Preparation and Properties of Creatine Kinase from the Breast Muscle of Normal and Dystrophic Chicken (*Gallus domesticus*)

BY B. P. ROY,* J. F. LAWS AND A. R. THOMSON

United Kingdom Atomic Energy Authority, Atomic Energy Research Establishment, Harwell, Berks., U.K.

(Received 3 June 1970)

1. The purification of creatine kinase from normal and genetically dystrophic chicken breast muscle is described. Enzyme recovery was significantly lower from dystrophic muscle. 2. Both enzymes had the same number of reactive and total thiol groups and had similar specific activities and similar amino acid compositions. 3. No significant differences were observed in sedimentation, electrophoretic or kinetic properties. 4. Peptide 'maps' showed no significant differences, and electrophoresis of partial acid hydrolysates of the labelled enzymes suggested that corresponding amino acid sequences around all the thiol groups were very similar. 5. The enzymes showed identical temperature stabilities. 6. No significant differences between the enzymes from normal and dystrophic muscle were observed.

The creatine kinases (ATP-creatine phosphotransferases, EC 2.7.3.2) catalyse the reaction:

in the presence of bivalent cations such as Mg^{2+} and Mn^{2+} . The enzymes occur in many vertebrate tissues and it is known that they have molecular weights of approx. 80 000 (Kuby, Noda & Lardy, 1954; Eppenberger, Dawson & Kaplan, 1967; Kumudavalli, Moreland & Watts, 1970) and consist of two very similar subunits (Dance & Watts, 1962) each with one highly reactive and 'essential' thiol group (Mahowald & Kuby, 1960; Watts, Rabin & Crook, 1961; Eppenberger *et al.* 1967). Creatine kinase is known to be intimately involved in normal muscle contraction and probably occurs in close physical association with the contractile elements.

In the development of normal muscle there is a progressive change in the creatine kinase isoenzyme pattern from a preponderance of the brain type in the foetus, generally via a hybrid form of intermediate electrophoretic mobility, to the enzyme of low mobility characteristic of the adult muscle (Eppenberger, Eppenberger, Richterich & Aepli, 1964; Dawson & Kaplan, 1965; Kendrick-Jones & Perry, 1967). Of the several genetically determined types of muscular dystrophy known, the Duchenne type, which is X-chromosome-linked and recessive and is characterized by progressive degeneration of the skeletal muscles in young males, has been

most widely studied (Walton & Gardner-Medwin, 1969). In this disease serum creatine kinase activities are generally greatly elevated, particularly in the early stages, and the elevated enzyme activities frequently observed in mothers who are carriers of the disease are of great diagnostic value (Ebashi, Toykura, Momoi & Sugita, 1959). In dystrophic human tissue the isoenzyme pattern is foetal rather than adult in character (Miyazaki *et al.* 1965) but it is not clear whether this is a result of a lack of development of the normal adult pattern or of a reversion to the foetal isoenzyme distribution. Investigation of the dystrophies has been hampered until recently by the lack of suitable experimental materials. The discovery of genetically linked dystrophies in a number of experimental animals, e.g. mouse (Michelson, Russell & Harman, 1955), hamster (Homburger, Baker, Nixon & Whitney, 1963), duck (Rigdon, 1966) and chicken (Asmundson & Julian, 1956), has greatly facilitated research in this group of diseases. Hooton & Watts (1966a) reported the presence of a defective form of creatine kinase in dystrophic mouse muscle. They isolated the enzyme from normal and dystrophic muscle tissue, and showed that the enzyme from the latter had half the specific activity of that of the normal and contained only one reactive thiol group compared with two in the normal. In addition, they observed a single difference on peptide 'maps' that they attributed to the replacement of one reactive cysteine residue by a glutamic acid residue. On the basis of these findings they suggested that dystrophic tissue contained a modified creatine kinase under genetic control, and that this was a possible

* Present address: Molecular Enzymology Laboratory, Department of Biochemistry, University of Bristol Medical School, Bristol BS8 1TD, U.K.

primary cause of the disease. If the presence of a defective creatine kinase is the primary cause of one type of genetically determined muscular dystrophy, it also might be expected to occur in other species affected with this type of disease. We decided to investigate this problem in further detail at the molecular level, but because of the great difficulty in obtaining sufficient muscle tissue from mice we used New Hampshire chickens with hereditary dystrophy. Part of this work has already been presented elsewhere (Roy, Laws & Thomson, 1969).

