Electrophoretic Analysis of Multiple Forms of Myosin in Fast-Twitch and Slow-Twitch Muscles of the Chick

By JOSEPH F. Y. HOH, PAUL A. McGRATH and ROSS I. WHITE
Department of Physiology, University of Sydney, Sydney, N.S.W. 2006, Australia

1. A method is described for the electrophoretic analysis of intact myosin in polyacrylamide gel in a buffer system containing 0.02M-pyrophosphate and 10% (v/v) glycerol, pH 8.8. 2. In this system chicken skeletal-muscle myosins reveal five distinct electrophoretic components, three components from the fast-twitch posterior latissimus dorsi muscle and two slower-migrating components from the slow-twitch anterior latissimus dorsi muscle. 3. The Ca^{2+}-activated ATPase (adenosine triphosphatase) activity of myosin components was measured by densitometric scanning of the gel for the $Ca_3(PO_4)_2$ precipitate formed during the ATPase reaction and subsequently for stained protein. Each component from the same muscle appears to have identical ATPase activity, but components from the fast-twitch muscle had an activity 2.2 times higher than those from the slow-twitch muscle. 4. On re-electrophoresis in the same buffer system, individual fractions of fast-twitch myosin did not reproduce the three-band pattern of the original myosin, but migrated at rates consistent with their original mobility. 5. Analysis of the mobility of the three fast-twitch myosin components in gels of different concentrations suggests that they are not stable oligomers of each other. 6. It is suggested that these components of fast-twitch myosin and slow-twitch myosin are isoenzymes of myosin.

Biochemical studies on myosin have revealed that this molecule plays a key role in regulating the dynamic properties of muscle. By measuring the ATPase* activities of myosins extracted from muscles whose speeds of shortening have previously been measured, Bárány (1967) showed that over a wide range of speeds, the ATPase activity of myosin was proportional to the speed of shortening. This finding is highly significant from the thermodynamic point of view, since it relates the rate of ATP utilization by the contractile machinery to the rate of power output of the muscle, the latter being proportional to the force and speed of contraction.

Differences in dynamic properties of muscles in the same animal are reflected in differences in structure and properties of myosin they contain. Avian (Canfield, 1971) and mammalian (Close, 1972) muscles may be broadly classified into fast-twitch and slow-twitch types, and myosin extracted from these types of muscles differ in ATPase activity (Bárány, 1967; Melichna *et al.*, 1974), light-chain composition (Sarkar *et al.*, 1971; Lowey & Risby, 1971) and electrophoretic mobility (d'Albis & Gratzer, 1973; Hoh, 1975a). The possibility that myosin in skeletal muscles of adult animals may be more heterogeneous than described above is suggested by a number of observations on the physiological

and biochemical properties of muscle. By using the time-course of the isometric twitch as the criterion, there are three types of motor units in the hindlimb of the rat (Close, 1967). Some fast-twitch muscles, e.g. extra-ocular muscles of a number of species (Cooper & Eccles, 1930; Close & Luff, 1974), the thyroarytenoid muscle of the rabbit (Hall-Craggs, 1968) and jaw muscles of the cat (Taylor *et al.*, 1973), are faster than the fastest hindlimb muscles of the same animal. Further, the ATPase activity of myofibrils or myosin extracted from rabbit thyroarytenoid muscle is 30–55% higher when compared with that of the rabbit fast-twitch extensor digitorum longus muscle (Syrovy & Gutmann, 1971).

In attempting to resolve further heterogeneity of skeletal-muscle myosins, we have improved on the method of electrophoretic analysis of intact myosin in polyacrylamide gel previously developed in this laboratory (Hoh, 1975a). The original method used ATP or pyrophosphate to solubilize myosin in low-ionic-strength buffer during electrophoresis. The ATP medium has the advantage that Ca^{2+}-activated ATPase reaction can be performed in the gel immediately after electrophoresis, but the pyrophosphate medium gives better resolution and is more economical. The improved method described in the present paper has higher resolution, and permits the quantification of myosin ATPase activity in the gel. We have used it to analyse myosins from the slow-

* Abbreviation: ATPase, adenosine triphosphatase.

twitch anterior latissimus dorsi and the fast-twitch posterior latissimus dorsi muscles of the chick. This has led to the resolution of five electrophoretically different forms of chicken skeletal-muscle myosin; two in anterior latissimus dorsi and three in posterior latissimus dorsi.

Materials and Methods

Materials

In the present work the anterior latissimus dorsi and posterior latissimus dorsi muscles of adult female white Leghorn chickens were used. The muscles were dissected from animals anaesthetized by an intra-peritoneal injection of sodium pentobarbitone (50 mg/kg).

ATP (disodium salt, grade II), 2-mercaptoethanol (type 1) and Coomassie Brilliant Blue R were obtained from Sigma (St. Louis, MO, U.S.A.). Acrylamide and methylenebisacrylamide were purified by the method of Loening (1967). Apparently identical results have been obtained by using commercially purified acrylamide ('for electrophoresis'; Eastman, Rochester, NY, U.S.A.) and methylenebisacrylamide (Ceres brand, Scientific and Research Equipment Co., Sydney, N.S.W., Australia). All other reagents were of analytical grade.

