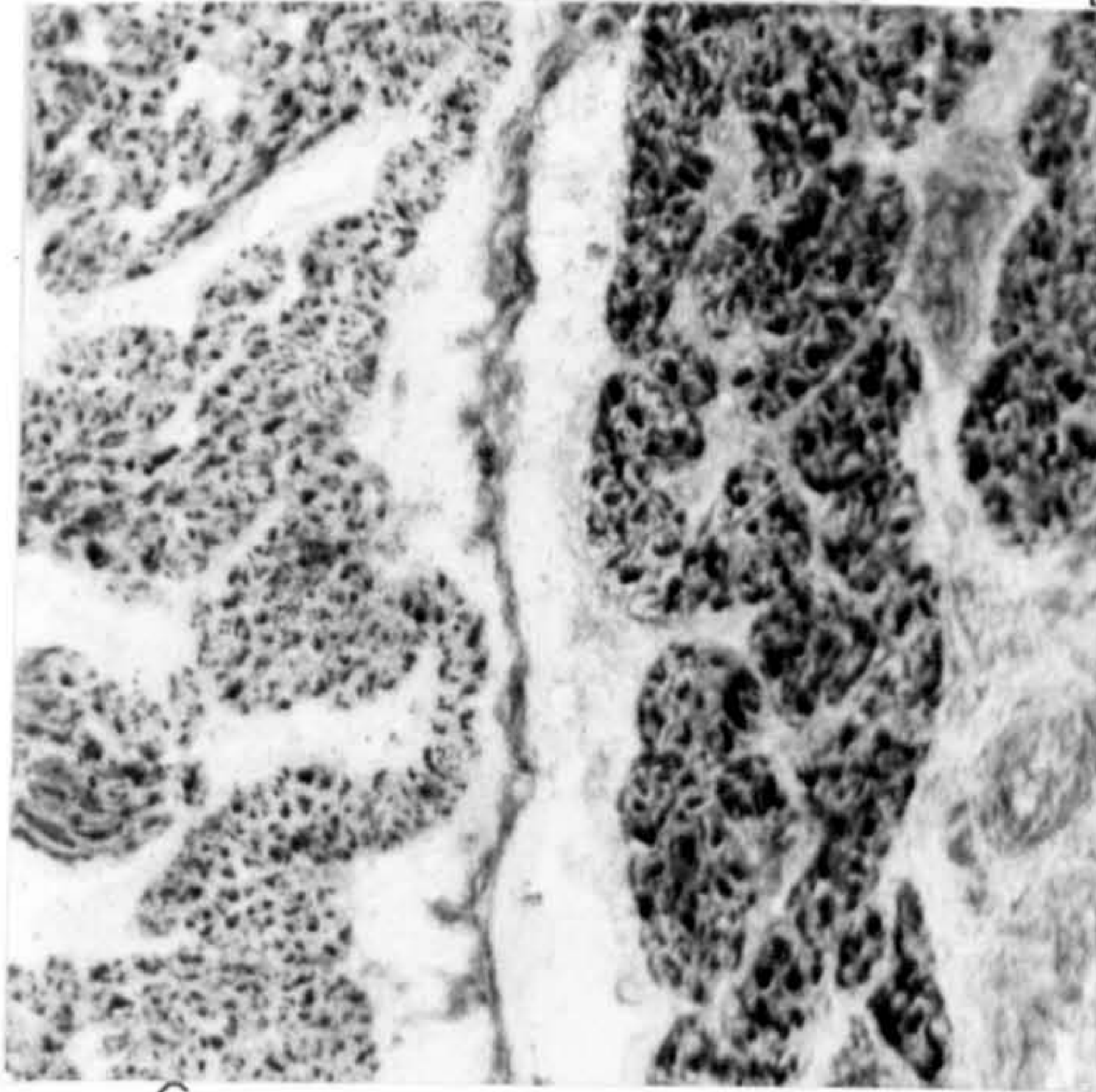
a. acid pre-treatment (pH 4.6) x250

b. alkaline pre-treatment (pH 10.1) x400



a.

b.



c.

Fig.55 Terrapin atrium (left) and ventricle (right) stained for:

a.  $\alpha$ -glycerophosphate dehydrogenase activity x250.

b. succinate dehydrogenase activity x100.

c. NADH -tetrazolium reductase activity x160.

