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Evidence for Myosin Heterogeneity in *Drosophila melanogaster*

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Summary. Electrophoresis of myosin extracts from larvae and adult tissues of *Drosophila melanogaster* under non-dissociating conditions indicate that two of the bands seen are myosins. They stain for Ca^{2+} ATPase activity and when cut and re-run under dissociating conditions are found to contain a myosin heavy chain that co-migrates with rabbit skeletal muscle myosin heavy chain. One of the forms of myosin seen is found primarily in extracts from the leg. The other is common to the adult fibrillar flight muscles and the larval body wall muscles.

The electrophoretic evidence for two myosin types is strengthened by the histochemical demonstration of two myofibrillar ATPases on the basis of their lability to acid or alkali preincubation. The myofibrillar ATPase in the leg and the Tergal Depressor of the Trochanter (TDT) are shown to be relatively acid labile and alkali stable. The larval body wall muscles and the adult fibrillar flight muscles have an ATPase which is acid stable and alkali labile. This distribution of the two myofibrillar ATPases coincides with that predicted by electrophoresis of extracts from whole tissue and also locates the two myosins to specific muscle types.

Key words: Myosins – *Drosophila* – muscle

Introduction

Myosin, a major contractile protein, is present in multiple forms in vertebrate muscle (Weeds 1978). The molecule in its native state contains two heavy chain and four light chain sub-units non-covalently bound together. Heterogeneity in one or more of these subunits gives rise to polymorphic forms of myosin which have been called isozymes (D'Albis et al. 1979). In this study we examine the muscles of the fruit fly, *Drosophila melanogaster* for myosin polymorphism.

The development and morphology of the muscles of *Drosophila* has been reviewed by Crossley (1978). In the adult, the indirect flight muscles are fibrillar; the direct flight muscles and the muscles of the leg are tubular. The major muscles in the larva are the supercontractile body wall muscles. Several flightless mutants of *Drosophila* are defective in the development and maintenance of muscles (Kaono and Hotta 1977; Deak 1977a); these along with the known neurological mutants (reviewed by Hall and Greenspan 1979) can be used for a genetic dissection of myogenesis and the role, if any, played by the nervous system in muscle development (Neusch 1968; Jan and Jan 1978). In mammals the specificity of nerve connections to muscle is one of the factors that decides the tissue-specific expression of myosin isozymes (Weeds 1978). In invertebrates, while myosin heterogeneity has been demonstrated in the nematode *Caenorhabditis elegans* (Epstein et al. 1974) the relation between myosin synthesis and functional nerve connections has not been examined. We are examining the role of the nervous system of *Drosophila* in myosin synthesis. We show here by electrophoretic and histochemical methods, that *Drosophila melanogaster* has at least two types of myosins and that these can be located in morphologically distinguishable muscle fiber types.

Materials and Methods

Myosin Extraction. Myosin extracts were prepared from various adult parts and from whole larvae and adult flies. Legs and heads were separated from the thorax and abdomen as follows. Vials containing flies were repeatedly dipped in liquid nitrogen and shaken vigorously. This results in the detachment of limbs and head from the body. These organs can then be separated by sieving.

2.5 g whole adults, adult thorax and abdomen (henceforth referred to as adult body) or larvae were homogenised in 2 vol homogenisation buffer (50% glycerol, 0.1 M KCl, 5 mM EDTA, 5–10 mM Na-Azide. 5 mM phenylmethyl sulfonyl fluoride, 20 mM Na-K phosphate buffer pH 7.0). The homogenate was centrifuged at 3,000 g for 15 m, the pellet resuspended in 2 vol wash buffer (0.1 M KCl, 5 mM $MgCl_2$, 5 mM EGTA, 5–10 mM Na-azide, 5 mM phenylmethyl sulfonyl fluoride, 20 mM Na–K phosphate buffer pH 7.0) and centrifuged at 3,000 g for 15 m. After another identical washing and centrifugation the pellet was suspended in 1 vol extraction buffer. (1.0 M KCl, 1.0 mM $MgCl_2$, 10 mM tetrasodium pyrophosphate, 1 mM EDTA, pH 7.0). Equivalent results were obtained when 80 mM sodium pyrophosphate, 1 mM EDTA, 0.1% 2-mercaptoethanol pH 8.5 was used for extraction. The suspension was centrifuged at 40,000 g for 3 h and the supernatant myosin fraction was collected. Extracts from legs were prepared by directly homogenising in 1 vol of extraction buffer using 10–30 mg tissue as starting material. All operations were carried out between 0 and 5° C. Samples could be stored in 50% glycerol for up to a month at −20° C.