Methods

Myosin preparation. Myosin was prepared by a modified procedure of Baril *et al.* (1966). All steps were carried out on ice. Muscle tissue (50–300 mg) was finely minced with scissors and washed in phosphate-buffered NaCl (40 mM-NaCl/3 mM-sodium phosphate, pH 7.0, at 2°C). The washed muscle was homogenized in the same buffer by using a Sorvall Omni-Mixer with a micro-attachment for a total of 90 s, i.e. three periods of 30 s homogenizing, 30 s rest. The homogenate was then centrifuged at $3640g$ in an International centrifuge, model HR-1, for 30 min at 1°C. The supernatant was discarded, and the pellet washed and centrifuged for a second time. The pellet was again collected and immersed in an extraction solution containing 40 mM-$Na_4P_2O_7$/1 mM-2-mercaptoethanol, adjusted to pH 8.8 at 2°C with conc. HCl, and homogenized for 30 s. The homogenate was left for 60 min, after which it was centrifuged at $78000g$ in a type 30 rotor in a Beckman L5-65 ultracentrifuge for 180 min at 1°C. The supernatant was collected, mixed with an equal volume of ice-cold glycerol and stored at −20°C.

Preparation of gels. Polyacrylamide gels [25 ml of 4% gels; T = 4%, C = 5%, on the notation of Hjerten (1962)] were prepared with the following composition (final concns.): $Na_4P_2O_7$, pH 8.8 (20 mM); acrylamide (38 g/l); methylenebisacrylamide (2 g/l); glycerol (10%, v/v); NNN'N'-tetramethyl-ethylenediamine (0.15%, v/v).

The solution was de-aerated, and polymerization was initiated by adding 0.1 ml of freshly prepared ammonium persulphate solution (125 g/l). Gel tubes used were 7 cm (long) × 5 mm (int. diam.). The gels polymerized in 10–15 min, and were left for a further 15 min before use.

The mobility of the myosin bands at different gel concentrations was analysed. Gels were prepared with total acrylamide concentrations, of 6, 5.08, 4.67, 4.30 and 3.95% (C = 3% in all cases), other constituents being unchanged.

Electrophoresis. Electrophoresis was carried out in a Lab-Quip analytical-electrophoresis apparatus (Scientific and Research Equipment Co.) equipped to accommodate twelve gel tubes. Electrophoresis buffer contained 20 mM-$Na_4P_2O_7$ and 10% (v/v) glycerol, pH 8.8, at 2°C ($I = 0.144$ mol/l) in both upper and lower buffer reservoirs. To neutralize the products of electrolysis during the run, buffer was recirculated by pumping it from the lower to the upper reservoir with a centrifugal pump (Eheim, Hamburg, West Germany) at the rate of 1 litre/min. Two of the gel tubes were not filled with gel so as to allow buffer to return from the upper to the lower reservoir. By the completion of the run the pH had dropped to about 8.6, presumably because of the absorption of atmospheric CO_2. The temperature of the electrophoresis buffer was maintained at 1–2°C by a refrigerated thermostat. The coolant from this unit was circulated through a glass coil immersed in the lower reservoir of the electrophoresis apparatus. The lower reservoir was magnetically stirred to promote heat exchange.

A pre-run of 30 min was carried out under conditions identical with those of electrophoresis. Myosin samples (1–20 μl) in 50% (v/v) glycerol were loaded directly on top of the gels and electrophoresed overnight (approx. 16 h) under a constant voltage gradient of 0.14 V/m (the upper reservoir is cathodic) by using a regulated power supply (All Power, Sydney, N.S.W., Australia). Power dissipation under the conditions of electrophoresis ranges between 5.5 and 6.5 W.

Staining of gels. Gels were stained for protein in 20 ml of Coomassie Brilliant Blue R solution (0.25 g/l) for 2 h at 70°C. The stain was dissolved initially in 20 ml of methanol, and 70 ml of acetic acid was then added and the final volume brought to 1 litre with water; under these conditions the stain was completely dissolved. Gels were then de-stained in 7% (v/v) acetic acid by diffusion for 48 h in a Hoefer de-staining apparatus (Polysciences, Warrington, PA, U.S.A.). Densitometer tracings of the gels were obtained by using a Gilford model 240 spectrophotometer equipped with a linear transport and a chart recorder.

