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Electrophoretic Differentiation of Myofibrillar Proteins in the Pig

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1. Starch-gel electrophoretograms of myosin and tropomyosin preparations in 8M-urea, from longissimus dorsi and psoas muscles of the pig, were characterized by laser densitometry. 2. The typical pattern for freshly prepared myosin from both muscles was similar, there being five electrophoretically distinct components. 3. The number of electrophoretically distinct components in both muscles increased after freeze-drying, but the effect of freeze-drying was more marked in longissimus dorsi. 4. Extraction with 8M-urea containing 2% β-mercaptoethanol decreased the number of major electrophoretically distinct components of the fresh myosin of both muscles to four. 5. Although there was also some simplification of the patterns after freeze-drying the greater susceptibility of the myosin from longissimus dorsi was still evident. 6. The typical pattern for freshly prepared tropomyosin in 8M-urea differed in the two muscles: in each case it was more complex than that of the corresponding myosins. 7. The pattern of tropomyosin from neither longissimus dorsi nor psoas was altered significantly after freeze-drying. 8. Electrophoretograms of pig longissimus dorsi tropomyosin in 8M-urea differed from those of longissimus dorsi tropomyosin from sheep, ox and rabbit. 9. Extraction of the tropomyosins in 8M-urea and 2% β-mercaptoethanol simplified the electrophoretic pattern to two major components with samples from pig, sheep and ox, and to one major component with samples from rabbit. 10. It was concluded that classification of skeletal muscles as 'red' or 'white' is insufficient to account for the degree of functional specialization which the electrophoretograms suggest.

So-called 'red' and 'white' muscles have predominant capacities for aerobic and anaerobic metabolism respectively (Lawrie, 1953*a*,*b*, 1968; Scopes, 1968); and the myosin of the frankly red muscles of the rabbit is distinguishable from that of the frankly white muscles in this species, having, for example, a lower Ca^{2+}-activated adenosine triphosphatase activity (Sréter, Seidel & Gergely, 1966). Even between muscles that are not superficially distinguishable by myoglobin concentration, there are other biochemical differences. Thus in l. dorsi* and psoas major of the ox, which have comparable myoglobin content, a cycle of freezing and thawing causes a much greater loss of water-binding capacity in the former, notwithstanding similar rates of post-mortem glycolysis and identical values for the final pH thereby attained, in both muscles (Howard, Lawrie & Lee, 1960). Again, in both ox and pig, freeze-drying causes significantly different degrees of damage to these two muscles, as indicated by the relative capacity of myofibrils isolated from each to reabsorb water on reconstitution (Penny, Voyle & Lawrie, 1963, 1964).

* Abbreviation: l. dorsi, longissimus dorsi.

The myofibrillar proteins (and particularly the myosins) of mammalian and avian muscle are markedly less susceptible to denaturation than are those of fish (Connell, 1961), but within the mammalian group those of the pig appear to be especially susceptible to change *post mortem*, as can be deduced from the severe exudation of fluid from porcine muscles frequently detected soon after death (Ludvigsen, 1954; Bendall & Lawrie, 1964). For this reason the investigations of differences between muscles in myofibrillar proteins described in the present paper were confined to samples from l. dorsi and psoas muscles of the pig. In both, the myoglobin content is less than 0·1% (Lawrie, Pomeroy & Cuthbertson, 1963): they are not clearly differentiated as 'red' or 'white'. It was hoped to establish if, notwithstanding the apparent similarity of their mode of generation of energy, the contractile proteins of these two muscles might be distinguishable and might thus reflect their differing water-binding capacities after being subjected to processes such as freeze-drying (Penny *et al.* 1964).

Myosin and tropomyosin, respectively taken to represent the more labile and less labile myofibrillar

components, were examined by electrophoresis on starch gel before and after freeze-drying. The electrophoretograms were scanned by a laser beam in the hope of obtaining a more accurate assessment of the relative concentration of the resolved components than is possible with incoherent light. In addition to consideration of such differences between muscles, the electrophoretic pattern of tropomyosin from l. dorsi of the pig was compared with that obtained from the tropomyosin of l. dorsi muscles of ox, rabbit and sheep.