Electrophoresis of Myosin Extracts Under Non-Dissociating Conditions. Polyacrylamide gel electrophoresis of native myosin was done by the method of d'Albis et al. (1979). The electrophoresis buffer (40 mM sodium pyrophosphate, pH 8.5, 1 mM EDTA and 0.01% 2-mercaptoethanol was recirculated between the two

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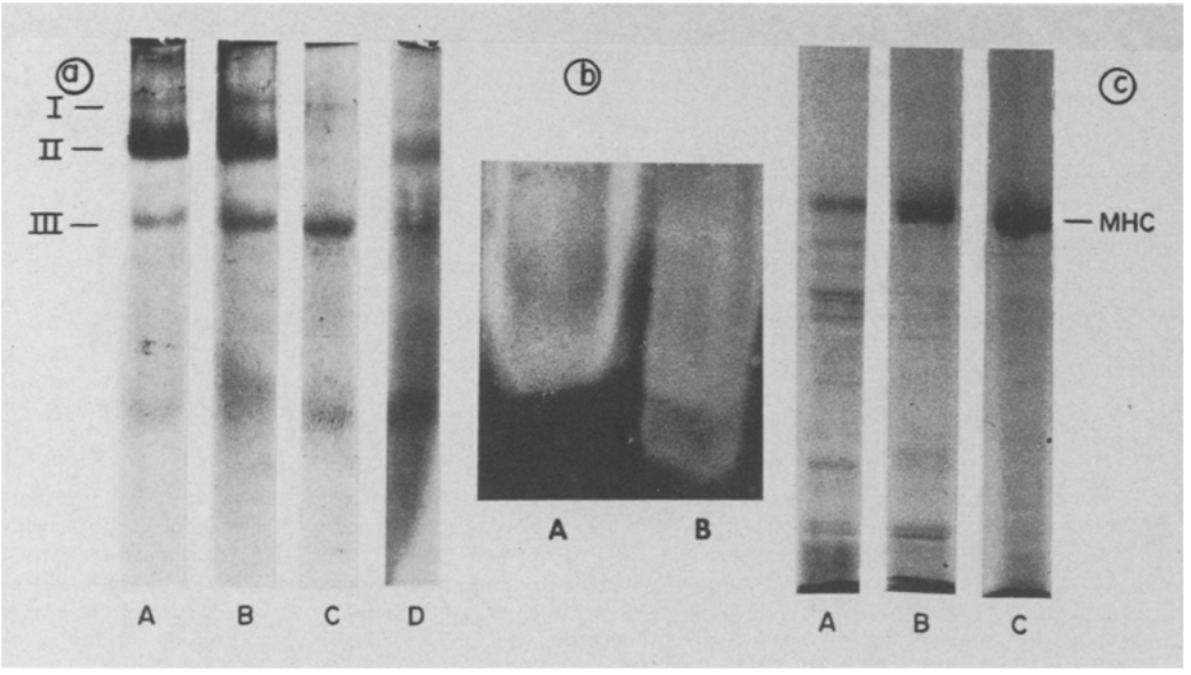

Fig. 1a–c. Extracts of myosin after electrophoresis under non-denaturing conditions (a and b) and in the presence of SDS (c). **a** Adult myosin (lane A), Larva and adult mixtures (lane B) larva (lane C) and legs (lane D). **b** Ca^{2+} ATPase activity on native gels: Rabbit skeletal muscle myosin (lane A) and larval myosin (lane B). **c** Native gel bands from (a) above cut and rerun on 5% SDS gels. Lane A: *Drosophila* myosin extract. Lane B: Band I, and Lane C: Band II cut and re-run. MHC indicates the myosin heavy chain

electrode chambers and all runs were performed at cold room temperature (5–10° C).

Polyacrylamide slab gels were prepared in 3.2% acrylamide, 40 mM sodium pyrophosphate, 0.115% methylenebisacrylamide and polymerized with 0.033% v/v tetramethylenediamine and 0.033% ammonium persulfate. Other acrylamide concentrations from 3.2% to 4% were also tried and the results were equivalent. Gels were run at a constant current of 3 mA/lane for 17–24 h. Gels were stained for protein in 1% coomassie brillant blue 45% methanol, 45% acetic acid and destained in a 10% methanol, 10% acetic acid solution.