Protein determination. Protein concentration in solutions was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin
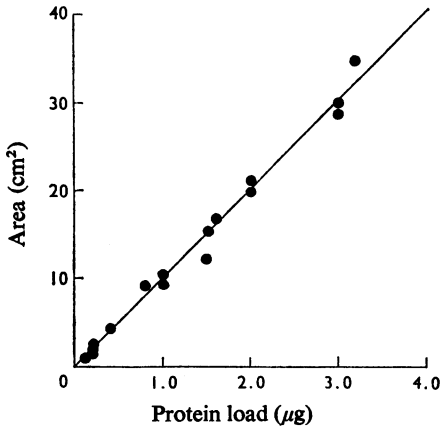
Fig. 1. *Calibration curve for determining protein in gel*

The line drawn ($y = 10.17x–0.146$, $r = 0.994$) was determined by regression analysis by the method of least squares. Bovine serum albumin (used as standard) was electrophoresed for 1.5–2.0h under conditions identical with those for myosin, during which time it migrated a distance comparable with that of myosin after an overnight run.

as standard. The amount of protein under each absorbance peak in the gel after electrophoresis was determined by the method described below. A known volume of either a 0.1% or a 0.01% solution of bovine serum albumin was electrophoresed for 1.5–2h under conditions identical with those for the electrophoresis of myosin. These gels were stained and de-stained as described above. The gels were scanned at 550nm under a standard set of recording conditions (ordinate, $0.3E$ unit/2.54cm of chart paper; abscissa, 0.4cm of gel/2.54cm of chart paper). The area under the absorbance peaks was measured by planimetry. Fig. 1 shows a plot of the absorbance area against protein load; the regression line was used to determine the amount of protein in myosin bands scanned under identical conditions. With loads of bovine serum albumin in excess of $4\mu g$, the absorbance peaks exceed the range of the instrument at 550nm, but scanning gels at 650nm (the absorbance of stained proteins at this wavelength is 0.28 times that at 550nm) extends the linear range of this method up to at least $10\mu g$ of protein.

Measurement of Ca^{2+}-activated myosin ATPase in gels. Since Ca^{2+} is precipitated in the presence of the pyrophosphate ions of the gel buffer, the Ca^{2+}-activated ATPase reaction cannot be carried out on the gel immediately after electrophoresis. A convenient method of removing pyrophosphate ions by electrophoresis was developed to overcome this problem. Electrophoresis of myosin was carried out

in the manner described for 16h; the upper reservoir was then emptied, washed with water and filled with a buffer comprising 20mM-Tris, 0.19M-glycine, 5mM-ATP and 10% glycerol, adjusted to pH8.8 at 2°C with 1M-NaOH. There was no recirculation of buffer and the level of the two empty gel tubes was raised above the level of the buffer in the upper bath to prevent mixing of upper- and lower-reservoir buffers. Electrophoresis was continued for a further 1.5–2h at the same constant voltage gradient. At the end of electrophoresis the gels were removed from the tubes and placed in test tubes containing 10ml of ATPase incubation solution at 37°C (0.6M-KCl/5mM-ATP/5mM-2-mercaptoethanol / 15mM-CaCl₂ / 25mM-Tris/0.19M-glycine, adjusted to pH8.8 at 20°C with 1M-NaOH). The test tubes were maintained at 37°C in a water bath with gentle agitation. The P_i released forms a white precipitate of calcium phosphate in the presence of Ca^{2+}. Gels were scanned at 550nm after various times of incubation, and subsequently stained for protein as described above, the calcium phosphate bands being dissolved in the acid medium. The ATPase of the myosin bands was calculated by plotting the ratio of the absorbance area of the calcium phosphate to the absorbance area of the stained protein against incubation time. The slope of the line obtained from linear-regression analysis was taken as the ATPase activity in arbitrary units.

Results

Gel electrophoresis of intact myosins from chicken anterior latissimus dorsi and posterior latissimus dorsi muscles

Fig. 2 shows superimposed tracings of densitometer scans of gels containing anterior latissimus dorsi and posterior latissimus dorsi myosins. Posterior latissimus dorsi myosin migrated faster than anterior latissimus dorsi myosin; the former can be resolved into three components (PLD_1, PLD_2 and PLD_3 in order of decreasing mobility), whereas the latter shows a minor fast (ALD_1) and a major slow (ALD_2) component. Components PLD_3 and ALD_1 show clearly distinct mobilities when analysed in separate gels, suggesting that they are distinct from each other. In mixtures containing anterior latissimus dorsi and posterior latissimus dorsi myosins, the minor ALD_1 component can be resolved from component PLD_3, as shown in Fig. 3. Both myosins occasionally showed trailing proteins, especially when gels were heavily loaded with protein. This is shown for anterior latissimus dorsi myosin in Fig. 3 and for posterior latissimus dorsi myosin in Fig. 6 (----).

The electrophoretic pattern of anterior latissimus dorsi and posterior latissimus dorsi myosins described above was observed in six preparations from three different chickens for anterior latissimus dorsi myosin and seven preparations from five different chickens for

posterior latissimus dorsi myosin, by the method described above. Myosins prepared from anterior latissimus dorsi and posterior latissimus dorsi muscles of other chickens by the method of Bárány & Close (1971) by using 0.3 mM-KCl/5 mM-ATP in phosphate buffer, pH 6.6, gave identical results. In no case has an anterior latissimus dorsi component been found in detectable amounts in posterior latissimus dorsi
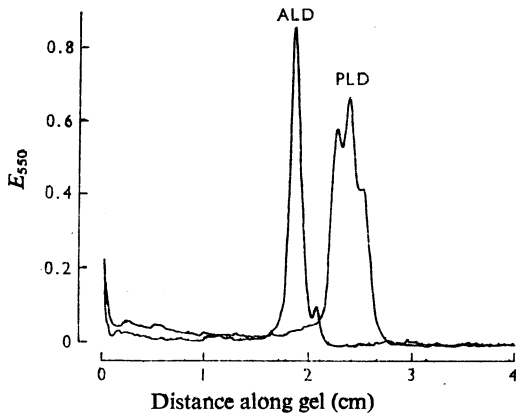
muscle and vice versa. There were slight variations in the proportions of the various components of anterior latissimus dorsi and posterior latissimus dorsi myosin prepared from different animals. Table 1 gives the mean percentage composition of each component.

