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Methods for Starch-Gel Electrophoresis of Sarcoplasmic Proteins

AN INVESTIGATION OF THE RELATIVE MOBILITIES OF THE GLYCOLYTIC ENZYMES FROM THE MUSCLES OF A VARIETY OF SPECIES

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1. Details of an improved method for starch-gel electrophoresis of water-soluble muscle proteins are given. 2. Methods are described for detecting enzyme activities on the starch gel after electrophoresis, by using pieces of filter paper. 3. Compositions of incubation mixtures suitable for detecting any of the enzymes of glycolysis, and certain other enzymes, are given. 4. A comparison of the various enzymes in extracts of several muscles from one rabbit was made; most differences are quantitative only. 5. A detailed comparison of the mobilities of various enzymes in extracts of muscles from a wide variety of species was made. Each species was found to have a characteristic pattern of proteins on the starch gel, and the mobilities of individual enzymes varied considerably. 6. Potential uses and extensions of the methods are discussed.

The comparative composition of skeletal muscle has been the subject of many investigations, most work having concentrated on the protein components. It has been found that whereas the structural proteins, at least of the vertebrates, show a universal similarity, and often are indistinguishable regardless of source, the proteins of the sarcoplasm vary both qualitatively and quantitatively even between closely related species (Hamoir, 1955). The proteins of many species, including mammals, reptiles and fish, have been investigated in the Laboratories of Professor M. Dubuisson (e.g. Jacob, 1947; Crepax, 1952; Henrotte, 1960; Focant & Pechère, 1965). Also, Connell (1953), using free-boundary electrophoresis, compared the sarcoplasmic proteins of 20 fish species, and Nikkila & Linko (1955) used zone electrophoresis on paper in a study of ten fish species.

Gel electrophoresis has proved to be ideally suited for such comparative work. Giles (1962) presented starch-gel-electrophoresis patterns of rabbit, ox, pig and sheep sarcoplasmic proteins; Tsuyuki, Roberts & Vanstone (1965) compared about 50 fish, and Baker (1966) some avian species. One of the advantages of this technique is that it is possible to identify the protein bands by several methods. These include elution from the gel and assaying for enzyme activity (Hartshorne & Perry, 1962; Tsuyuki, 1963), parallel running of purified enzyme preparations (Tsuyuki & Wold, 1964; Scopes, 1964*a*) and the use of specific staining techniques (e.g. Markert & Møller, 1959; Spencer, Hopkinson & Harris, 1964; Sjorvall & Voigt, 1964; Scopes, 1964*b*). By using a combination of these techniques, Scopes (1964*c*, 1966) identified all the enzymes of the glycolytic sequence in the starch-gel-electrophoresis pattern of pig muscle sarcoplasmic proteins. Further development of the specific staining techniques is discussed in the present paper, together with the results of comparisons first of several muscles from one rabbit and secondly of muscles from a variety of species.

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EXPERIMENTAL

Materials. Muscle was obtained as soon as possible after death; extracts were made within 3hr., usually within 15min., and before rigor mortis had set in. The kangaroo muscle, however, was frozen 1hr. *post mortem*, and kept frozen for 2 weeks before extraction. Previous work (Scopes, 1964*a*; Aberle & Merkel, 1966) has shown that the starch-gel-electrophoresis pattern of the sarcoplasmic proteins is little changed up to 24hr. *post mortem*, provided that the muscle is well cooled.

Sources of enzymes were as described in Newbold & Scopes (1967). Substrates and cofactors were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

Preparation of extracts. Extracts were made by homogenizing muscle in 2vol. of iso-osmotic glycerol (about 2%,

v/v) containing 2mM-EDTA and 10mM-tris (pH8·7). Glycerol was used rather than sucrose (Czok & Bücher, 1960) so that the subsequent dialysis of the extract against a sucrose-containing buffer would result in a substantial concentration of the protein solution. The homogenates were adjusted to pH7·0±0·3 with M-tris if necessary, before centrifuging at 25000*g* for 10min. The pH of the extract was then lowered to 5·5 with N-acetic acid, and the extract was centrifuged again, the temperature being kept below 10° during these manipulations. This procedure removed any particulate material still present, and some minor nucleoproteins that did not contribute substantially to the final electrophoretic pattern (Scopes, 1964*a*). Phosphofructokinase was also precipitated (Parmeggiani, Luft, Love & Krebs, 1966), but in only one species, the codling (*Gadus callarias*), did this treatment cause any marked denaturation of proteins. The supernatant pH was finally adjusted to about 7·5 with M-tris, snd the solution dialysed overnight against a buffer containing 3mM-phosphoric acid and 0·6M-sucrose, adjusted to pH7·8 with tris. Dialysed samples were stored at −22° until required.

Apparatus and procedure. The salient features of the vertical apparatus and the buffer systems employed have been described previously (Scopes, 1963, 1964*a*, 1966). Full details of the methodology are given here, including several minor points of procedure that are important for achieving the best results.

With a vertical apparatus as originally described by Smithies (1959), a considerable proportion of the gel is involved in support for the inner part in which the migration of proteins occurs. If attempts are made to combine the beneficial effects of a discontinuity in the gel buffer (Poulik, 1957) with the advantages that the vertical gel system has for sample application, it is found that the quantity of supporting gel at the cathode is so great that, by the time the discontinuity reaches the central portion, many of the more mobile proteins are already migrating into the lower supporting gel. Two methods of overcoming this difficulty are possible. First, by designing an apparatus that does away with the supporting blocks, the discontinuity can be made to commence closer to the insertion slots. Alternatively, with the normal apparatus a buffer discontinuity can be built into the gel by pouring the gel in two parts, the supporting (outer) part being made up in a borate buffer, and the central (inner) part in a citrate (or other polyanion, see below) buffer. The first of these methods poses difficulties in preventing the gel from slipping while in the vertical position, and there are additional problems in controlling the conductivity and buffering in the gel during the electrophoretic run. These difficulties could probably be overcome by suitable designs. The author has adopted the two-gel method, which has proved to be very satisfactory for the electrophoresis of sarcoplasmic proteins, and readily adaptable when some other type of extract has demanded use of different buffer and pH conditions.

To minimize diffusion and create maximum reproducibility, an efficient cooling system must be included in the design of apparatus. A box that will take crushed ice can be built on to the back of Smithies's (1959) design. The apparatus used for the experiments described below was similar to this, consisting of a rectangular box (for the ice) on one side of which were built the containing walls for the gels. It is illustrated in Fig. 1. The outer gel was poured in the ends suitably blocked in by removable pieces of Perspex

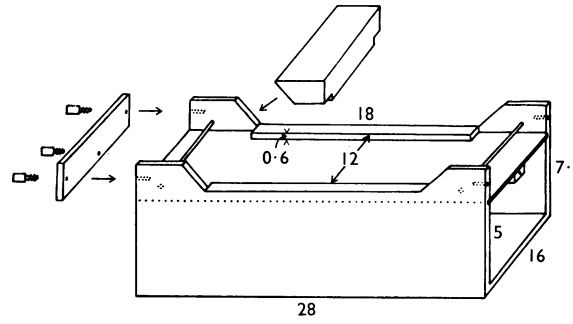


Fig. 1. The starch-gel-electrophoresis apparatus, in position for pouring the gels. Removable blocking pieces for one end are illustrated. Dimensions are in cm.

as shown; there were Perspex rods passing through the centre of these end gels to aid their support. The total volume of gel poured was about 100ml. at each end. After the outer gel had set, the blocks were removed and any leakage into the inner section was cleaned away. The surface to take the inner gel was given a very thin coating of a light oil, and the second gel was then made and poured into the rectangular hole, until the level was slightly above the sides. A slot-former (which did not cover the whole gel) made ten slots 6cm. from the cathode end of the inner gel. When this gel had partly set, exposed parts were covered with a thin sheet of polyethylene, and the apparatus was left in the cold for several hours, usually overnight. The two gels stuck together well, a discontinuity in the buffers being produced at their junction.