MATERIALS AND METHODS

Starch was obtained from the Connaught Medical Research Laboratories, Toronto, Ont., Canada. All other reagents were of analytical grade. Deionized distilled water was used throughout.

Preparation of myosin. Myosin was prepared from porcine l. dorsi and psoas muscles (region of fourth, fifth and sixth lumbar vertebrae), immediatley *post mortem*, by the method of Perry (1955). In order to delay the onset of rigor mortis, and thus to enhance the yield of myosin, pigs were injected before slaughter with sufficient $MgSO_4$ solution (50%, w/v) to cause relaxation of muscles (Howard & Lawrie, 1956). The myosin preparations, after purification by reprecipitation four times from solution in 0·5 M-KCl by a tenfold dilution with water, had E_{280}/E_{260} ratios 1·6–1·7. All myosin samples were stored in 0·6 M-KCl at 0° for no longer than 1 week.

Preparation of tropomyosin. Tropomyosin was prepared from l. dorsi muscles of lamb, pig, ox and rabbit and from porcine psoas muscle by the method of Bailey (1948): post-rigor muscle was used in all cases. The $(NH_4)_2SO_4$ precipitation cycle was repeated three times between the limits 53–60% saturation (Hartshorne & Mueller, 1968) before the product was dissolved and the protein solution was dialysed against several changes of water. The absence of actomyosin as an impurity was shown by heating samples of all preparations at 60° for 5 min. (Hamoir & Laszt, 1962), when the solutions showed no signs of cloudiness or precipitate.

Accelerated freeze-drying. Myosin and tropomyosin preparations from l. dorsi and psoas porcine muscles were freeze-dried at a plate temperature of +50° in a Vickers–Armstrong pilot plant accelerated freeze-drier, the drying cycle being followed by a Honeywell multi-pen recorder.

Preparation of samples for electrophoresis. As subjected to electrophoresis, myosin preparations contained 2·5–2·7 mg. of protein/ml. and tropomyosin preparations 2·8–3·0 mg/ml. Protein determinations were made by the micro-Kjeldahl method, a value of 16·7% of N in protein being used for converting N contents into protein contents. Samples (2 ml.) of preparations of myosin and tropomyosin were pipetted into 6 ml. of 8 M-urea in 25 ml. conical flasks. The flasks were sealed with Parafilm and left for 24 hr. at 0° with occasional shaking (Scopes, 1964). Freeze-dried samples of myosin and tropomyosin were rehydrated to the concentration of the original preparations and the same extraction procedure was followed. In view of the work of Woods (1966, 1967), which indicated that incorporation of a thiol-protecting agent suppressed polymerization of tropomyosin subunits in 8 M-urea, myosin samples from l. dorsi and psoas of pig and tropomyosins from l. dorsi of pig, sheep, ox and rabbit were also extracted with 8 M-urea containing 2% β-mercaptoethanol. All mixtures were centrifuged at 20000g for 15 min. after extraction and the supernatants were retained for electrophoresis.

Starch-gel electrophoresis. A vertical apparatus as described by Smithies (1959) was employed with the following non-discontinuous buffer system of Neelin & Rose (1964): inner and outer gel buffer, 0·01 M-sodium barbitone–5 mM-HCl, pH 7·5 at 0°; upper and lower tray buffer, 0·1 M-sodium borate, pH 7·5 at 0°.

The starch content of the gel was 16% and 8 M-urea was added to prevent polymerization of the proteins under study (Mueller & Perry, 1962). Electrophoresis was carried out at 220 v for 5½ hr., 25–30 mA, in a cold-room kept at 0°. After being sliced, the gels were stained with a solution of 1% Naphthalene Black 10B plus 2% Nigrosine and cleared with 10% acetic acid to provide a translucent background.