Ca²⁺-activated ATPase activity of anterior latissimus dorsi and posterior latissimus dorsi myosins

The Ca²⁺-activated ATPase activity of anterior latissimus dorsi and posterior latissimus dorsi myosins have been compared at 37°C after a short gel-electrophoretic run (which did not clearly resolve the different components) as well as after an overnight run. In Fig. 4 the ratio of the absorbance area after ATPase incubation to the absorbance area after pro-

Fig. 2. *Superimposed densitometer tracings of individual gels from the same run in which typical electrophoretic profiles of anterior latissimus dorsi and posterior latissimus dorsi myosins are illustrated*

The mobilities of the anterior latissimus dorsi (ALD) components are distinct from those of the three posterior latissimus dorsi (PLD) components. Protein loads were 0.3 μg for anterior latissimus dorsi and 0.45 μg for posterior latissimus dorsi. Electrophoresis was carried out at a voltage gradient of 0.14 V/m at 1°C for 16.25 h.

Table 1. *Mean percentage composition of anterior and posterior latissimus dorsi myosins in terms of components*

The two anterior latissimus dorsi components were sufficiently well resolved to allow accurate measurement of areas under the absorbance peaks. The posterior latissimus dorsi components, however, were not sufficiently well resolved to allow accurate determination of the area under each absorbance peak and consequently the relative peak heights were taken as an estimate of their relative proportions. Mean percentage compositions ±S.E. are given.

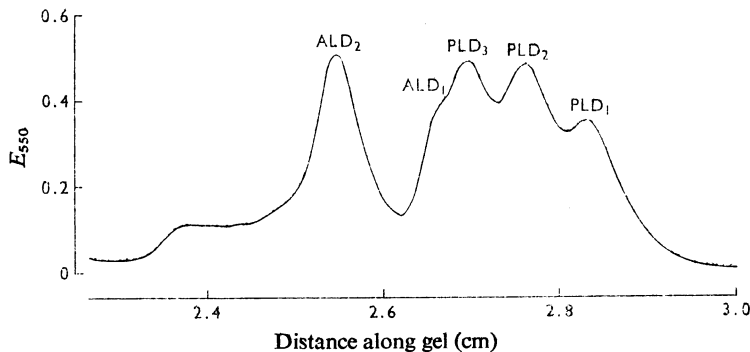| Component	No. of chickens used	No. of myosin preparations	Mean percentage composition
PLD₁	5	7	22.8 ± 1.3
PLD₂	5	7	38.4 ± 0.6
PLD₃	5	7	38.8 ± 1.8
ALD₁	3	6	13.5 ± 3.6
ALD₂	3	6	86.5 ± 3.6

Fig. 3. *Densitometer tracing of a mixture of anterior latissimus dorsi and posterior latissimus dorsi myosins run in the same gel, to show that the anterior latissimus dorsi components are distinct from those of posterior latissimus dorsi*

Note the broad trailing band of anterior latissimus dorsi myosin, which is sometimes observed particularly at high protein loads. This was also seen in gels from the same run loaded only with anterior latissimus dorsi myosin. Gels loaded only with an equal amount of posterior latissimus dorsi myosin in this run showed no trailing protein.
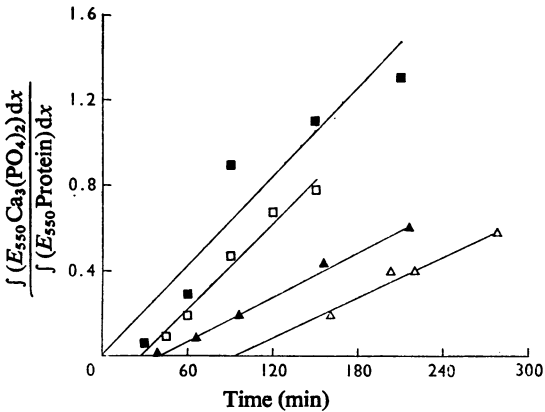
Fig. 4. *Comparison of Ca^{2+}-activated ATPase activity of anterior latissimus dorsi and posterior latissimus dorsi myosins after electrophoresis*

The ratio of the area under the absorbance peaks for $Ca_3(PO_4)_2$ precipitate, $\int(E_{550}Ca_3(PO_4)_2)\,dx$, to the area under the absorbance peaks for the stained protein, $\int(E_{550}$ Protein$)\,dx$, was plotted for each gel against time of incubation. Gels were incubated at 37°C. The data were obtained from two separate electrophoretic runs of 2h ($\square$, $\triangle$) and 16h ($\blacksquare$, $\blacktriangle$) duration. Each point represents data from a single gel. Regression lines drawn were calculated by the method of least squares. The slope of posterior latissimus dorsi myosin for each run is 2.2 times higher than that for anterior latissimus dorsi myosin and this difference is statistically significant ($P < 0.05$). $\blacktriangle$, $\triangle$, myosin from anterior latissimus dorsi muscle; $\blacksquare$, $\square$, myosin from posterior latissimus dorsi muscle.