The relative metabolic capacities of atrium and ventricle were also examined in this work. In Figs.55a-c are shown terrapin atrium and ventricle stained for oxidative and glycolytic enzyme activities. The oxidative enzymes used were succinate dehydrogenase (SDH) and NADH tetrazolium reductase (NADH-TR), the glycolytic enzyme used was  $\alpha$ -glycerophosphate dehydrogenase ( $\alpha$ -GPDH). Figs.55a & b show that  $\alpha$ -GPDH and SDH activity are evenly distributed between atrium and ventricle. Fig.55c shows, however, that the same is not true for NADH-TR; the stain for this enzyme being heavier in the ventricle than in the atrium.

In the crocodile the situation is somewhat different. Figs.56a & b show that  $\alpha$ GPDH and SDH are unevenly distributed whereas NADH-TR is evenly distributed in the atrium and ventricle. SDH activity is higher in the ventricle than in the atrium whereas  $\alpha$ GPDH is higher in the atrium than in the ventricle.

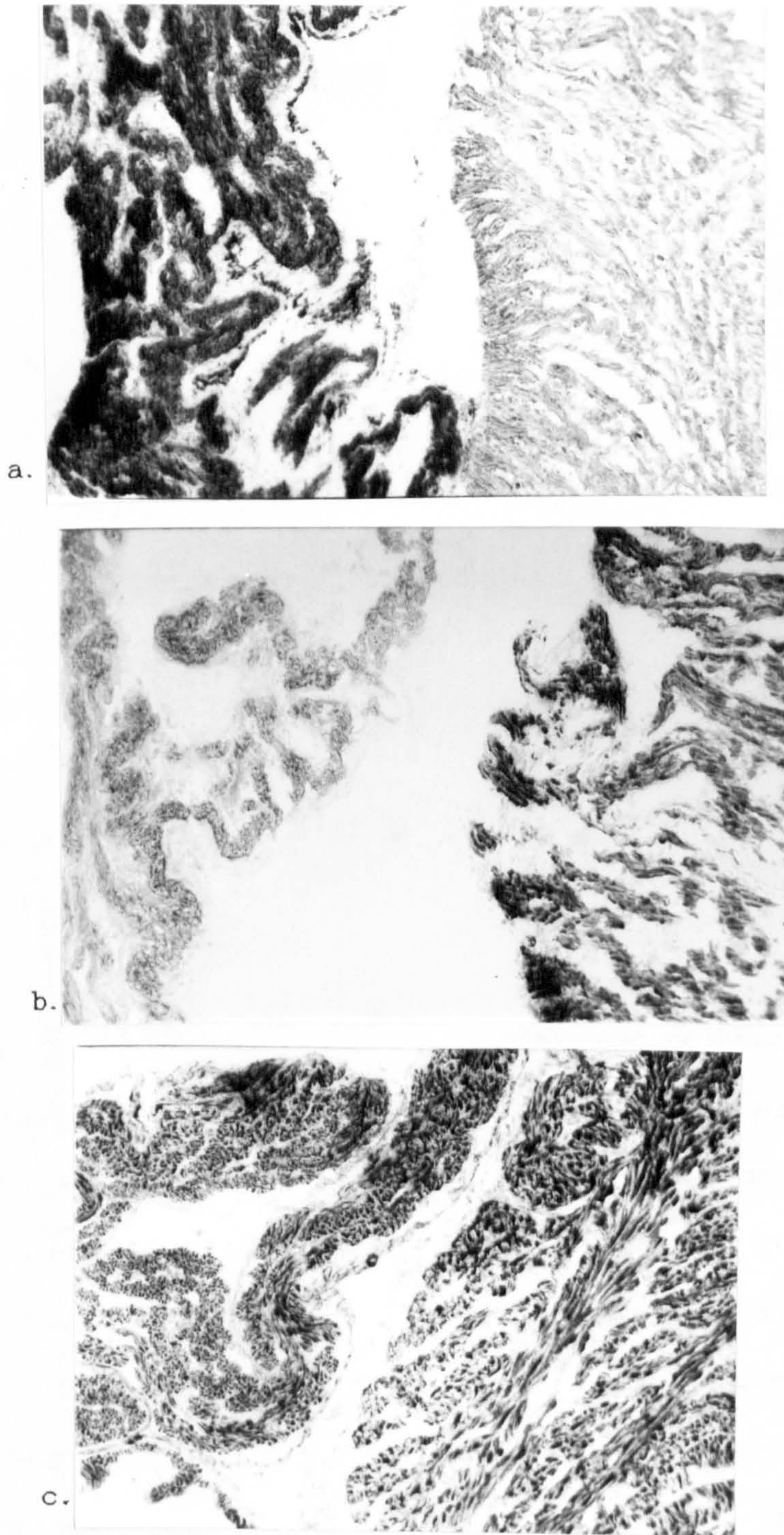


Fig.56 Crocodile atrium (left) and ventricle (right) stained for:

a.  $\alpha$ -glycerophosphate dehydrogenase activity x250.