Densitometry. Starch-gel electrophoretograms were scanned by a transmission-type densitometer, with a G3 helium gas laser as a light-source. Because laser light is so coherent, with very little beam divergence, starch gels, cleared of background stain with 10% acetic acid, were sufficiently transparent for this type of densitometry. The laser beam was directed into a slit of 1 mm. width. The image of the slit was focused in the centre of the gel being scanned, by a converging lens. The beam was turned through 90°, by a prism mounted after the lens, to pass vertically through the horizontal gel. The gel was contained between two glass slides on a mechanically driven traverse moving at 15 mm./min. Light transmitted through the gel was received by an EEL selenium photocell connected to a Rikadenki potentiometric recorder. All the components of the densitometer were fixed on an optical bench.

RESULTS

Typical values for the relative densities of stained protein components, separated by starch-gel electrophoresis and scanned by a laser beam, from myosins of l. dorsi and psoas muscles of the pig are illustrated in Figs. 1 and 2. Fig. 1 shows the results with 8 M-urea alone. Fig. 2 illustrates the effect of including 2% β-mercaptoethanol in 8 M-urea. Peaks are numbered from the origin (the cathode).

From Figs. 1(*a*) and 1(*b*) it is apparent that, as freshly prepared, the myosins of both muscles were similar in having three major and two minor electrophoretically distinct components. Of these, the two most electropositive were present in greatest quantity (peaks 1 and 2). Freeze-drying clearly effected a considerable change in myosins from both sources (Figs. 1*c* and 1*d*). As judged by the increased number of components (e.g. peak 6) and by the development of a markedly less mobile component (peak 1), that of l. dorsi was more extensively altered. The laser traces in Fig. 2 indicate that the inclusion of β-mercaptoethanol with the 8 M-urea simplified these electrophoretic patterns to some extent both before and after freeze-drying. Five peaks were evident in the patterns of fresh myosins of l. dorsi and psoas (Figs. 1*a* and 1*b*) in the absence of the thiol, but these were decreased to four

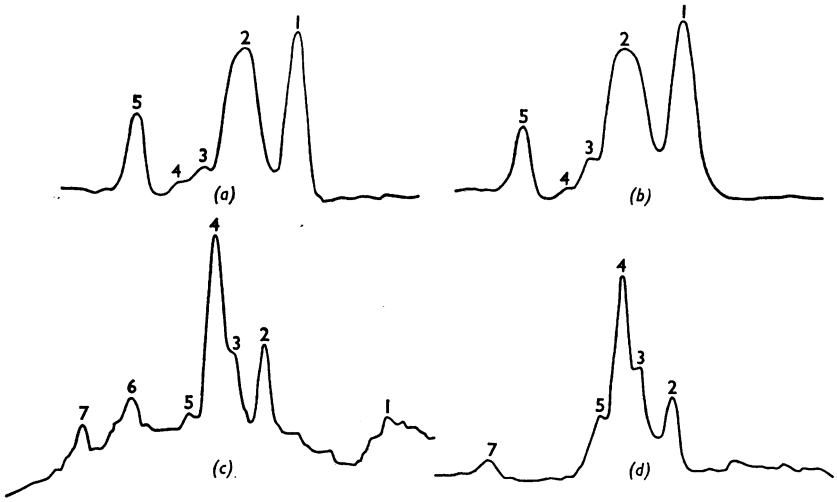

Fig. 1. Densitometer tracings (by laser beam) of typical starch-gel electrophoretograms in 8 M-urea of myosins of the pig: (*a*) l. dorsi myosin (fresh); (*b*) psoas myosin (fresh); (*c*) l. dorsi myosin (freeze-dried); (*d*) psoas myosin (freeze-dried). Peaks are numbered in order of increasing electronegativity.