Fig. 5. *Comparison of profiles of posterior latissimus dorsi myosin after ATPase and protein staining*

The extinction scale on the left refers to $Ca_3(PO_4)_2$ profile and that on the right to the protein profile. The gel was one of those described in Fig. 4 (16h run, after 90min incubation). Note the similarity of the two profiles, suggesting that the Ca^{2+}-activated ATPase activity of each component was the same.

tein staining is plotted against duration of incubation. Straight lines have been fitted to the data by the method of least squares. The slopes of the lines give a measure of the relative Ca^{2+}-activated ATPase activity of the myosins. Posterior latissimus dorsi myosin gave slopes 2.2 times higher than that for anterior latissimus dorsi myosin in both short and long runs, the difference in slope for the myosins in these runs being statistically significant at the 1% and 5% level respectively by analysis of covariance (Snedecor, 1956). Densitometric profiles of gels after the ATPase reaction is virtually identical with that after protein staining. This is shown for posterior latissimus dorsi myosin in Fig. 5. When the ratios of the absorbance peaks ($Ca_3(PO_4)_2$/protein) for each posterior latissimus dorsi component after a long run were plotted against time, the slopes obtained were not significantly different from each other. These results suggest that the Ca^{2+}-activated ATPase activity of each posterior latissimus dorsi component is the same. For anterior latissimus dorsi myosin, the two components are sufficiently well resolved after a long run to analyse the ATPase activity of each component separately by measuring areas under absorbance peaks. The slopes obtained for the absorbance ratios of the two anterior latissimus dorsi components, when separately plotted against time of incubation, were identical, showing that these two components have the same Ca^{2+}-activated ATPase activity.

The relative Ca^{2+}-activated ATPase activity of the posterior latissimus dorsi components was also investigated at room temperature (23–25°C). In these experiments, five gels from two different runs were each scanned at various times after commencement of incubation. The ratios of the absorbance peaks ($Ca_3(PO_4)_2$/protein) for each posterior latissimus dorsi component were plotted against time. The slopes obtained for each band were not significantly different from each other (paired t test).

Electrophoresis of isolated components of posterior latissimus dorsi myosin

Since homogeneous proteins may produce multiple band patterns on zone electrophoresis owing to protein–buffer or protein–protein interactions (Cann, 1970), fractionation experiments were done on posterior latissimus dorsi myosin to investigate the possibility that the three-component pattern is due to these interactions. After electrophoretic analysis of posterior latissimus dorsi myosin, an 8mm segment of a gel containing the three components was sectioned serially in 1mm slices in the cold-room. This was done several hours after the end of electrophoresis, to allow possible local variation of buffer concentration in the protein zones to re-equilibrate with the rest of the gel buffer. Each slice was subsequently re-electrophoresed in another run in which a sample of the original myosin was also analysed. Fig.
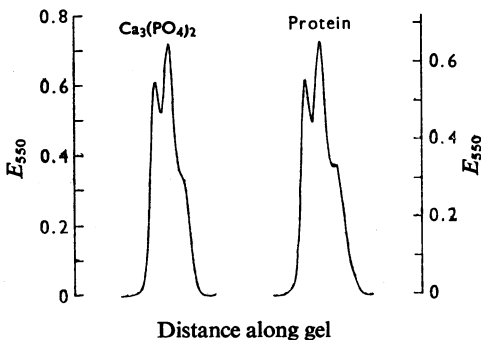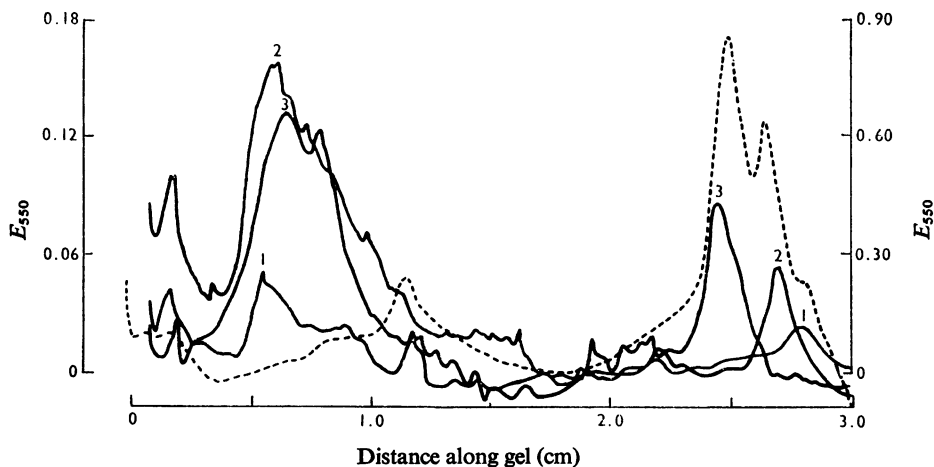
Fig. 6. *Re-electrophoresis of individual bands of posterior latissimus dorsi myosin after fractionation*