b. succinate dehydrogenase activity x160.

c. NADH -tetrazolium reductase activity x160.

Discussion

The work which has been done in recent years on cardiac myosin isoforms has concentrated mainly on mammalian hearts. Although some of the pioneering work in this field was done in avian hearts (Sartore et al., 1978) the situation in lower vertebrates such as reptiles is largely unknown. The present work was carried out in order to investigate whether multiple cardiac myosin isoforms are found in reptiles. The results presented here suggest that reptilian hearts are similar to mammalian hearts in respect of myosin distribution.

In both terrapin and crocodile the ventricle contains a less alkali-stable myosin than the atrium and therefore stains less darkly than the atrium after alkaline pre-treatment. The difference in alkali-stability seen here is an indication that different myosins exist in reptilian atria and ventricle(s) and is very similar to the situation found in mammalian species e.g. all the species examined in this thesis. The difference in acid-stability between terrapin atrium and ventricle seen in Fig.52 is further evidence that there are different myosins (or different proportions of the same myosins) in these muscles. The lack of differentiation between crocodile atrium and ventricle after acid pre-treatment indicates only that the myosins involved have similar acid-stabilities; the difference in alkali-stability is enough to allow one to conclude that different myosins are present in the crocodile atrium and ventricle.

In mammalian species the fact that different myosins are found in the atrium and ventricle can be attributed to the different workloads of the chambers. If workload can be defined as :

$$\text{Volume moved} \times \text{Pressure}$$

then the workload of the atrium is lower than that of the ventricle as both the pressure in and the volume moved by the atrium during a contraction are lower than in the ventricle (atrial systole only increases the volume in the ventricle by a very small amount; Milnor, 1974). Although the anatomical arrangement of the ventricles in mammals is different to that in reptiles i.e. reptiles have only one ventricle (Young, 1981) this does not alter the fact that the ventricle works under higher pressure than the atrium and hence works harder.

This difference in workload is reflected in the metabolic capacities of the atria and ventricles of the rabbit heart (this thesis p.47) in which the harder working ventricle has a higher SDH activity. In order to test whether the differences in myosin type found here in reptilian hearts were also caused by differences in workload, sections were stained for activities of SDH, NADH-TR (oxidative enzymes) and  $\alpha$ GPDH (a glycolytic enzyme).

In the crocodile heart two of the three enzymes were not equally active in the two muscles i.e. SDH and  $\alpha$ GPDH. SDH is, as in the rabbit heart, higher in the harder-working ventricle than in the atria, but  $\alpha$ GPDH is higher in the atria. These differences in metabolic capacity between crocodile atrium and ventricle indicate that, as in mammalian species, the atrio-ventricular difference in myosin distribution is due to a difference in workload.

In the terrapin heart SDH and  $\alpha$ GPDH staining intensities are equally high in atrium and ventricle, however, NADH-TR (which showed no atrio-ventricular difference in the crocodile) is higher in the ventricle than in the atrium. While it is not clear why one oxidative marker should show the inter-chamber difference in one species, and the other marker in the other species, the general indication surely remains that, as in the rabbit, oxidative capacity is higher in the harder working ventricle. It thus appears, from the evidence available, that the atrio-ventricular differences in myosin distribution in both the crocodile and terrapin can be explained in terms of the different workloads of these chambers.

In the terrapin it is also possible to show myosin heterogeneity between individual fibres of the ventricle (Figs. 54a & b). Similar heterogeneity has been shown to exist in rabbit, ferret and guinea-pig ventricles in this thesis and in the rabbit ventricle by Weisberg et al., (1982). It is not possible to tell from the evidence here how many ventricular myosin isoforms exist, only that there is more than one.