The samples were applied to the slots by means of a fine pipette, and were sealed in with a molten mixture of petroleum jelly and CCl_4, which was sufficiently soft at 0° to stick to the gel and not flake away. The whole gel was finally covered with a sheet of thin polyethylene, and the reservoir on the back of the apparatus filled with crushed ice and water. Electrophoresis was carried out in the cold-room, to minimize temperature gradients across the gel.

After completion of electrophoresis, the inner gel was removed, sliced in half and stained for 30sec. with a solution of 10g. of Amido Black 10B and 20g. of Nigrosine/l. of methanol–acetic acid–water (5:1:4, by vol.), then washed with water, followed by several changes of the above-mentioned solvent. The complementary half was stained for various enzymes as described below.

In the author's experience, the most important features of the system are the buffers used, not only in the gels, but also in the electrode trays. Other workers have commonly used a sodium borate buffer at both anode and cathode, with or without Ag–AgCl electrodes in reservoirs of KCl solution. During electrophoresis, Na^+ ions migrate into the gel, and are major contributors to the current, but do not buffer at all. At the very low ionic strengths occurring with discontinuous systems, this can result in very poor buffering in the gel. In the present system, a tris buffer was used at the anode and for making up the outer gel; thus only $tris^+$ ions could carry cationic current through the gel, and being in equilibrium with neutral tris at all times, contributed substantially to the buffering. With a sodium borate buffer at the cathode, however, it was found that the current fell

continuously during the electrophoresis as borate ions were neutralized by $tris^+$ ions. Erratic and often unrepeatable results were obtained as a result of the very low conductivity in the gel. Inclusion of some chloride in the cathode buffer could prevent this; the very mobile Cl^- ions penetrated the outer gel block, and moved through the inner gel, keeping the current up; since the only cations were $tris^+$, the higher current reflected a higher buffering in the gel. Inclusion of chloride in the buffer for making up the outer gel was not a satisfactory alternative. The best results were obtained with 50mM-NaCl in the cathode buffer (in practice $Na_2B_4O_7$ was partly neutralized with HCl). With the buffers described below, the current was about 30mA when 400v was applied to the system, and fell to about 20mA after 1·5hr., and remained at that value for the next 2hr., by which time the discontinuity had reached the end of the inner gel. Attempts to duplicate these current characteristics in a one-gel system by adjusting the amount of NaCl in the cathode buffer were unsuccessful.

The composition of the buffers for use in the inner gel has been extensively investigated. Diethylenetriamine pentaacetic acid was used, as a polyanion with strong complex-forming powers to remove any traces of heavy metals in the gel (Scopes, 1964*a*). However, basically similar patterns were obtained with other polyanions such as citrate, EDTA, phosphate or pyrophosphate. Most of the results described below were achieved with a 3·5mM-phosphate buffer containing EDTA in the inner gel. The estimated pH in the gel during electrophoresis, taking into account the slight acidity of the starch and the temperature effect on the pK of the tris buffer, was 8·4. Homogenates of inner gel taken before or after electrophoresis differed little in pH. The buffers were made up as follows: outer-gel buffer, 36g. of tris and 30g. of H_3BO_3 in 1l., diluted fivefold for use; inner-gel buffer, 2·3ml. of 85% (w/v) H_3PO_4, 18g. of tris and 370mg. of disodium EDTA in 100ml., diluted 100-fold for use; cathode buffer, 95g. of $Na_2B_4O_7$ and 25ml. of 10N-HCl in 5l.; anode buffer, 60g. of tris and 8ml. of 36N-H_2SO_4 in 5l. A platinum anode was used, and a stainless-steel or platinum cathode. About 200ml. of each electrode buffer was used, and between runs these buffers were drained off and replaced, but the filter-paper bridges retained without washing.

A buffer system for operating at a lower pH was preferable in certain cases; with the same electrode buffers as above, the gels were made up in the following: outer-gel buffer, 5·5g. of diethylbarbituric acid and 1·5g. of imidazole in 1l., used undiluted; inner-gel buffer, 6·3g. of citric acid monohydrate, 12g. of imidazole and 370mg. of disodium EDTA in 100ml., diluted 100-fold for use. The estimated pH during electrophoresis was 7·2.

All illustrations in this paper are of gels run in the pH8·4 system.

It was found that gels left at temperatures close to 0° for several hours changed in some way so that they no longer gave satisfactory results. This effect could be avoided by inclusion of urea in the gel. 1M-Urea not only greatly improved the gel's keeping properties, but also improved its handling characteristics; it was included in all inner gels.

Outer gels were made with 20g. of starch and 200ml. of buffer, inner gels with about 18g. of starch (depending on the batch), 9g. of urea and 150ml. of buffer. The assembled system is illustrated in Fig. 2.

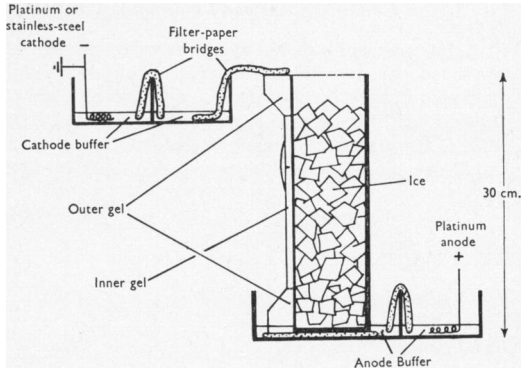


Fig. 2. Arrangement of the starch-gel-electrophoresis apparatus and buffer trays.

Methods for detecting certain enzymes on the gel surface have been described by many authors (e.g. Markert & Møller, 1959; Spencer *et al.* 1964; Sjorvall & Voigt, 1964; Thorne, Grossman & Kaplan, 1963; Fildes & Harris, 1966). The present work describes methods based on the reduction of nitro-blue tetrazolium by NAD(P)H, for all the enzymes of glycolysis, and some associated enzymes. By using sections of the glycolytic chain (plus glucose 6-phosphate dehydrogenase), all activities can be made ultimately to result in the oxidation or reduction of NAD(P). However, use of one or more coupling enzymes in free solution is not always satisfactory because of rapid diffusion of the products of reaction across the surface of the gel, before the coupling enzymes can complete the reaction chain. Diffusion time can be restricted by using a large excess of coupling enzymes; this demands a high purity of the enzymes, and often a large budget, to obtain satisfactory results.

The products of reaction can be partially fixed close to their generation point by using a sheet of filter paper soaked in the reaction mixture laid on the gel. Not only is diffusion across the surface restricted, but also high concentrations of enzymes and substrates can be used, as only a small volume of reaction mixture is required. This has proved to be very satisfactory for normal 'positive' methods involving one or more coupling enzymes to reduce $NAD(P)^+$. It produces bands of reduced nitro-blue tetrazolium at the sites of enzyme activity, mainly on the filter paper. The use of filter paper also enables 'negative' methods to be employed, in which the enzymes manifest themselves by oxidation of applied NADH, through coupling enzymes if necessary. After incubation the filter paper is removed; both the paper and the gel itself can then be treated with nitro-blue tetrazolium to detect unchanged NADH.