Densitometer tracings obtained from fractionated bands (——) compared with that of unfractionated sample (----) analysed in the same run. Densitometer tracings obtained from fractionated bands have been moved 1 mm to the right to allow for the extra 1 mm that these fractionated bands have to migrate. The ordinate axis on the left refers to the extinction of the fractionated bands and that on the right to the unfractionated sample. The duration of electrophoresis was 16.25 h. Densitometer tracings numbered 1, 2 and 3 refer to components PLD_1, PLD_2 and PLD_3 respectively. For a detailed explanation, see the text.
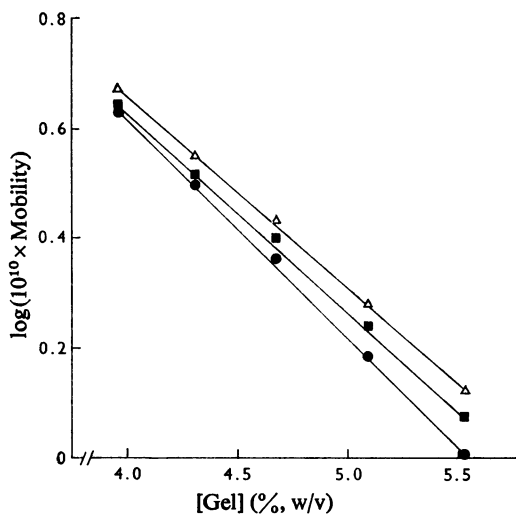

Fig. 7. *Effect of gel concentration on the mobility of posterior latissimus dorsi myosin components*

The logarithm of 10^{10} times the mobility $(m^2 \cdot V^{-1} \cdot s^{-1})$ of each component was plotted against total gel concentration. Each gel was loaded with the same amount of protein and electrophoresed for 15.5 h. The lines are fitted by regression analysis by the method of least squares, $r > 0.999$ in all cases. ▲, PLD_1; ■, PLD_2; and ●, PLD_3. At a gel concentration of 6.0% (w/v) the posterior latissimus dorsi myosin components did not move sufficiently far into the gel to be resolved.

6 shows superimposed densitometer scans for the original sample (----) and the three consecutive protein-bearing slices, which corresponded to the three posterior latissimus dorsi components. A large portion of the protein from each slice appeared to have formed slow-migrating aggregates, presumably as a result of handling. Nevertheless, in the zone corresponding to the original posterior latissimus dorsi components, each component remained single, and migrated at a rate consistent with its original mobility.

Effect of gel concentration on the mobility of posterior latissimus dorsi myosin components

The possibility that the three components of posterior latissimus dorsi may be due to stable oligomers of myosin was investigated by analysis of the effect of gel concentration on electrophoretic mobility. The mobilities of posterior latissimus dorsi components in gels ranging from 3.95% to 5.52% total acrylamide were found to fit the Ferguson relationship:

$$\log M = \log M_0 - K_R T$$

where M is mobility, M_0 is mobility at zero gel concentration, K_R is retardation coefficient and T is total gel concentration (Ferguson, 1964). This is shown in Fig. 7, in which $\log M$ for each component is plotted against T. The values of M_0 and K_R obtained for each posterior latissimus dorsi component are given in Table 2.

Table 2. *Free electrophoretic mobility (M_0) and retardation coefficient (K_R) obtained from Ferguson plots (see Fig. 7) of posterior latissimus dorsi myosin components*

Free mobility (M_0) was obtained by extrapolation of regression lines to zero gel concentration. The negative of the slopes of the regression lines in Fig. 7 [$-\mathrm{d}\log M/\mathrm{d}T$, where M is mobility and T is total gel concentration (%, w/v)] gave the retardation coefficients (K_R)

Component	$10^8 \times M_0$ ($m^2 \cdot V^{-1} \cdot s^{-1}$)	K_R ($-\mathrm{d}\log M/\mathrm{d}T$)
PLD$_1$	1.52	0.35
PLD$_2$	1.20	0.36
PLD$_3$	1.16	0.39

Discussion

The results described above reveal that anterior latissimus dorsi myosin may be resolved into two electrophoretic components that show the same ATPase activity, and posterior latissimus dorsi myosin may be resolved into three electrophoretic components, each with an ATPase activity about 2.2 times higher than that of the anterior latissimus dorsi components. Before considering that these components in anterior latissimus dorsi and posterior latissimus dorsi myosins are multiple forms of myosin, certain alternative explanations for the observed heterogeneity should be considered. Protein–buffer interaction (Cann, 1970) is unlikely in this case for the following reasons: First, the band patterns are seen even with extremely low protein load and remain unchanged for a wide range of load. Secondly, each fraction of posterior latissimus dorsi myosin, on re-electrophoresis in the same buffer system, did not reproduce the three-band pattern of the original myosin, but migrated as a single component at a rate consistent with its original mobility. Between the termination of the original run and the commencement of the re-electrophoresis there would have been ample time for re-equilibration of the fraction with the running buffer. It may thus be concluded that there is inherent heterogeneity in these myosins.