Myosin ATPase activity after electrophoresis was visualised by Hoh's procedure (Hoh et al. 1976). Sodium pyrophosphate was electrophoresed out of the gel by stopping recirculation and replacing the buffer in the upper (cathodic) chamber with an ATP buffer (0.025 M Tris, 0.10 M glycine, 0.005 M ATP, 0.035 M $CaCl_2$, 6 M KCl pH 8.5). At least 6–8 h electrophoresis after a 17 h run was required for the sodium pyrophosphate to be completely removed. ATPase activity was visualised as a white precipitate by incubating the gel in a $CaCl_2$-Tris-glycine buffer (5 mM $CaCl_2$, 0.5 M KCl, 2 mM ATP, 25 mM tris (Hydroxymethyl) aminoethane hydrochloride for $\frac{1}{2}$–1 h.

Electrophoresis Under Dissociating Conditions. Polyacrylamide gel electrophoresis in the presence of SDS was performed following the method of Laemmli and Favre (1973). Coomassie blue stained bands identified after electrophoresis under non-dissociating conditions were cut and incubated in SDS-Tris sample buffer before reelectrophoresis under dissociating conditions in 5% acrylamide.

Rabbit skeletal muscle myosin, extracted as above was used as a standard and was used to identify the *Drosophila* myosin heavy chain on SDS-polyacrylamide gels by comigration. Coomassie blue staining of proteins in these gels was done as for the non-dissociating gels.

Histochemical Staining for Ca^{2+} ATPase Activity. Unfixed cryostat sections 8–10 μm thick, of adult flies and larvae were stained for Ca^{2+} ATPase activity by the method of Padykula and Herman (1955). The staining solution contained 10 mM $CaCl_2$ and 4 mM ATP in 0.2 M sodium barbiturate buffer pH 9.4.

The pH lability of the myosin Ca^{2+} ATPase activity was examined by preincubating the sections in sodium acetate buffer (pH 4.0 through 5.0) or 0.1 M glycine buffer (pH 9.6 through 10.9) as described by Brooke and Kaiser (1969). The pre-incubation time was 5 min in all cases.

Results

Electrophoresis Under Native Conditions

Myosin was extracted as described in materials and methods from whole larvae, whole adults, adult thorax and abdomen (referred to as adult body) and adult legs.

Myosin extracts were electrophoresed in polyacrylamide gels in the presence of sodium pyrophosphate. This method, developed by d'Albis and Gratzer (1973) and Hoh (1975) allows native myosin molecules to be separated on the basis of their charge/molecular weight ratios. Figure 1a shows the coomassie blue stained protein pattern for larval, adult and leg extracts after a 17 h run. Band I is common to both larva and adult. Band II is present as a broad band in the adult and in the leg and barely detectable in the larva. Band III, the highest mobility band is common to both larva and adult. When extracts from