In practice, the procedures for staining were as follows. A piece of filter paper (Whatman no. 1) was soaked with the reaction mixture; 3ml. was sufficient for a paper covering the whole gel, but often smaller papers were used. The paper was laid on the gel, care being taken to exclude air bubbles. With positive methods, purple bands appeared after a few minutes at the sites of activity. The filter paper was removed, washed with water and dried, and could be

Table 1. *Composition of solutions used in filter-paper-staining techniques for specific enzymes*

Solutions were buffered at pH 7·8 with tris (50 mM) and included 5 mM-$MgSO_4$. All positive (+) methods also included nitro-blue tetrazolium (3 mg./ml.) and phenazine methosulphate (0·5 mg./ml.). All negative methods (−) included NADH at 3–5 mM. A mixture of nitro-blue tetrazolium (3 mg./ml.) and phenazine methosulphate (0·5 mg./ml.) was used to detect unchanged NADH. Coupling enzymes were at a concentration of 5–10 E.U./ml. (1 E.U. transformed 1 μmole of substrate/min. at 30°). Coupling enzymes in parentheses were not essential additions in cases where there was a high activity of the enzyme being sought in the gel.

Enzyme	Abbreviation	+ or −	Substrates, coenzymes etc.	Coupling enzymes*
Hexokinase† (EC 2.7.1.1)	HK	+	Glucose, 20 mM; ATP, 8 mM; NADP, 0·2 mM	G6PDH
Glucose 6-phosphate dehydrogenase† (EC 1.1.1.49)	G6PDH	+	Glucose 6-phosphate, 8 mM; NADP, 0·2 mM	—
α-Glucan phosphorylase† (EC 2.4.1.1)	PH	+	Glycogen, 1 mg./ml.; NADP, 0·2 mM; glucose 1,6-diphosphate, 0·01 mM	PGM, G6PDH
Phosphoglucomutase (EC 2.7.5.1)	PGM	+	Glucose 1-phosphate, 8 mM; glucose 1,6-diphosphate (present in glucose 1-phosphate); NADP, 0·2 mM	G6PDH
Phosphoglucose isomerase (EC 5.3.1.9)	PGI	+	Fructose 6-phosphate, 8 mM; NADP, 0·2 mM	G6PDH
Phosphofructokinase† (EC 2.7.1.11)	PFK	+	Fructose 6-phosphate, 8 mM; ATP, 5 mM; NAD, 1 mM; sodium arsenate, 5 mM	ALD, TPI, GAPDH
Aldolase (EC 4.1.2.7)	ALD	+	Fructose 1,6-diphosphate, 5 mM; NAD, 1 mM; sodium arsenate, 5 mM	TPI, GAPDH
Triose phosphate isomerase (EC 5.3.1.1)	TPI	+	Dihydroxyacetone phosphate, 5 mM; NAD, 1 mM; sodium arsenate, 5 mM	GAPDH
α-Glycerophosphate dehydrogenase (EC 1.1.1.8)	αGPDH	−	Fructose 1,6-diphosphate, 5 mM	ALD, TPI
Glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12)	GAPDH	+	Fructose 1,6-diphosphate, 5 mM; NAD, 1 mM; sodium arsenate, 5 mM	ALD, TPI
Phosphoglycerate kinase (EC 2.7.1.31)	PGAK	−	3-Phosphoglycerate, 8 mM; ATP, 8 mM	GAPDH, (TPI), (αGPDH)
Phosphoglycerate mutase (EC 2.7.5.3)	PGAM	−	2-Phosphoglycerate, 5 mM; 2,3-diphosphoglycerate, 0·1 mM; ATP, 5 mM; phosphoenolpyruvate, 5 mM; KCl, 0·1 M	GAPDH, PGAK, TPI, αGPDH, PK, LDH
Phosphopyruvate hydratase (EC 4.2.1.11)	PPH	+§	2-Phosphoglycerate, 8 mM; ADP, 5 mM; glucose, 20 mM; KCl, 0·1 M; NADP, 0·2 mM	PK, G6PDH, HK
Phosphopyruvate hydratase (EC 4.2.1.11)	PPH	−	2-Phosphoglycerate, 8 mM; ADP, 8 mM; KCl, 0·1 M; (glucose, 20 mM)	PK, LDH, (HK)
Pyruvate kinase (EC 2.7.1.40)	PK	+§	Phosphoenolpyruvate, 8 mM; ADP, 5 mM; KCl, 0·1 M; NADP, 0·2 mM, glucose, 20 mM	HK, G6PDH
Pyruvate kinase (EC 2.7.1.40)	PK	−	Phosphoenolpyruvate, 8 mM; ADP, 5 mM; KCl, 0·1 M; glucose, 20 mM	LDH, HK
Lactate dehydrogenase (EC 1.1.1.27)	LDH	−	Sodium pyruvate, 10 mM	—
Creatine kinase (EC 2.7.3.2)	CK	+§	Creatine phosphate, 8 mM; ADP, 5 mM; glucose, 20 mM; NADP, 0·2 mM	HK, G6PDH
Creatine kinase (EC 2.7.3.2)	CK	−	Creatine, 30 mM; ATP, 5 mM; phosphoenolpyruvate, 8 mM; KCl, 0·1 M‡	PK‡, LDH
AMP kinase (EC 2.7.4.3)	AMPK	+	ADP, 5 mM; glucose, 20 mM; NADP, 0·2 mM	HK, G6PDH

* Abbreviations of enzymes are defined in column 2.

† These methods were not always studied in the sarcoplasmic extracts.

‡ pH adjusted to 9·0 with 2 M-tris, and 30–40 E.U. of pyruvate kinase/ml. was used.

§ These methods also stain AMP kinase.

stored for future reference. By making appropriate measurements on the two halves of the gel, the location of the enzyme activity in the general protein pattern could be determined.

With negative methods, the progress of activity was not visible; the filter paper was removed after 2–15 min., depending on the enzyme activity. A streak of solution containing nitro-blue tetrazolium and phenazine methosulphate was placed on a sheet of polyethylene, about 1 ml. being sufficient for a full-sized filter paper. The paper was quickly passed through this streak of liquid, and the NADH remaining caused the appearance of a purple precipitate of reduced dye on the paper, with clear areas indicating the enzyme's location. As before, the paper was washed and dried. The enzyme's location could also be detected on the gel itself, by laying a filter paper soaked in the dye mixture on to the gel. This was more satisfactory if the first stage had been repeated, thus allowing more NADH to diffuse into the top surface of the gel. Most of the colour produced in this way was on the gel surface. However, some clearer areas sometimes resulted at positions where there was a high concentration of protein other than the enzyme being sought, because of lower penetration of NADH at this position; results obtained in this way should be compared with the filter paper before conclusions are reached.

The compositions of the staining mixtures for each enzyme are given in Table 1. Concentrations of substrates were not critical, and 2–3 mM-substrate transformed was sufficient to give a satisfactory colour in the positive methods. But for the negative methods, NADH was required at 3–4 mM to give a satisfactory background colour, and non-regenerated substrates to oxidize it were required in excess of this amount. Coupling enzymes were mostly added at a concentration of 5–10 E.U./ml.; but, for the negative creatine kinase method, the properties of the enzymes involved necessitated the use of pyruvate kinase at 30–40 E.U./ml. (1 E.U. is that amount of enzyme transforming 1 μmole of substrate/min. at 30°).

Comments on the individual methods are reserved for the Results section. Hexokinase, glucose 6-phosphate dehydrogenase and phosphofructokinase were generally not present in the extracts; the kinases were precipitated by the pH 5·5 treatment (see the section on preparation of extracts). Phosphorylase was strongly adsorbed in the starch, and either did not migrate into the gel or else formed a sharp band about 1 mm. from the insertion slot on the anode side. None of these four enzymes is discussed further. Myoglobin (together with other proteins with catalase activity) was stained with *o*-dianisidine as described by Owen, Silberman & Got (1958).