The possibility that the inherent heterogeneity in each type of myosin may be due to stable oligomers of myosin will now be considered. The retardation coefficient K_R, derived from the Ferguson plot, is a measure of the effective molecular surface area (Rodbard & Chrambach, 1971). Assume that myosin molecules are rods and that they undergo side-to-side interaction to form stable dimers. If the cross-sectional surface of the region of overlap of myosin monomers is circular, the surface area/unit length in this region would be $\sqrt{2}$ times that of the monomer. Higher values for the surface area/unit length in the region of overlap would be expected if cross-sectional area of this region is not circular. The surface area of

the myosin dimer compared with that of the monomer would therefore increase by a factor ranging from $\sqrt{2}$ (for complete overlap) to 2 (for minimal overlap). If myosin heads are considered to be free to move, the minimum ratio of the surface area of the myosin dimer to that of the monomer would be greater than $\sqrt{2}$. Thus K_R for a myosin dimer would be expected to be $\geqslant \sqrt{2}$ times or $\geqslant 41\%$ higher than that for a monomer. However, the observed K_R of component PLD$_2$ is only 3% higher than that of component PLD$_1$. Further, one likely cause of stable oligomers is disulphide-bridge formation. Electrophoresis of adult chicken myosins in pyrophosphate buffers containing 2mM-2-mercaptoethanol or 2mM-thioglycollic acid to prevent thiol oxidation did not alter the band patterns in any way. It is therefore improbable that the observed heterogeneity of the fast-twitch and slow-twitch skeletal-muscle myosin arose from the formation of stable oligomers.

It is conceivable that heterogeneity of anterior latissimus dorsi and posterior latissimus dorsi myosins may arise from complete or partial loss of light chains of a significant population of myosin molecules during preparative procedures. Against this possibility is the observation that essentially the same band patterns have been observed by using two different methods of myosin extraction, in which the extractant differed markedly in pH and ionic composition. Further, since half of the myosin light chains are necessary for ATPase activity (Gazith *et al.*, 1970), removal of light chains is likely to abolish or decrease ATPase activity of some of the components. The results showed, on the contrary, that each component of anterior latissimus dorsi myosin has equal ATPase activity. Each component of posterior latissimus dorsi myosin has also virtually identical ATPase activity. More direct evidence against the possibility that electrophoretic heterogeneity of myosin resulted from variations in light-chain composition is available for rat ventricular myosin which shows three electrophoretic components (Hoh, 1975*b*). Light chains from ventricular myosins which differed markedly in the relative amounts of each component are qualitatively and quantitatively identical on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (J. F. Y. Hoh & P. A. McGrath, unpublished work).

A further possible source of myosin heterogeneity which should be considered are variations in post-translational modification. Avian and mammalian fast-twitch myosins are known to contain 3-methyl-histidine and methylated lysine residues (Kuehl & Adelstein, 1970). Out of the four methylated amino acid residues per heavy chain of rabbit fast-twitch myosin, which also shows electrophoretic heterogeneity (White & Hoh, 1973), three of these have been conclusively shown to be homogeneously methylated (Huszar & Elzinga, 1971; Huszar, 1972). The 5,5'-dithiobis-(2-nitrobenzoic acid) light chain of

rabbit fast-twitch myosin contains a serine residue which may be phosphorylated (Perrie *et al.*, 1972). In view of the large size and the expected large net charge on the myosin molecule, it would seem unlikely that the possible small variations in the extent of methylation or phosphorylation described above could lead to such large differences in free mobility (Table 2).

In view of all the evidence cited above, we suggest that electrophoretic heterogeneity of chicken myosins arose from the presence of two myosin isoenzymes in anterior latissimus dorsi muscle and three myosin isoenzymes in posterior latissimus dorsi muscle. This proposition raises interesting questions with regard to the functional significance and subunit structure of the various forms of myosin.

The existence of different forms of skeletal-muscle myosin may explain the considerable functional diversity of skeletal muscles within the same animal which was referred to in the introduction. In this connexion, it is surprising that the ATPase activities of the multiple forms of chicken myosin within the same muscle appear to be identical. Differences in myosin ATPase activity have been observed in the multiple forms of rabbit fast-twitch myosin (J. F. Y. Hoh, unpublished work), so that shifts in the distribution of these multiple forms of myosin may be expected to correlate with differences in contractile properties of muscle fibres. Shifts in the distribution of multiple forms of myosin of the rat ventricle are correlated with changes in functional characteristics of the myocardium (Hoh, 1975b).

Current models of myosin structure envisage the molecule to be made up of two heavy polypeptide chains and four light chains (Gershman *et al.*, 1969; Gazith *et al.*, 1970; Lowey & Risby, 1971; Sarkar *et al.*, 1971). Evidence for structural differences between subunits of fast-twitch and slow-twitch muscle myosins has been briefly reviewed (Hoh, 1975a). The present work requires that models of the subunit structure of fast-twitch and slow-twitch muscle myosins explain the heterogeneity of both types of myosin. Heterogeneity may arise from differences in light chains, heavy chains or both. A model for three isoenzymic forms of fast-twitch muscle myosin in which each isoenzyme has its own complement of light chains has been proposed (Sarkar, 1972). Whether these postulated variants of myosin molecules correspond to the three electrophoretically distinct forms in fast-twitch muscle reported here remains a matter for direct experimentation. However, lack of differences in light-chain composition of ventricular myosins with different electrophoretic patterns suggests that differences reside in heavy-chain structure.