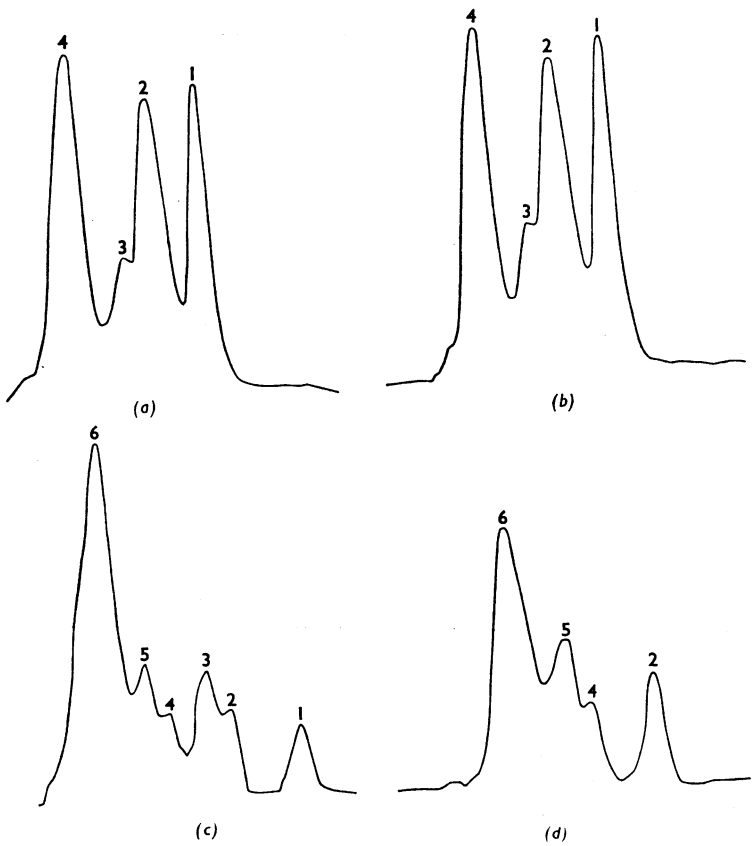

Fig. 2. Densitometer tracings (by laser beam) of typical starch-gel electrophoretograms in 8 M-urea containing β-mercaptoethanol of myosins of the pig: (*a*) l. dorsi myosin (fresh); (*b*) psoas myosin (fresh); (*c*) l. dorsi myosin (freeze-dried); (*d*) psoas myosin (freeze-dried). Peaks are numbered in order of increasing electronegativity.

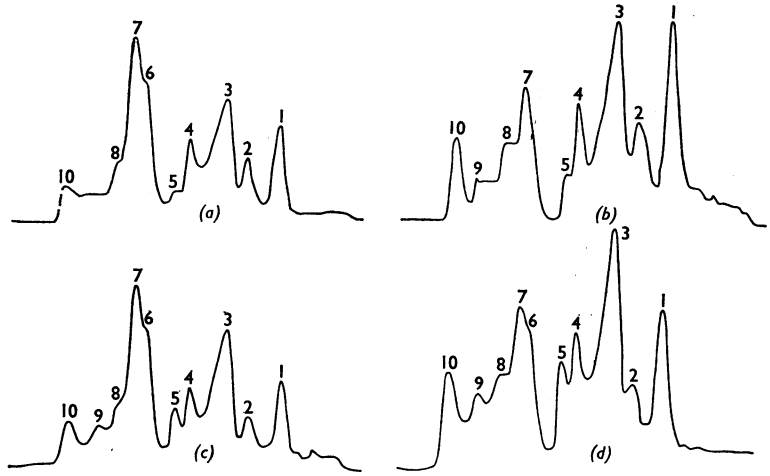

Fig. 3. Densitometer tracings (by laser beam) of typical starch-gel electrophoretograms in 8M-urea of tropomyosins of the pig: (*a*) l. dorsi tropomyosin (fresh); (*b*) psoas tropomyosin (fresh); (*c*) l. dorsi tropomyosin (freeze-dried); (*d*) psoas tropomyosin (freeze-dried). Peaks are numbered in order of increasing electronegativity.