The fact that no heterogeneity was visible in the crocodile ventricle does not necessarily mean that only one ventricular myosin exists in this species. It is quite possible that the crocodile resembles the rat in this respect i.e. that more than one myosin isoform exists but that all fibres contain the same mixture. It is also possible that, if only one myosin isoform is present in the crocodile ventricle, it is due to the age of the particular animal studied - c.f. the fact that in the young rat only one



ventricular myosin exists (V<sub>1</sub>) which is gradually replaced by V<sub>2</sub> and V<sub>3</sub> as the animal ages.

The evidence presented suggests that all forms of cardiac myosin ATPase heterogeneity demonstrable in the hearts of small mammals also exist in reptilian hearts. In addition, the distributions of metabolic enzymes strongly suggest that the differences in myosin isoform between atrium and ventricle in reptilian hearts are due, as is the case in mammalian hearts, the different workloads of these chambers.

SUPPLEMENTARY CHAPTER 3

ISOLATED MYOCYTES

### Introduction

There are now many techniques for the isolation of cardiac myocytes from mammalian hearts (e.g. Powell et al., 1980; Isenberg & Klockner, 1982; Lundgren et al., 1984), most of which have much in common. Retrograde perfusion of the coronary arteries with a low calcium (nominally calcium-free) medium appears to be an almost universally used first step in the isolation of cardiac myocytes (one of the very few exceptions Isenberg & Klockner (1982) have isolated single myocytes from bovine hearts without perfusion, presumably the size of the bovine heart prohibited this procedure). The decalcification step is apparently responsible for separating myocytes from each other at the intercalated discs (Muir, 1966); the critical process seems to be the removal of calcium ions from the desmosomes which hold these structures together.

Calcium-free perfusion is normally followed by perfusion of the heart with an enzyme-containing medium. The most commonly used enzyme is collagenase, although it is often used in conjunction with other enzymes e.g. hyaluronidase (Glick et al., 1974) or trypsin which is to be found in the crude collagenase used by many workers (e.g. Powell et al., 1980). Myocytes are normally released from hearts following this stage by mincing and mechanical agitation, although some techniques require a further incubation in 'storage solutions' to confer 'calcium tolerance' on myocytes.

'Calcium tolerance' refers to the ability of myocytes to retain their rod-like morphology when returned to physiological concentrations of calcium (the enzyme perfusion is also carried out

at low calcium concentrations). Cells which are not calcium tolerant go into irreversible contracture and become rounded. This seems to be a consequence of membrane hyperpermeability to calcium brought about by the exposure to very low calcium concentrations (Reiser et al., 1979) and is similar to the phenomenon noted in whole hearts called the 'calcium paradox' (Zimmerman & Hulsman, 1966). Calcium sensitivity has been linked to the loss of the external lamina of the glycocalyx from the sarcolemma (Borgers et al., 1985) although Isenberg & Klockner (1980) had previously shown that the glycocalyx is not essential for calcium tolerance. Myocytes are most often isolated in order to study their electrophysiology; calcium intolerance, of course, makes this sort of study impossible.

An immunohistochemical study of the myosin content of isolated myocytes of the rat heart, has been carried out by Samuel et al. (1983). In this they show that more than one ventricular myosin can be present in individual ventricular myocytes notwithstanding the fact that cells from one heart all appear to have the same distribution of myosins. To my knowledge no studies have looked at myosin ATPase activity in isolated cardiac myocytes. Reported here are techniques, developed as part of a study to look for a correlation between myosin type and electrophysiology in single cells, which would allow myosin ATPase activity to be investigated in single cells.

Methods

Isolation Techniques

New Zealand White rabbits (2-2.5kg) were killed by a blow to the back of the head and their hearts rapidly removed and transferred to ice-cold Tyrode-like (henceforth referred to as Tyrode) solution. The aorta was then cannulated and the heart attached to the base of a Langendorff perfusion apparatus. The heart was then retrogradely perfused through the coronary arteries with Tyrode solution at 37°C. The Tyrode solution is listed below:

|                                  |         |
|----------------------------------|---------|
| NaCl                             | 136.9mM |
| NaHCO <sub>3</sub>               | 11.9mM  |
| KCl                              | 5.4mM   |
| CaCl <sub>2</sub>                | 1.8mM   |
| MgCl <sub>2</sub>                | 0.53mM  |
| NaH <sub>2</sub> PO <sub>4</sub> | 0.33mM  |
| HEPES                            | 5.0mM   |
| glucose                          | 11.0mM  |
| pH adjusted to 7.2 with NaOH     |         |