There is some evidence in the literature for heterogeneity of heavy chains of myosin. Bechtel *et al.* (1971) showed that heavy chains derived from rabbit

back muscles are heterogeneous on isoelectrofocusing. It is probable that this heterogeneity is due to fast-twitch muscle myosin alone, since electrophoretic analysis of myosin extracted from the white longissimus portion of the erector spinae muscles of Laboratory White Castle Hill-strain rabbits revealed three components (J. F. Y. Hoh & C. G. dos Remedios, unpublished work), characteristic of vertebrate fast-twitch muscle myosins (White & Hoh, 1973), and this preparation was virtually free of contamination by slow-twitch myosin. Starr & Offer (1973) showed the presence of two types of *N*-terminal sequences, which occurred in the ratio of 1.8:1 in heavy chains of rabbit back and leg-muscle myosin. Other studies, using myosins of unknown electrophoretic behaviour, have also revealed heterogeneity of heavy chains (Florini & Brivio, 1969; Florini *et al.*, 1971; Hale & Beecher, 1971).

References

Bárány, M. (1967) *J. Gen. Physiol.* **50**, 197–218

Bárány, M. & Close, R. I. (1971) *J. Physiol. (London)* **213**, 455–474

Baril, E. F., Love, D. S. & Herrmann, H. (1966) *J. Biol. Chem.* **241**, 822–830

Bechtel, P. J., Pearson, A. P & Bodwell, C. E. (1971) *Anal. Biochem.* **43**, 509–514

Canfield, S. P. (1971) *J. Physiol. (London)* **219**, 281–302

Cann, J. R. (1970) *Interacting Macromolecules*, Academic Press, New York

Close, R. (1967) *J. Physiol. (London)* **193**, 45–55

Close, R. (1972) *Physiol. Rev.* **52**, 129–197

Close, R. I. & Luff, A. R. (1974) *J. Physiol. (London)* **236**, 259–270

Cooper, S. & Eccles, J. C. (1930) *J. Physiol. (London)* **69**, 377–385

d'Albis, A. & Gratzer, W. B. (1973) *FEBS Lett.* **29**, 292–296

Ferguson, K. A. (1964) *Metab. Clin. Exp.* **13**, 985–1002

Florini, J. R. & Brivio, R. P. (1969) *Anal. Biochem.* **30**, 358–367

Florini, J. R., Brivio, R. P. & Battelle, B. A. M. (1971) *Anal. Biochem.* **40**, 345–350

Gazith, J., Himmelfarb, S. & Harrington, W. F. (1970) *J. Biol. Chem.* **245**, 15–22

Gershman, L. C., Stracher, A. & Dreizen, P. (1969) *J. Biol. Chem.* **244**, 2726–2736

Hale, R. G. & Beecher, G. R. (1971) *FEBS Lett.* **18**, 245–248

Hall-Craggs, E. C. B. (1968) *J. Anat.* **102**, 241–255

Hjerten, S. (1962) *Arch. Biochem. Biophys. Suppl.* **1**, 147–151

Hoh, J. F. Y. (1975a) *Biochemistry* **14**, 742–747

Hoh, J. F. Y. (1975b) *Proc. Aust. Biochem. Soc.* **8**, 105

Huszar, G. (1972) *J. Biol. Chem.* **247**, 4057–4062

Huszar, G. & Elzinga, M. (1971) *Biochemistry* **10**, 229–236

Kuehl, W. M. & Adelstein, R. S. (1970) *Biochem. Biophys. Res. Commun.* **39**, 956–964

Loening, U. E. (1967) *Biochem. J.* **102**, 251–257

Lowey, S. & Risby, D. (1971) *Nature (London)* **234**, 81–85

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Melichna, J., Gutmann, E. & Syrovy, I. (1974) *Physiol. Bohemoslov.* **23**, 511–520

Perrie, W. T., Smillie, L. B. & Perry, S. V. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 17–18

Rodbard, D. & Chrambach, A. (1971) *Anal. Biochem.* **40**, 95–134

Sarkar, S. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 14–17

Sarkar, S., Sreter, F. A. & Gergely, J. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 946–950

Snedecor, G. W. (1956) *Statistical Methods*, 5th edn., p. 394, Iowa State University Press, Ames

Starr, R. L. & Offer, G. W. (1973) *J. Mol. Biol.* **81**, 17–31

Syrovy, I. & Gutmann, E. (1971) *Experientia* **27**, 248

Taylor, A., Cody, F. W. J. & Bosley, M. A. (1973) *Exp. Neurol.* **38**, 99–109

White, R. & Hoh, J. F. Y. (1973) *Proc. Aust. Physiol. Pharmacol. Soc.* **4**, 179–180