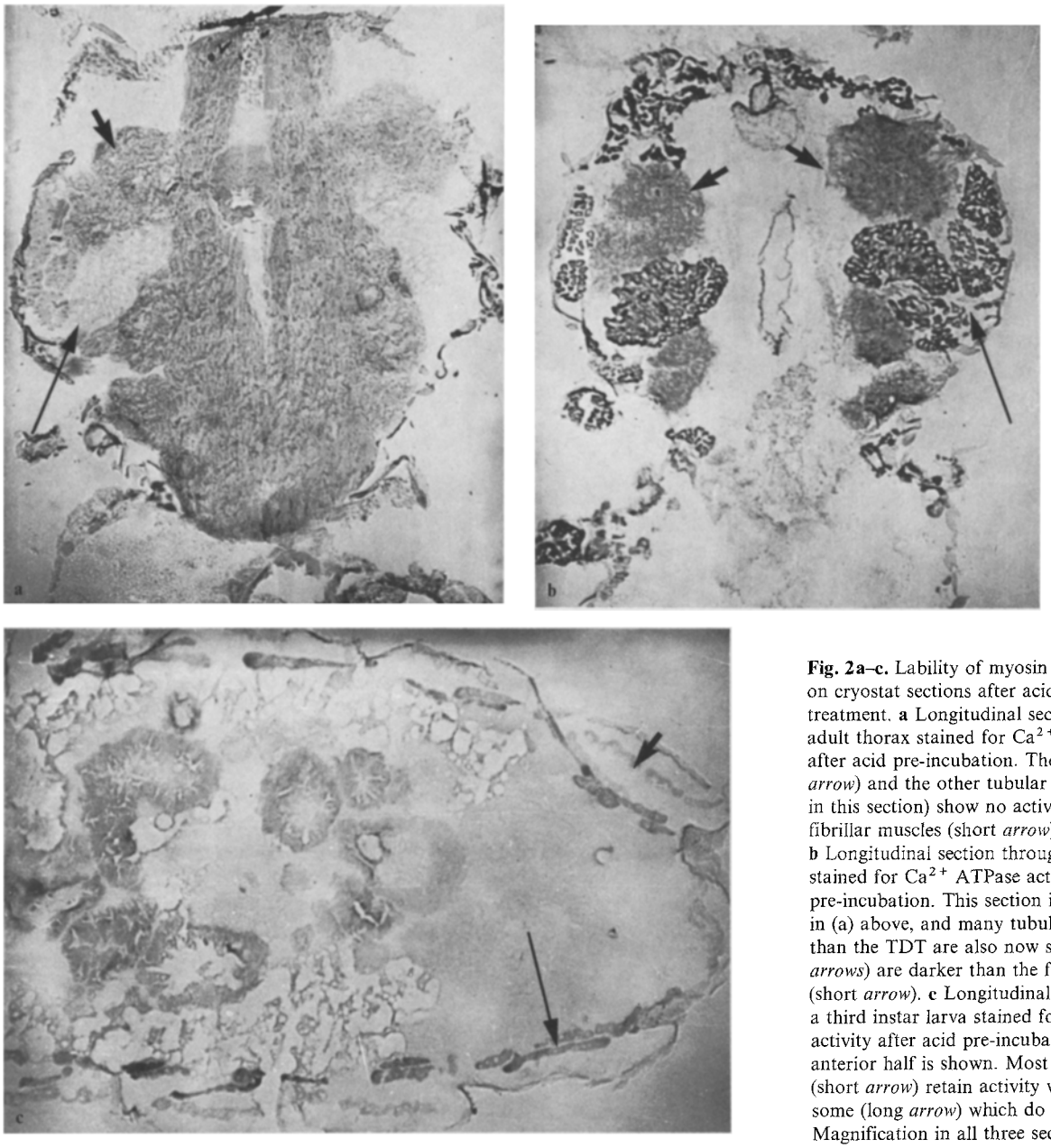

Fig. 2a–c. Lability of myosin ATPase viewed on cryostat sections after acid or alkali pre-treatment. **a** Longitudinal section through an adult thorax stained for Ca^{2+} ATPase activity after acid pre-incubation. The TDT (long *arrow*) and the other tubular muscles (not seen in this section) show no activity while the fibrillar muscles (short *arrow*) retain activity. **b** Longitudinal section through an adult thorax stained for Ca^{2+} ATPase activity after alkali pre-incubation. This section is ventral to that in (a) above, and many tubular muscles other than the TDT are also now seen. These (long *arrows*) are darker than the fibrillar muscles (short *arrow*). **c** Longitudinal section through a third instar larva stained for Ca^{2+} ATPase activity after acid pre-incubation. Only the anterior half is shown. Most of the muscles (short *arrow*) retain activity while there are some (long *arrow*) which do not show activity. Magnification in all three sections: ×100

adult bodies were run on native gels Band I was seen. The background streaking obscured the possible presence of Band II. When samples were eluted through a Sephadex G-200 column to remove the background we could not get a good separation of Bands I and II in the whole adult extracts. The adult band was however consistently broader than the larval band. Extracts from the adult body showed that Band I was predominant.

The native myosin bands (Fig. 1a) were cut after staining with coomassie blue and re-run on SDS gels after incubation in SDS sample buffer. When Band II was cut care was taken to cut the lower region so that no contaminating Band I was included. Several bands were pooled and re-electrophoresed in each case. The myosin heavy chain was assigned by co-migration with *Drosophila* myosin heavy chain from crude extracts. The *Drosophila* myosin heavy chain co-migrates with rabbit skeletal muscle myosin heavy chain. Figure 1c shows that the myosin heavy chain is present in Bands I and II.