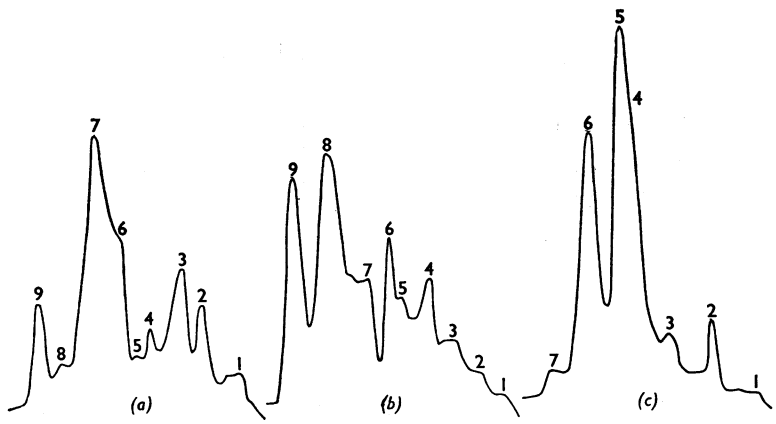

Fig. 4. Densitometer tracings (by laser beam) of typical starch-gel electrophoretograms in 8M-urea of tropomyosins from l. dorsi of (*a*) sheep, (*b*) ox and (*c*) rabbit. Peaks are numbered in order of increasing electronegative character.

in both muscles when β-mercaptoethanol was present. However, the general impression given by Fig. 1 was confirmed. Thus, though the fresh myosins of both muscles gave almost identical patterns (Figs. 2*a* and 2*b*), freeze-drying increased the number of electrophoretically distinct components from both muscles, but to a greater degree with those from l. dorsi (Figs. 2*c* and 2*d*).

In spite of the expectations that tropomyosins would polymerize in the absence of β-mercaptoethanol (Woods, 1966, 1967), it is noteworthy in Figs. 3(*a*) and 3(*b*) that the freshly prepared tropomyosins from pig showed a greater degree of electrophoretic complexity in 8M-urea than did the corresponding myosins, there being three major and seven minor components in tropomyosin samples from both muscles. The patterns for the freshly prepared tropomyosins from the two muscles differed more markedly from one another than did those for the corresponding myosins. However,

freeze-drying had relatively little effect on the components of the tropomyosins of either muscle, as the differences seen between the fresh samples were largely unchanged (Figs. 3*c* and 3*d*).

Characteristic patterns for freshly prepared tropomyosin in 8M-urea from l. dorsi muscles of sheep, ox and rabbit are shown in Figs. 4(*a*), 4(*b*) and 4(*c*) respectively. On comparing these with that of pig (Fig. 3*a*) it is clear that the tropomyosins of the four species are different. The patterns for pig, sheep and ox were not dissimilar but there was a tendency for the more electronegative components to be less in sheep than in pig, and to be less in ox than in sheep. The pattern for rabbit tropomyosin was simpler and more distinct than those of the other three species, there being only seven components, of which two were predominant (Fig. 4*d*, peaks 5 and 6). Although extraction of tropomyosin from l. dorsi with 8M-urea containing β-mercaptoethanol greatly diminished the number of electrophoretically separable components, the distinction between the tropomyosins of pig, sheep and ox on the one hand, and rabbit on the other, remained, there being two major peaks in the former group and one major peak in the latter (Fig. 5).

It may be significant that even with the simplified pattern in the presence of β-mercaptoethanol there appeared to be slight differences in the relative electrophoretic mobilities and in the relative peak heights of the two major components of the tropomyosins of pig, sheep and ox.