Once the blood had been washed out of the heart the perfusate was changed to calcium-free Tyrode i.e. the above solution with CaCl<sub>2</sub> omitted. After a three minute perfusion of calcium-free Tyrode the perfusate was changed again to a calcium-free Tyrode plus 0.4mg/ml collagenase (Sigma Type I). This solution was recirculated using a Watson-Marlow peristaltic pump to a continuously oxygenated reservoir approximately 1.5m above the level of the heart. This head of pressure gave an initial flow-rate

of about 25ml/min which increased as the heart was digested and resistance to flow decreased. Perfusion of the heart with the enzyme-containing solution was continued for 40min. At the end of this time, the collagenase solution was washed out with the storage solution shown below .

Storage Solution

|                                 |       |
|---------------------------------|-------|
| KCl                             | 25mM  |
| KH <sub>2</sub> PO <sub>4</sub> | 10mM  |
| glutamic acid                   | 70mM  |
| taurine                         | 10mM  |
| oxalic acid                     | 10mM  |
| glucose                         | 11mM  |
| EGTA                            | 0.5mM |

pH adjusted to 7.2 with KOH

The heart was then taken off the perfusion apparatus and the atria removed. The ventricles were cut into small pieces and placed in the above storage solution at 4°C for 60min. The calcium sensitivity of the ventricular cells was then tested by placing one piece in the normal calcium-containing Tyrode solution. Once calcium tolerance had been achieved, suspensions of myocytes were prepared by mechanical agitation of the pieces of ventricle in the calcium-containing Tyrode.

Single Cell Staining Techniques

Two methods were developed for the histochemical 'staining' of isolated cardiac myocytes. The first of these methods involved the preparation of a 'smear' of myocytes on a glass slide. A drop of a suspension of isolated myocytes was placed at one end of a glass

slide and drawn out by another slide to form a thin film. Glass slides treated in this way were then left to dry in air. During this drying process myocytes became attached to the slide and could then be stained for myosin ATPase activity.

Drying is, of course, an important step in ATPase staining of sections; ensuring proper adhesion of the section to the slide. Allowing the cells to dry out, therefore should not have any deleterious effect on the subsequent demonstration of myosin ATPase activity.

Another technique was developed for the staining of single, identified cells in response to problems with the above method. A drop of a dilute suspension of myocytes is placed on a 1-3mm thick sheet of 7% agarose (Marine Colloids Inc.). The fluid was then allowed to evaporate or removed with a tissue. As agarose is almost completely clear, single cells could when illuminated from below be seen on the surface of the sheet at moderate magnification (e.g. x20) using a dissecting microscope. Pieces of agarose to which a cell (or several cells) was adhering were then cut and shaped in such a way that, when the piece was mounted in a frozen block, transverse sections of the cell could be cut. If several cells were to be mounted in a block in this way agarose sheets thinner than 2mm were used to keep the block manageable. The thicker sheets were used when only one or two layers were to be in each block, so that blocks would be broad enough to stand on their own before freezing.

Agarose sheets, to which cells were adhering, were mounted in frozen blocks as described in Methods p.20.

Smears and sections of single cells were stained for myosin ATPase activity as described p. 21. No pre-treatments were used.





a.



b.



c.



d.

Fig.57 Isolated myocytes stained for myosin ATPase activity using the 'smear' technique; a-c x1000, d x250.

Results

Two methods for staining isolated cardiac myocytes were developed in this work. Results of the first of these is shown in Fig.57a-d. Each photograph (magnification x1000) in Fig.57 contains a myocyte (photograph d contains two myocytes; x250) stained for myosin ATPase activity. Myocytes retain their rod-like morphology during the staining procedure. Stain is largely confined to within the myocytes, although sometimes stain could be seen outside cells e.g. Fig.57c. From Fig.57a-d. it is clear that myocytes differ in their proportions and from Fig.57d. that they can stain differently with this technique. As there were several problems with this method of staining myocytes (see Discussion) another method was developed.

Fig.58a-d shows two myocytes stained for myosin ATPase activity using this second method. Shown are four serial sections (x100) of two myocytes imbedded in 7% agarose, the block containing these cells was oriented such that the upper cell would be cut transversely (the increasing distance between the myocytes in successive sections indicates that the lower myocyte was lying at an angle to the upper myocyte).