Ca^{2+} ATPase Activity on Myosin Bands

Myosin crude extracts were electrophoresed under non-dissociating conditions and stained for Ca^{2+} ATPase activity as described in materials and methods. Bands I and II (Fig. 1a) show Ca^{2+} ATPase activity. In the larva Band II is barely visible by coomassie blue staining but can be seen when stained for enzyme activity (Fig. 1b). The other band (Band III, Fig. 1a) seen on native gels does not exhibit enzyme activity. However, there is some activity seen in the Band III region but it is not clear whether or not it is associated with any of the coomassie blue stained bands seen. Furthermore only Band I and II clearly have the myosin heavy chains as shown on SDS gels.

Lability of Ca^{2+} ATPase

We have examined the lability of the myofibrillar Ca^{2+} ATPase to acid and alkali preincubation. If there are multiple myosins

then their sensitivity to acid or alkali preincubation may be different and can be easily visualised histochemically if they are located in different muscle types.

Acid or alkali preincubation of frozen sections of whole flies and larvae were done as described in materials and methods. After preincubation at pH 4.0 the tergal depressor of the trochanter (TDT) and the other tubular muscles in the leg show no activity while the fibrillar flight muscles and the larval body wall muscles still retain significant activity (Fig. 2a, c). On alkali preincubation at pH 9.6 for 5 min the TDT and other tubular muscles show higher activity than the fibrillar muscles (Fig. 2b). In all cases heterogeneity amongst the tubular muscles themselves was observed. The TDT generally shows lower activity than the other tubular muscles as found by Deak (1977a) earlier. Further there are a few larval muscles (Fig. 2c, long arrow) that show no activity after acid preincubation.

Discussion

The separation of myosin on the pyrophosphate-polyacrylamide gels developed by d'Albis et al. (1979) is based on the different charge/molecular weight ratios of multiple forms of the molecule. These forms can arise from subunit differences which quite often are not resolved on SDS-PAGE where the separation is based on molecular weight alone. On electrophoresis of myosin extracts in the presence of SDS we find only one form of the heavy chain. The light chain sub-units are not easy to identify because *Drosophila* myosin is easily degraded and insect myosin is known to have light chain sub-units different from those of rabbit myosin (Bullard et al. 1973) and unlike the heavy chain, comigration with vertebrate myosin subunits cannot be used for identification of myosin light chains after electrophoresis in the presence of SDS. Electrophoretic separation of the native molecules based on differences in the charge/molecular weight ratios thus has certain advantages. Differences which are not apparent on molecular weight based fractionation alone can be visualised. Since enzyme activity can also be stained for, degradation and the presence of contaminants do not obscure the results.

Bullard et al. (1973) found that insect myosin had light chains distinct from that of rabbit skeletal muscle myosin on SDS gels. We therefore expected that on the pyrophosphate system *Drosophila* myosin will not have the same mobility as rabbit skeletal muscle myosin. We, in fact, find one band on native gels which co-migrates with rabbit skeletal muscle myosin, shows Ca^{2+} ATPase activity and has the intact myosin heavy chain. This band is present in extracts from all tissues (Band I, Fig. 1a). Band II also shows Ca^{2+} ATPase activity, and contains the myosin heavy chain. However, it has a mobility greater than that of rabbit myosin on native gels. Band II is present strongly in extracts from legs indicating that it could be a component of tubular muscles. Given that the cause of the heterogeneity between Bands I and II has not been demonstrated, the possibility that Band II is a degradation product of Band I must be considered. The fact that *Drosophila* myosin is easily degraded supports this viewpoint. At the same time certain other observations are relevant: Band II retains enzyme activity. It is present in both fresh and old samples and its amount relative to Band I does not change with sample age as viewed by coomassie blue staining. Both fresh and old larval extracts do not have Band II. It can still be maintained that Band II, though a breakdown product, retains enzyme activity and that the degradation is confined to adult extracts and not to the larva. This possibility, though unlikely, can be ruled out only by a subunit analysis of the two bands. However, the histochemical results independently support the existence of two myosin types. Wherever we see two myosins on native gels we see heterogeneity in the ATPases histochemically.

For example, Band II is visible on enzyme staining in larval extracts (Fig. 1b) but the coomassie blue staining barely shows it. Histochemically there are muscles in the larva (Fig. 2c) which show no activity after incubation at pH 4.0 while most of the larval muscles retain activity.

The histochemical results taken together with our detecting enzyme activity in Band II argues against it being a degradation product. While only a subunit analysis can demonstrate the cause of the heterogeneity we observe, our results indicate that there are at least two myosins in *Drosophila melanogaster*.

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