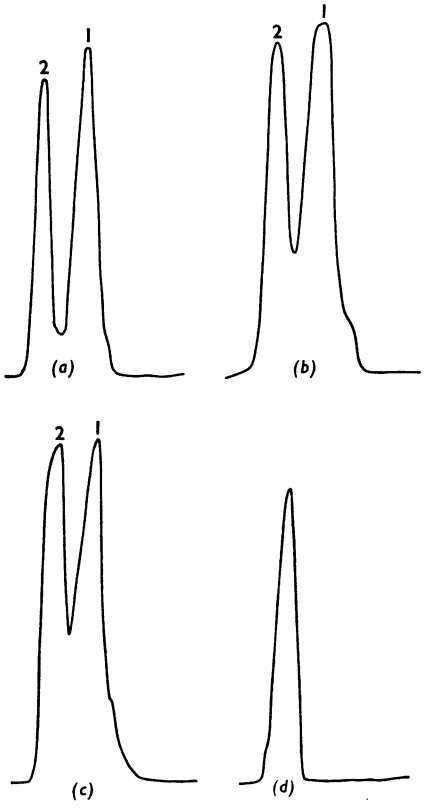

Fig. 5. Densitometer tracings (by laser beam) of typical starch-gel electrophoretograms in 8M-urea containing β-mercaptoethanol of tropomyosins from l. dorsi of (*a*) pig, (*b*) sheep, (*c*) ox and (*d*) rabbit. Peaks are numbered in order of increasing electronegativity.

DISCUSSION

The degree of complexity of the composition of myofibrillar proteins indicated by the electrophoretograms is not unexpected. Thus Locker & Hagyard (1968) demonstrated that five subunits of low molecular weight are derivable from acetylated myosin in so-called 'mixed' rabbit muscle (i.e. a muscle having both 'red' and 'white' fibres). The porcine muscles investigated fall within such a category, neither l. dorsi nor psoas being markedly 'red' or 'white' in character.

The major intention of the present study was to establish whether or not there were characteristic differences between the electrophoretic patterns of the myofibrillar proteins of l. dorsi and psoas of the pig. Such differences might reflect their known differences in susceptibility to extraneous manipulation *post mortem*. It was realized that myofibrillar proteins, dissociated by 8M-urea, may reassociate in various ways, and hence that a multiplicity of electrophoretically distinct components need not signify a corresponding multiplicity of structurally distinct entities. This fact is attested by the simplification of the patterns of the tropomyosins (to one major component with preparations from rabbit and to two major components with those from pig, sheep and ox) that is effected by β-mercaptoethanol. The findings on rabbit tropomyosin accord with those of Woods (1967). He showed that, after incubation of rabbit tropomyosin with *N*-ethylmaleimide, the number of protein components separable by electrophoresis on acrylamide gel was lowered from eight or nine to one. Nevertheless, if under identical conditions of preparation and electrophoresis, even in the absence of a reagent protecting thiol groups, proteins from different sources produce characteristically and consistently different patterns, this must signify some distinction between them, however subtle. Although interpretation of the pattern is necessarily complicated in the presence of 8M-urea by the possibility of polymerization of subunits dissociated from the original protein, the fact of distinction itself is not altered.

Moreover, with regard to the myosin preparations in the present experiments, inclusion of β-mercaptoethanol with 8M-urea produced relatively little change in the overall effect obtained in its absence. Under both experimental conditions, the electrophoretic patterns of the freshly prepared myosins of l. dorsi and psoas of the pig were very similar; freeze-drying altered these patterns, and this alteration was considerably more marked with l. dorsi. This accords with the greater susceptibility of the intact l. dorsi muscle to lose water-binding capacity when processed. The tropomyosins of the two muscles, although more clearly differentiated as freshly prepared samples in 8M-urea than are the corresponding myosins, show little change on freeze-drying, which accords with their known higher resistance to denaturing conditions.

Although differences between the Ca^{2+}-activated adenosine triphosphatase of the myosins of 'red' and 'white' muscles have been demonstrated (Sréter *et al.* 1966), there was no reason to suppose that the contractile proteins of muscles having such comparable energy-yielding mechanisms as do l. dorsi and psoas of the pig should differ. The distinction that obviously exists between the myosins and tropomyosins of these two muscles presumably reflects a more subtle degree of functional specialization in muscle at the biochemical level than is presently recognized.

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