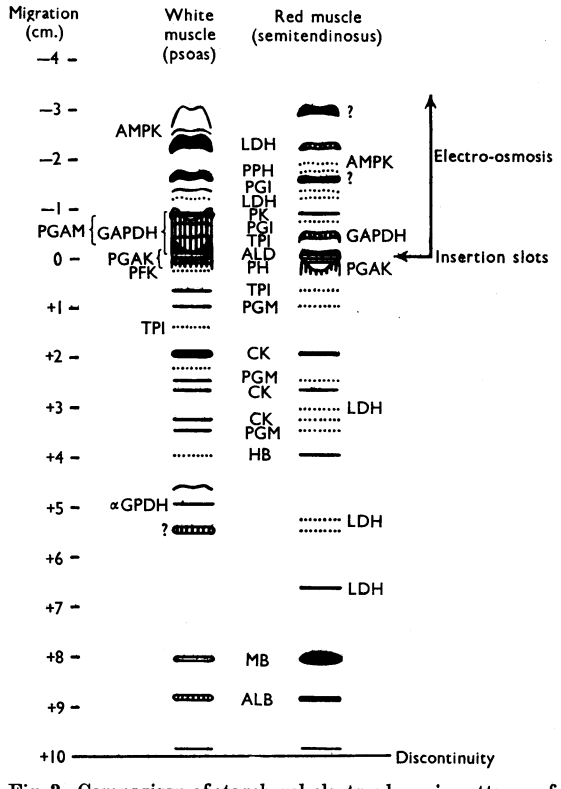


Fig. 3. Comparison of starch-gel-electrophoresis patterns of typical white (psoas) and red (semitendinosus) muscles from rabbit. HB, Haemoglobin; MB, myoglobin; ALB, albumin. For enzyme abbreviations, see Table 1. In the centre, the positions of the enzymes that have identical mobilities in both white and red muscles are given; on either side are given the identities of bands that only appear in the one muscle type in that particular position. Phosphofructokinase position was determined with an extract that had not been adjusted to pH 5·5 (see the text). Major unidentified bands indicated by ?.

RESULTS

Comparisons of the starch-gel-electrophoresis patterns of the sarcoplasmic proteins (general protein stain) from several different muscles from the same rabbit are shown in Plate 1(*a*) and Fig. 3. It can be seen that the white muscles (longissimus dorsi, semimembranosus, psoas) have considerably more of the glycolytic enzymes (Weber & Meyer, 1933), most of which migrate towards the cathode, than the red muscles (semitendinosus, intertransversarius). The red muscles have a strong myoglobin band, more albumin, more heart-type lactate dehydrogenase, less creatine kinase, and two cathodic bands that have not been identified (indicated by ?). The unidentified band at + 5·5 cm. in the white muscles, particularly marked in the psoas, corresponds to the unidentified nucleotide-containing protein described by Czok & Bücher (1960).

The mobilities of most of the individual enzymes did not differ between the white and red muscles. However, certain unsharp bands moved more towards the anode in the red-muscle extracts; this was a function of relative concentration, since in diluted samples of white-muscle extracts these proteins again moved more towards the anode. Thus there was little evidence of structurally different enzymes in the two types of muscle, with

the exception of the well-documented case of lactate dehydrogenase; the main differences were quantitative only.

Eight mammals, one bird (chicken), 23 fish species, three reptiles (tortoise and turtles), three amphibians (frog and toads), and one invertebrate (crayfish) have been studied at the time of writing. Detailed results are given below for all eight mammals (pig, rabbit, ox, sheep, white rat, grey kangaroo, possum and bandicoot, the last three being Australian marsupials), together with the chicken, frog (*Rana temporaria*), turtle (*Pseudemys scripta*), carp (*Cyprinus carpio*) and Tasmanian crayfish (*Jasus lalandii*). Extracts were made from several animals each of the species pig, rabbit, ox, chicken, carp and crayfish; no intraspecies variation was found, except in carp (not described here). The muscles used were: for the mammals, longissimus dorsi (except ox, biceps femoris, and kangaroo, gastrocnemius); chicken, pectoral; frog, mixed; turtle, mixed; carp, white muscle; crayfish, tail muscle.

Photographs of Amido Black–Nigrosine-stained gels including all these species, and a few others, are shown in Plates 1(*b*) and 2. Slight differences in the positions of bands in the same sample but on different gels reflect minor variations in the electrophoresis conditions, for the gels illustrated were produced over a period of several months. Adherence to the methodology described eliminated day-to-day variation, but over longer periods changes in opened batches of starch, and sometimes quite large fluctuations in quality of the different batches, made exact reproducibility difficult.

Rather than present each species individually with its enzyme identifications, as is done for rabbit in Fig. 3, the mobilities of the individual enzymes are compared in Fig. 4, with myoglobin in Fig. 5. Most of the strongly staining protein bands of each species were identified, although in many cases there were one or two bands, amounting by visual estimation to up to 5% of the total protein, that were not assigned any enzyme activity.

It can be seen that each species has a characteristic protein pattern; the two most similar were the sheep and ox, which are the only two species of the same family represented. Nevertheless there were characteristic differences between them. All three marsupials had a greater proportion of protein migrating towards the anode than the other mammals, but differed considerably from each other; the invertebrate had almost no detectable protein migrating towards the cathode.

Comparison of specific enzymes

In Figs. 4 and 5 the mobilities of the various proteins in the species studied in detail are compared. In the discussion of the separate enzymes below, 'cathodic' means present on the cathodic side of the insertion slot; in almost every case the distance migrated was less than the electro-osmotic movement, and so the term does not necessarily imply that the enzyme was positively charged at pH 8·4. Similarly 'anodic' means that the band was on the anodic side of the insertion slot, and it can be assumed that such a protein is isoelectric below pH 8·4. In Figs. 4 and 5, ○ indicates that activity in this species was not detected under conditions when the reaction was successful with other species, and × indicates that no successful attempt was made.

Phosphoglucomutase (*Fig.* 4*a*). The reaction catalysed is:

Glucose 1-phosphate ⇌ glucose 6-phosphate

A glucose 1-phosphate preparation containing sufficient glucose 1,6-diphosphate was used (Spencer *et al.* 1964). The enzyme was very simple to detect, and active bands often appeared within 1 min. The diagram of the different patterns shown in Fig. 4(*a*) includes both phospho- and dephospho-forms of the enzyme, which migrate approx. 1 cm. apart; the relative amounts of these forms differed between extracts from animals of the same species. Only one cathodic phosphoglucomutase has been found, in an extract of the fish *Trigla lucerna* L. (gurnet); most fish had fast anodic bands similar to that shown for the carp.