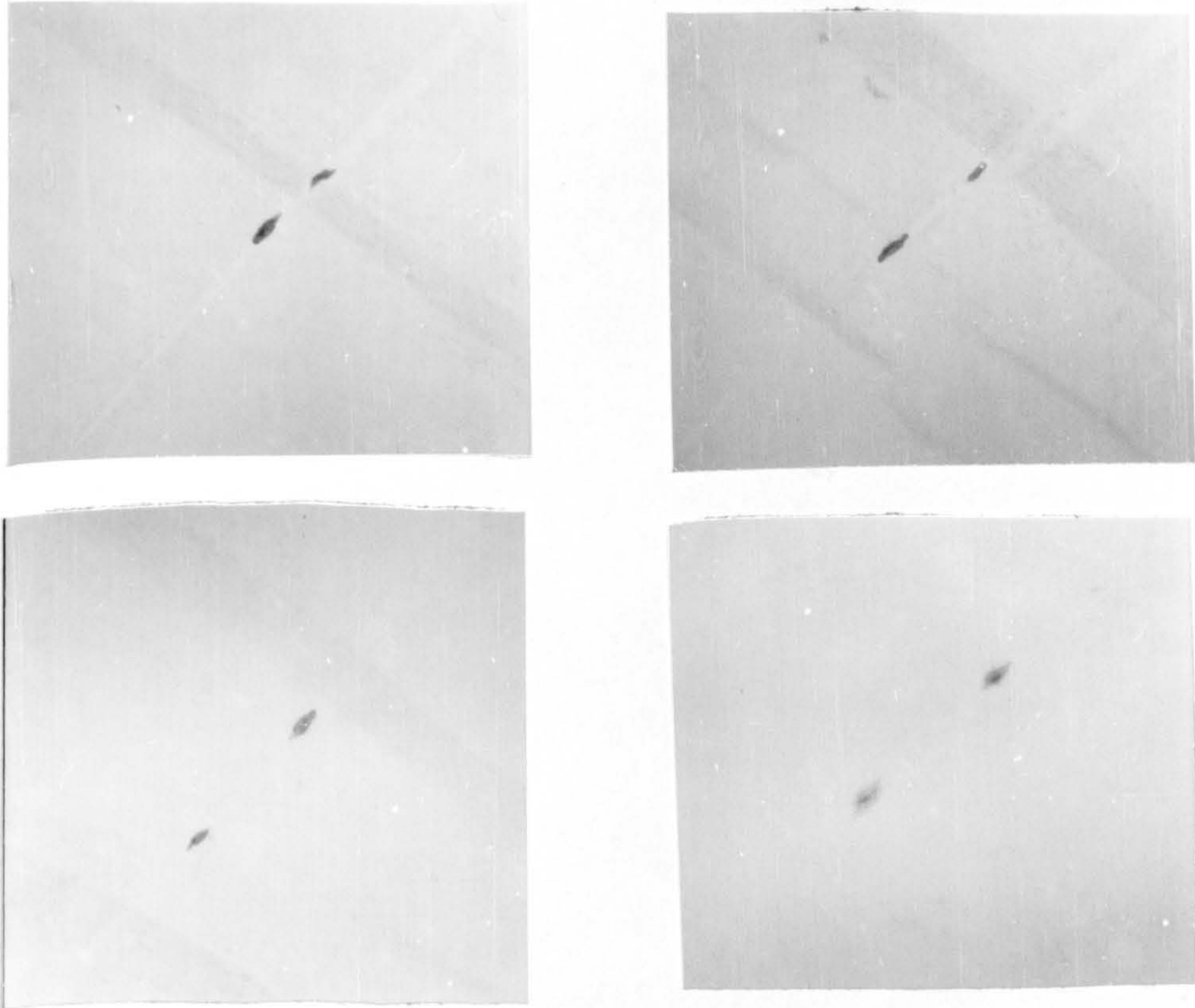


Fig.58 Four serial sections of two isolated myocytes stained for myosin ATPase activity. The myocytes are sandwiched between two layers of 7% agarose.

Fig.59a & b are electron micrographs of normal, intact rabbit cardiac muscle (x10000) and isolated rabbit myocyte (x8317) respectively. In Fig. 59a mitochondria size and cristae, myofilament spacing, sarcolemma and glycocalyx all appear to be normal. In Fig.59b however the mitochondria appear to be swollen and have large gaps between their cristae and the myofilaments are less densely packed than in Fig 59a. Also there are large gaps in the sarcolemma which appears to have lost its glycocalyx.