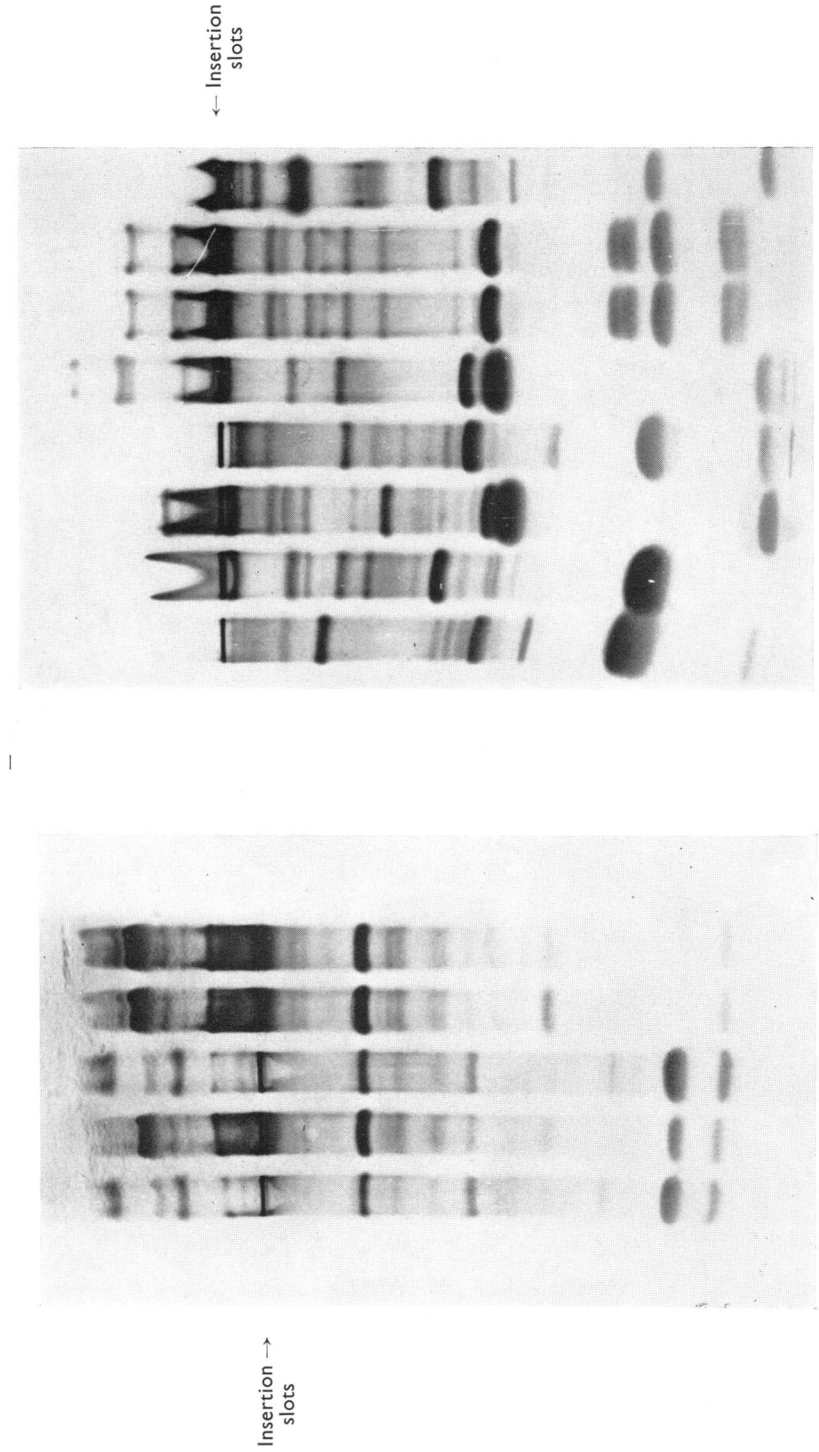
Phosphoglucose isomerase (*Fig.* 4*b*). The reaction catalysed is:

Fructose 6-phosphate ⇌ glucose 6-phosphate

This method was satisfactory provided that a suitable fructose 6-phosphate preparation was used. If the solution contained much glucose 6-phosphate, reduced nitro-blue tetrazolium was formed on making up the reaction mixture; the precipitated dye could be removed by centrifugation, more nitro-blue tetrazolium added and the solution then used. The reaction was rapid; several bands of isomerase activity were detected in most species (cf. Roberts & Tsuyuki, 1963). The possum, bandicoot, turtle and crayfish differed from the others illustrated in having wholly anodic bands. Rabbit red muscles also had some anodic isomerase bands, which were very weak or non-existent in white muscles. In fish, phosphoglucose isomerase usually gave several bands, either cathodic or anodic.

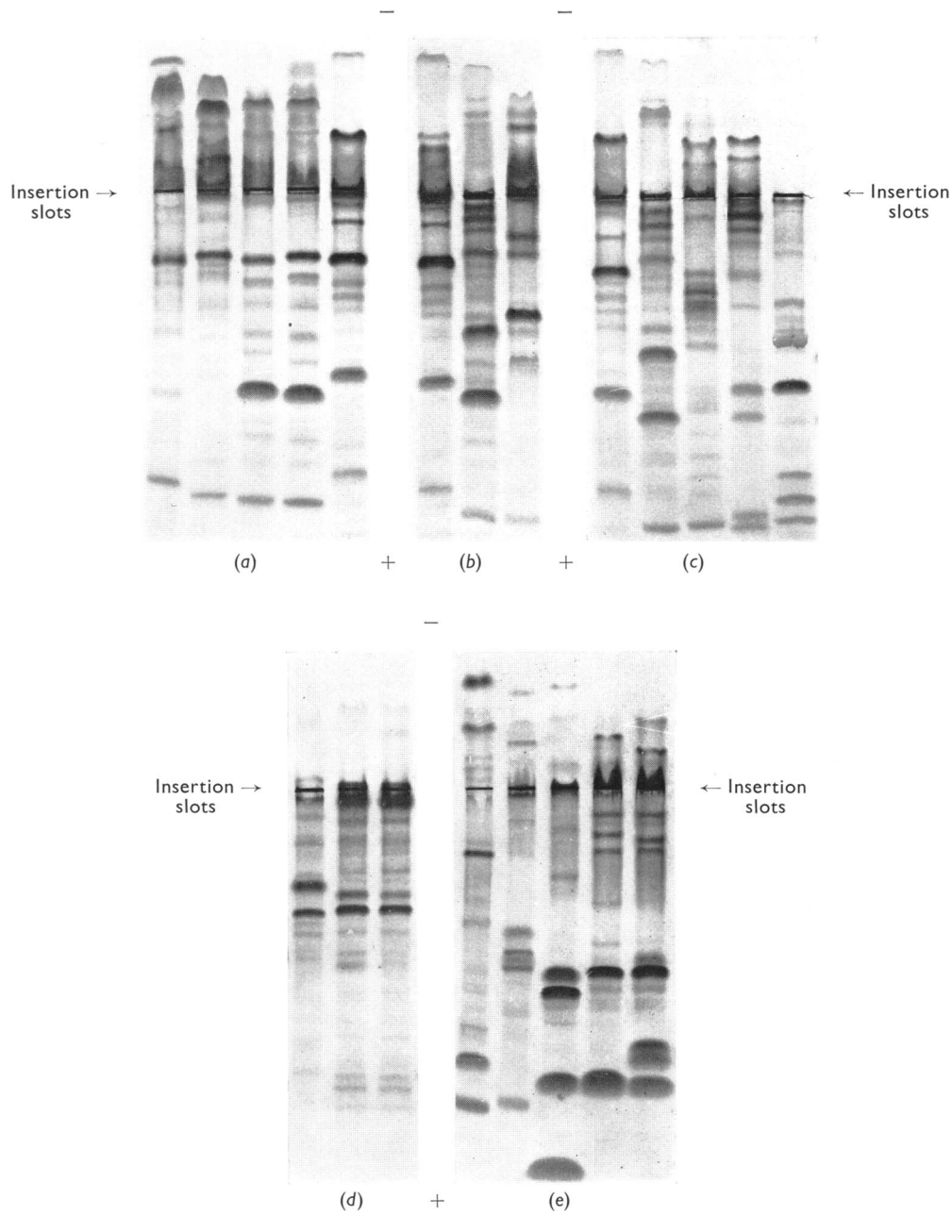
Aldolase (*Fig.* 4*c*). The reaction catalysed is:

Fructose 1,6-diphosphate ⇌
dihydroxyacetone phosphate + glyceraldehyde 3-phosphate



EXPLANATION OF PLATE I

(*a*) Starch-gel-electrophoresis patterns of some rabbit muscles (left to right): intertransversarius, semimembranosus, semitendinosus, psoas major, longissimus dorsi. (*b*) Starch-gel-electrophoresis patterns of some fish species (left to right): *Cottus cottus*, *Micropterus salmoides* (black bass), *Astronotus ocellatus*, *Gadus callarias* (codling), *Trigla lucerna* (gurnet), *Tilapia guineensis*, *Tilapia guineensis* (same as previous extract), *Anguilla anguilla* (common eel).



EXPLANATION OF PLATE 2

Starch-gel-electrophoresis patterns, including the 13 species compared in detail in Figs. 4 and 5 (left to right): (*a*) pig, rabbit, ox, sheep, rat; (*b*) rat, kangaroo, chicken; (*c*) rat, kangaroo, possum, bandicoot, crayfish; (*d*) tortoise (*Testudo graeca*), turtle, turtle; (*e*) rabbit (red muscle), frog, carp, and two fish of the *Tilapia* genus (*Tilapia macrochir* and *Tilapia guineensis*).

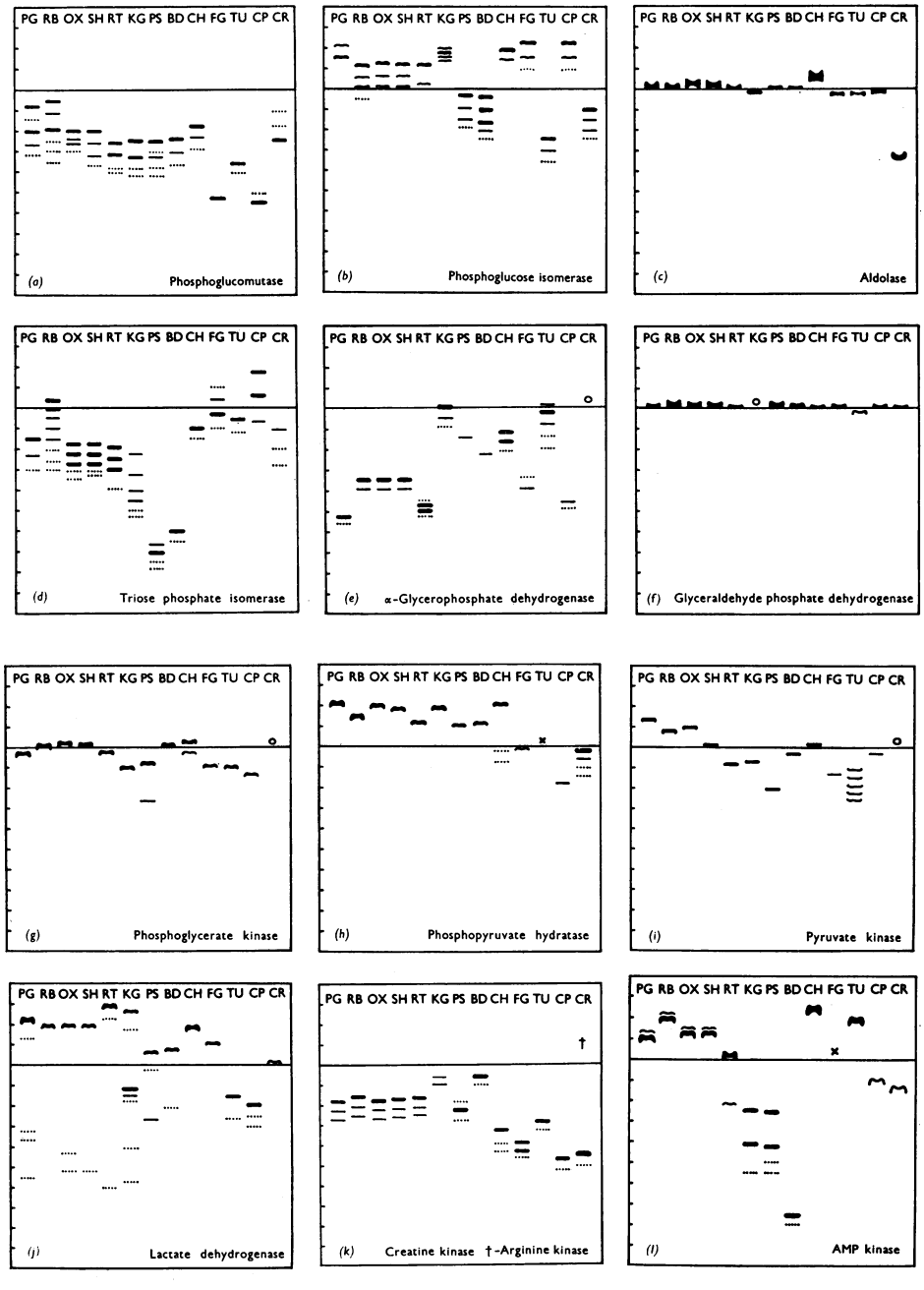


Fig. 4. Comparison of the mobilities of some glycolytic enzymes, creatine kinase and myokinase. Species compared are: pig (PG), rabbit (RB), ox (OX), sheep (SH), rat (RT), kangaroo (KG), possum (PS), bandicoot (BD), chicken (CH), frog (FG), turtle (TU), carp (CP) and crayfish (CR). On the left of each rectangle, the scale represents migration (cm.). The lower boundary represents the position of the discontinuity (+10 cm). and the horizontal line represents the line of the insertion slots. Electro-osmotic movement was −3 cm. ○ and × indicate that the enzyme in this species was not located (see the text).

With the exceptions of those of the kangaroo and crayfish, all aldolases formed curved bands that migrated only a small amount from the insertion slot. The kangaroo aldolase was comparatively sharp; the crayfish enzyme gave the irregular curved band at +3·5cm. clearly identifiable in Plate 2(*c*). Only a single band was detected in all species (cf. Anstall, Lapp & Trujillo, 1966). The electrophoretic behaviour suggests a partial adsorption of the enzyme in the starch gel.

Triose phosphate isomerase (*Fig.* 4*d*). The reaction catalysed is:

Dihydroxyacetone phosphate ⇌ glyceraldehyde 3-phosphate

This enzyme produced some of the most varied and complex patterns. The results illustrated in Fig. 4(*d*) were obtained by the method described elsewhere (Scopes, 1964*b*). However, equally clear results were obtained on filter papers, with the same dihydroxyacetone phosphate solution (produced by dismutation between α-glycerophosphate and pyruvate) and a large amount of highly purified glyceraldehyde phosphate dehydrogenase. The rabbit white-muscle extracts contained eight forms of the enzyme, of equal spacing and diminishing intensity, the strongest being the only mammalian triose phosphate isomerase found that was cathodic, although many fish had cathodic bands of this enzyme. In contrast, the possum enzyme bands were almost as anodic as serum albumin. In areas where isomerase activity overlapped that of α-glycerophosphate dehydrogenase, the result was difficult to interpret, for here the NADH produced by the reaction was oxidized faster by the dihydroxyacetone phosphate present than by the phenazine methosulphate–nitro-blue tetrazolium system.

α-Glycerophosphate dehydrogenase (*Fig.* 4*e*). The reaction catalysed is:

Dihydroxyacetone phosphate + NADH ⇌ α-glycerophosphate + NAD^+

The negative method for staining this enzyme was superior to the simple positive method; the latter gave ambiguous results and was very slow. About 15min. with the negative method was sufficient to show up the weaker bands. In most species the enzyme was anodic, with the greatest mobility in the pig and rat, and least in the kangaroo and turtle. Several species showed two bands a few millimetres apart, and the greatest activity was in the chicken, corresponding to two strongly staining protein bands which are visible in Plate 2(*b*). The mobilities of the enzymes from the rabbit, ox and sheep were identical. Fish extracts frequently contained three or more bands of activity, but these were generally very weak.