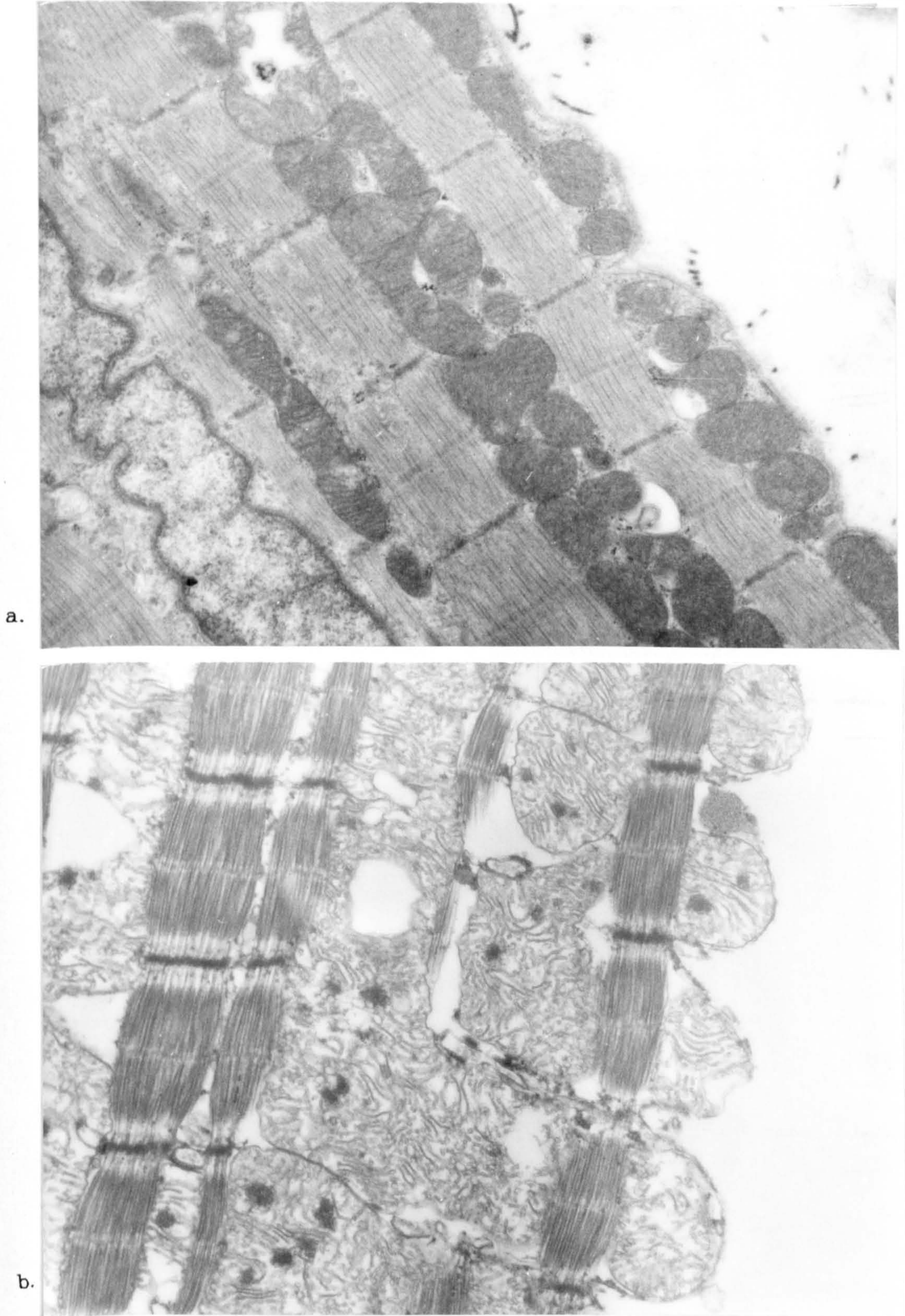


Fig.59a. Electron micrograph of normal, intact cardiac muscle of the rabbit (x10000).

b. Electron micrograph of isolated myocyte of rabbit heart (x8317).