Glyceraldehyde phosphate dehydrogenase (*Fig.* 4*f*). The reaction catalysed (in the presence of arsenate) is:

Glyceraldehyde 3-phosphate + NAD^+ → 3-phosphoglycerate + NADH

This enzyme has been prepared crystalline by the same procedure from many species; thus it is not surprising that staining on the starch gel did not suggest any great qualitative differences between species. It was not detected in the kangaroo, probably because of the treatment of this muscle before extraction. In fish there was a little more variation in this enzyme's mobility; it ran at positions between +1cm. and −1cm. As with aldolase, its limited mobility and spreading character suggest partial adsorption in the starch gel, and so there may be more variation in properties than is indicated by these results.

Phosphoglycerate kinase (*Fig.* 4*g*). The reaction catalysed is:

3-Phosphoglycerate + ATP ⇌ 1,3-diphosphoglycerate + ADP

The negative method coupled through glyceraldehyde phosphate dehydrogenase was successful for this enzyme; even better results were obtained when further enzymes were included to pull the reaction through to α-glycerophosphate; removal of glyceraldehyde phosphate prevented inhibition of the dehydrogenase, and 2mol. of NADH were oxidized/mol. of 3-phosphoglycerate phosphorylated. Most species had only one band of the enzyme, which migrated a short distance from the insertion slot. With only one exception (a faint anodic band in the possum) the bands were curved, with a sharp edge towards the cathode and considerable streaking towards the anode. The enzyme from the pig was previously identified as a cathodic band (Scopes, 1966). Reinterpretation of these results in light of the present findings indicates that the band then labelled 'D' was phophoglycerate kinase; the cathodic band was in fact AMP kinase (see below).

Phosphoglycerate mutase. The reaction catalysed is:

2-Phosphoglycerate ⇌ 3-phosphoglycerate

This enzyme has proved to be one of the most difficult to locate with precision; the reason seems to be that, with the present buffer system, it does not form a sharp band and is located mostly in areas where the presence of large amounts of other enzymes introduces several possibilities of erroneous interpretation. The reaction mixture listed in Table 1 includes all enzymes between α-glycerophosphate dehydrogenase and lactate dehydrogenase, with the exception of enolase and mutase. Methods including enolase were unsuccessful only because the enolase preparation used contained

traces of mutase. Theoretically, 3mol. of NADH should be oxidized/mol. of 2-phosphoglycerate converted into 3-phosphoglycerate. Clear areas rapidly developed close to the insertion slot; since several enzymes overlapped in this area, it was possible that insufficient coupling enzymes had been included. However, this result was repeatedly obtained under various conditions. No indication of any activity just ahead of phosphoglucomutase, which was expected in view of the reported isoelectric points and molecular weights of the rabbit enzymes, was found, except in chicken. That the clear area for chicken did indeed represent phosphoglycerate mutase was proved by parallel running of a crystalline preparation of the enzyme (Torralba & Grisolia, 1966). Thus it appears that phosphoglycerate mutase interacts considerably with the buffer ions, and so does not give a sharp band. Definite determinations of the position of the enzyme were made only with chicken, rabbit and ox, and so a diagram is not given. The previous localization of the enzyme in the pattern for pig (Scopes, 1966) is now known to be incorrect.

Phosphopyruvate hydratase (*enolase*) (*Fig.* 4*h*). The reaction catalysed is:

$$\text{2-Phosphoglycerate} \rightleftharpoons \text{phosphoenolpyruvate}$$

The negative method was preferable, as often enolase was close to myokinase, both being stained in the positive method. With an enolase-free preparation of phosphoglycerate mutase present, 3-phosphoglycerate could be used as substrate. Addition of hexokinase and glucose regenerated the ADP (see below), but this was not essential. All mammals had one strong cathodic band, with some extracts showing a fainter more mobile band on aging. Chicken had a strong cathodic band, but also two faint nodica ones. Crayfish enolase was anodic, as were most fish enolases. The mobility of enolase relative to lactate dehydrogenase in the mammals, both strong cathodic bands, was sometimes reversed, depending on the exact conditions of electrophoresis.

Pyruvate kinase (*Fig.* 4*i*). The reaction catalysed is:

$$\text{Phosphoenolpyruvate} + \text{ADP} \rightleftharpoons \text{pyruvate} + \text{ATP}$$

As with enolase, the negative method was unambiguous, and so preferred. With added enolase and phosphoglycerate mutase, 3-phosphoglycerate could be used as substrate. ADP was regenerated with hexokinase and glucose; although this was not always essential, it avoided inhibition of the enzyme by ATP. In all species, with the exception of the turtle, only a single band was found; the relative mobilities varied considerably. The turtle extract gave five bands of activity; one interpretation of this would be that pyruvate kinase normally contains four identical sub-units, but that this particular individual was a heterozygote with one allele producing a sub-unit of different charge from the other. Pyruvate kinase was not detected on the filter paper in the crayfish extract.

Lactate dehydrogenase (*Fig.* 4*j*). The reaction catalysed is:

$$\text{Pyruvate} + \text{NADH} \rightleftharpoons \text{lactate} + \text{NAD}^+$$

The negative method for lactate dehydrogenase was quicker than the traditional method of Markert & Møller (1959); the results were the same. The strong muscle-type enzymes are seen in the white muscles of pig, rabbit and chicken, and in the ox and sheep though these muscles are rich in myoglobin (however, there are fainter anodic bands in these red-muscled species). The red muscle of rabbit contained all five isoenzymes of lactate dehydrogenase (Fig. 3). The pictures for the rat and the marsupials were more complex and contained anodic as well as the strong cathodic bands. The crayfish extract had very little of this enzyme. Most fish muscle lactate dehydrogenases were anodic, and often quantitatively much less than in the mammals.

Creatine kinase (*and arginine kinase*) (*Fig.* 4*k*). The reactions catalysed are:

$$\text{Creatine} + \text{ATP} \rightleftharpoons \text{creatine phosphate} + \text{ADP}$$
$$\text{Arginine} + \text{ATP} \rightleftharpoons \text{arginine phosphate} + \text{ADP}$$

Sjorvall & Voigt (1964) detected four bands of activity of creatine kinase in extracts of human muscles. In general the species investigated here have shown three main bands, the faster band described by Sjorvall & Voigt (1964) not being found (but with prolonged incubation a more mobile fourth and even a fifth band could be seen in aged extracts, with the same spacing as the other three bands). In nearly all cases the creatine kinase main band was characteristic in the general protein pattern; it was very strong and clearly defined. The mobilities were very similar in the non-marsupial mammals, but no two were exactly the same. Although the other marsupial preparations stained strongly, the kangaroo extract had very little of this enzyme, probably a result of the treatment of this muscle before extraction. The arginine kinase of the crayfish was detected by a similar procedure, arginine (phosphate) being substituted for creatine (phosphate) in the reaction mixtures in Table 1. In nearly all fish, creatine kinase ran between +3cm. and +5cm.; it was quantitatively even more important than in the mammals. It has been shown that the 'myogen I' crystals of carp (Henrotte, 1952), corresponding to a major peak in free-boundary electrophoresis, is creatine kinase; it

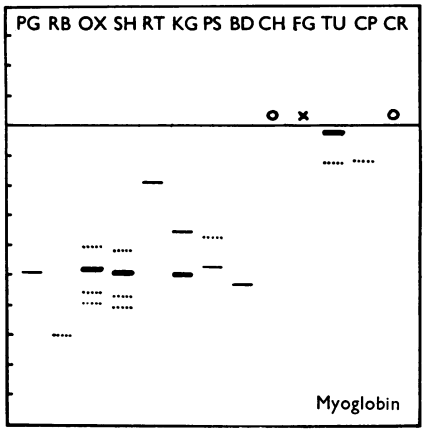


Fig. 5. Comparison of the mobilities of myoglobins. For explanations, see the legend to Fig. 4.

has been estimated that this peak makes up about 25% of the total sarcoplasmic protein in the carp, although varying with age.