### Discussion

The work presented in this chapter was done in order that single, identifiable myocytes could be compared in terms of both electrophysiology and myosin type (similar to the comparison made in pieces of cardiac muscle reported earlier in this thesis). Unfortunately the myocytes I was able to isolate, although they met some of the criteria used in assessing myocyte viability, were not electrophysiologically viable. I will discuss possible reasons for the non-viability of these cells after dealing with the techniques developed for their myosin ATPase staining.

The first technique tried involved the preparation of a monolayer of myocytes on a glass slide. This was done by allowing a 'smear' of a myocyte suspension to dry out onto the slide. During the drying process myocytes become attached to the slide and could then be stained. The drying should not have any adverse effect on the myosin ATPase activity as a very similar process takes place after sectioning of intact muscles.

This method had the advantage that it was very simple but had several important disadvantages. It was noticeable if one compared the number of myocytes attached to the slide before and after staining that myocytes were lost from the slide during staining. This is not surprising when one considers that the staining procedure involves several changes of solution which would almost certainly wash cells from the slide. This is no great disadvantage if one is interested only in myocytes in general, as it can be overcome simply by increasing the number of myocytes on the slide.

If, however, one wishes to stain identified myocytes then loss of cells during staining is a severe disadvantage.

Another disadvantage of the 'smear' technique can be seen by looking at the stained myocytes shown in Fig.57a-d. The irregular shape of cardiac myocytes means that one cannot be sure of the thickness of cells. Unless one knows that two myocytes are the same thickness, then relative staining intensities of the two cannot be interpreted in terms of myosin type (this is analagous to the problem of thickness variation encountered in conventional histochemistry of sectioned tissue).

Other problems associated with this method were that myocytes had to be viewed longitudinally (which would have presented problems with distribution error if photometric readings were required) and a brown deposit covering most but not all of the slide (this too would have presented problems in photometry as the layer of deposit was not uniform over all myocytes). This deposit is probably due to incomplete washing of the inorganic chemicals used in the final staining procedure. For these reasons a method involving sectioning of single myocytes was developed.

Fig.58a-d shows that it is possible to cut and stain for myosin ATPase activity serial sections of oriented single cardiac myocytes. Only the upper of the myocytes shown is cut transversely the other is cut obliquely (this is suggested by the increasing distance between the myocytes in each section). The fact that four serial sections can be cut from one myocyte means that each section can be given a different pH pre-treatment before staining. This would ensure optimal differentiation between myocytes containing

different myosin distributions. This pilot study was, however, terminated before this was attempted as it was realised that although 'calcium-tolerant' the myocytes obtained here were electrophysiologically non-viable, as they did not respond to electrical stimulation.

In order to find a possible explanation for the non-viability of the myocytes, an electron microscopic study comparing isolated myocytes with intact cardiac muscle was done. Several obvious differences existed between the preparations: the appearance of the mitochondria; the number and size of vacuoles; the appearance of the myofibrils and the continuity of the sarcolemma.

The large gaps in the sarcolemma of the isolated myocytes would mean that the myofilaments would be exposed to very much higher than normal concentrations of calcium. The obvious damage to the mitochondria and sarcolemma suggests how these cells could retain their rod-like morphology under these conditions. It is probable that these cells would have been unable to maintain ATP concentrations, through lack of functioning mitochondria and loss of glycolytic enzymes and ATP through the sarcolemma. With no ATP available, cross-bridge cycling and hence hypercontracture would be impossible. This implies that the damage to mitochondria and the sarcolemma was done before the re-introduction of physiological levels of calcium, as were the membrane to become leaky in the presence of calcium, hypercontracture would occur because ATP would not have fallen to zero. This does not however explain how the damage occurs.



One area of damage, the cause of which is known, is the loss of the glycocalyx. Those portions of the sarcolemma still to be seen appear to have no glycocalyx. It has been shown by Muir (1966) and Crevey et al. (1978) that exposure to low calcium concentrations cause the glycocalyx to be lost. As most of the cell isolation procedure is done at low calcium concentrations this would appear to explain the loss of the glycocalyx from the isolated myocyte in Fig.59b. Isenberg & Klockner (1980) have shown, however, that the glycocalyx is not required for electrophysiological viability and so it is unlikely that the low calcium concentrations are the only source of damage. Another candidate is the enzyme, as it is well known that batches of enzyme vary in their ability to produce viable cells (Powell 1980).

Although the electron micrographs shown here give no clue as to what has caused the damage to the myocytes, they do suggest how the cells could be 'calcium-tolerant' yet electrophysiologically non-viable.

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