AMP kinase (*myokinase*) (*Fig.* 4*l*). The reaction catalysed is:

$$2\,\mathrm{ADP} \rightleftharpoons \mathrm{ATP} + \mathrm{AMP}$$

A wide variety of mobilities of this enzyme was observed; the three marsupials had anodic bands, and the other species mainly cathodic. Callaghan (1956) reported an isoelectric point of 4·3 for the rabbit enzyme, and Noda & Kuby (1957) a value of 6·1. Both authors considered that these values were likely to be largely affected by binding of anions during electrophoresis. The starch-gel-electrophoresis result is strongly suggestive of a much higher isoionic point, about 8·0. Similar anion-binding effects have been noted with aldolase (Velick, 1949). Nevertheless the myokinase of the bandicoot had a high anodic mobility on starch gel, almost as fast as the serum albumin. Two equally strong bands of myokinase were found in both kangaroo and possum preparations (together with some minor components), but the mobilities in the two species were not quite the same. The patterns for these two marsupials were similar to, though more anodic than, those of the human phenotype 2 (Fildes & Harris, 1966). The fish enzymes generally resembled the rabbit ones in giving curved cathodic bands, but the carp enzyme was anodic.

Myoglobin (*Fig.* 5). The myoglobin content of the muscles was a direct reflection of their colour, and so the pale muscles (pig and rabbit longissimus dorsi, chicken pectoral and most fish muscles) had little or no myoglobin. Only one band is shown in Fig. 5 for pig and rabbit, but in the redder muscles of these species faster minor components are also found. Oxidation of the myoglobin to metmyoglobin resulted in a slower band than the main component; this is included in Fig. 5 in the patterns for ox, sheep, possum and kangaroo. No *o*-dianisidine-staining band was found in the chicken pectoral or crayfish tail muscles.

DISCUSSION

The best set of conditions for electrophoresis of a protein mixture depends largely on the composition of that mixture. Consequently there has been a wide variety of buffers and pH values described for use in starch-gel electrophoresis. Neelin (1963) investigated a variety of conditions with chicken sarcoplasm, and Tsuyuki, Roberts & Gadd (1962) changed the pH of the buffers to obtain the best conditions for the separation of salmon muscle extracts. Each concluded that values close to pH 8 gave the best results. In the present work, a buffer system giving good separation of the proteins from mammalian muscle extracts was developed, and it was suitable also for the sarcoplasms from non-mammalian species. Although the conditions were not ideal for certain proteins isoionic close to the pH of the gel buffer, or those interacting with the buffer ions, it was a suitable compromise for separation of all the various proteins present. The electro-osmotic effect was used to advantage, as it was mainly responsible for moving the cathodic proteins away from the insertion slot, allowing the filtration effect of the gel to cause a separation. This is one advantage that starch gel has over polyacrylamide gel (in which there is little electro-osmosis) for electrophoresis of sarcoplasmic proteins.

Interaction of proteins with buffer ions can cause curvature of the bands, often characteristic of the particular protein, because the concentration of the various ions differs between the protein area and the spaces between samples. This is particularly marked with the cathodic bands, although some enzymes, e.g. phosphoglucose isomerase and pyruvate kinase, do form sharp, almost straight, bands on the cathode side. Most anodic bands are straight and sharp, although phosphoglycerate kinases and mutases never give sharp bands, whether cathodic or anodic. Experiments with other buffer systems have shown that (pig) enolase streaks with borate buffers, but forms a sharp cathodic band in veronal systems. This indicates that enolase will bind borate ions, but not diethylbarbiturate ions. The passage of the discontinuity is partly responsible for streaking towards the anode; this can be explained in terms of the increased mobility of the proteins as the discontinuity passes. Nevertheless, with non-discontinuous systems, most bands were more diffuse and the results were less satisfactory.

The methods for staining individual enzymes on filter paper are generally an improvement over standard techniques, for reasons outlined in the Experimental section. Other workers have used filter-paper or agar-gel overlays on starch gel for detecting enzymes, e.g. Virden & Watts (1964), Fildes & Harris (1966) and Pietruszko & Baron (1967). The use of filter paper allows development of methods otherwise very difficult, expensive, or impossible by other techniques. One advantage is the rapidity of reaction; in particular the contrast with lactate dehydrogenase is very marked, because the equilibrium of the enzyme reaction is so far in favour of formation of lactate. Short reaction times lessen diffusion of the enzymes in the gel, and active bands 2mm. apart are clearly resolved. Finally, there is the convenience of having a permanent record without having to copy, measure or photograph the result. The methods are applicable to the same enzymes in other types of extract, and similar procedures can be evolved for staining many other enzymes, provided that suitable preparations of coupling enzymes are available. Not all methods are free from interference by other enzymes in the gel. In particular any method relying on the detection of ATP production from ADP using the glucose–hexokinase–glucose 6-phosphate dehydrogenase system will stain myokinase or other nucleotide monophosphate kinases. Also, the interference by α-glycerophosphate dehydrogenase in the triose phosphate isomerase method has been noted.

Two general uses of these methods are of particular value. The first is in procedures of protein fractionation, for it enables the worker to know, not only how much of an enzyme is present in a particular fraction, but also what is the nature of the main contaminants. For example, after purification of arginine kinase from crayfish muscle by DEAE-cellulose chromatography (Virden, Watts & Baldwin, 1965) the main protein contaminants were bands corresponding to triose phosphate isomerase. The molecular weight of lobster arginine kinase is 37000 (Virden, Watts, Watts, Gammack & Raper, 1966). If the lobster triose phosphate isomerase has the same molecular weight as that from the rabbit, i.e. 43000 (Burton & Waley, 1966), gel filtration would not be likely to remove the contaminants. Similarly, in a preparation of enolase contamination by creatine kinase could be removed by denaturation at pH 5·5 (Scopes, 1965).

The second general use is in comparative biochemistry. Many different species can be compared on one gel, and all stained for a particular enzyme, e.g. malate dehydrogenase (Kitto & Wilson, 1966) and creatine kinase (Eppenberger, Eppenberger & Kaplan, 1967). The electrophoretic pattern of the sarcoplasmic enzymes, although characteristic of a species, may show some variation between individuals. This genetic polymorphism has been found in some herring enzymes (Odense, Allen & Leung, 1966) and in human myokinase (Fildes & Harris, 1966). Consequently, sufficient individuals should be examined to establish a 'normal' pattern for a species. Starch-gel electrophoresis distinguishes enzymes that differ in their net charge and, to a smaller extent, size and shapes. A single amino acid substitution may not necessarily have to be a residue of different charge to cause a difference in electrophoretic mobility. Different steric or hydrophobic effects of the new residue may affect the pK of an adjacent residue (e.g. histidine) that is not fully ionized at the pH of electrophoresis. Certainly the small differences in mobility between, for example, the creatine kinases of rabbit and rat, or the myoglobins of ox and sheep, represent much less than one charge per molecule, and either are results of direct substitutions involving a partially ionized residue, or of secondary effects of charge-conserving substitutions.

Having established the normal patterns, species identification by the use of muscle extracts becomes feasible and comparison of different species within the same family or genus will indicate which enzymes exhibit the greatest variability. If it is assumed that mutation rate is the same for all genes, then a large variability would suggest that much of the enzyme's structure is relatively unimportant to its normal functioning. On the other hand, a constant mobility of an enzyme even when distantly related species are compared would suggest that most mutations affecting that enzyme are fatal or disadvantageous to the species. The overall comparisons of the enzyme patterns can indicate how closely related in evolutionary time are the various species, and generally provide a molecular basis for taxonomy.

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