MATERIALS AND METHODS

Dystrophic chickens (homozygous recessive, line 304) and control birds (line 200) were supplied by the Department of Poultry Husbandry, University of California, Davis, Calif., U.S.A. All chickens were male and were at least 6 months old. Chickens from a local source (Agricultural Research Council, Institute of Animal Diseases, Compton, Berks., U.K.) were used for preliminary investigations. Iodo[2-¹⁴C]acetic acid (specific radioactivity 10–30 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. It was diluted two- to five-fold with unlabelled iodoacetate and was recrystallized from light petroleum (b.p. 60–80°C). Other reagents used were Whatman DEAE-cellulose DE52 (W. and R. Balston Ltd., Maidstone, Kent, U.K.), enzyme-grade guanidine hydrochloride (BDH Chemicals Ltd., Poole, Dorset, U.K.), 5,5'-dithiobis-(2-nitrobenzoic acid) (Ralph N. Emanuel Ltd., Alperton, Middx., U.K.) and dithioerythritol [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.]. All other chemicals were of analytical or equivalent grade or the purest grade obtainable. Water was doubly distilled, the second distillation being from an all-quartz apparatus.

Purification of the enzymes. Creatine kinase was prepared from normal and dystrophic chicken muscle by a procedure essentially the same as that of Eppenberger *et al.* (1967). A number of significant modifications were made as follows. (1) The KCl extract was treated with cold ethanol without prior centrifugation. This speeded up the preparation without significantly affecting yields or purity of the preparation. (2) Dithioerythritol (80 mg/l) was incorporated in all solutions from the magnesium acetate extraction stage onwards to stabilize the enzyme. (3) The ammonium sulphate concentration step was omitted. (4) The magnesium acetate extracts were equilibrated by dialysis against 5 mM-tris-HCl buffer, pH 7.7, containing dithioerythritol (80 mg/l) before being loaded on to the column (4 cm × 45 cm), and the DEAE-cellulose used for chromatography was thoroughly equilibrated against the same buffer. After application of the magnesium acetate extract, the column was washed thoroughly with starting buffer to remove much loosely bound protein, and the enzymes were eluted with a linear gradient of 5–250 mM-NaCl in 5 mM-tris-HCl buffer, pH 7.7, containing dithioerythritol (80 mg/l). The eluate was monitored continuously at 254 nm (LKB Uvicord) and fractions were collected in a Baird and Tatlock Chromofrac fraction collector. Protein concentrations were determined from E_{280} values and the automated assay procedure described below was used to detect

Scheme 1. Purification of chicken breast muscle creatine kinase. Extractions were carried out at 2°C unless otherwise stated. Chickens were killed by cervical section and breast muscles were immediately frozen at –18°C. These were thawed out when required by keeping overnight in the cold-room at 4°C. Next morning the muscles were cut into pieces and minced with an electric mincer. The presence of dithioerythritol was required to maintain enzyme activity. On occasions 2-mercaptoethanol was used instead.

creatine kinase activity. The procedure used for the purification of chicken muscle creatine kinase is summarized in Scheme 1 and typical column elution profiles are shown in Fig. 1. Creatine kinase was prepared similarly from rabbit muscle (Kuby *et al.* 1954) and bovine brain (Wood, 1963) for comparison of electrophoretic mobilities.

Enzyme assay. (a) Phosphate method. The enzyme was assayed by the phosphate method of Kuby *et al.* (1954) by following the conversion of creatine into creatine phosphate. The standard assay mixture contained (final concentrations) creatine (80 mM), magnesium acetate (0.1 M) and glycine (0.4 M), and after addition of ATP (5 mM) the reaction mixture was equilibrated at 30°C. The diluted enzyme was then added to start the reaction. Adenosine triphosphatase activity was determined by incubation with ATP in the absence of creatine.

The creatine kinase reaction followed apparent second-order kinetics with respect to ATP, and velocity constants, K , were calculated either individually or read from a standard calibration curve (of μ mol of phosphate liberated against units of enzyme activity). Specific activities are expressed as units of enzyme activity/mg of protein, the unit being as defined by Kuby *et al.* (1954).

(b) Automated phosphate method. In the phosphate method of creatine kinase assay (Kuby *et al.* 1954) the amount of phosphate liberated from the creatine phosphate formed during the reaction is determined as ammonium phosphomolybdate. The Technicon Auto-Analyzer manifold for phosphate analysis was adapted for monitoring the creatine kinase activity (A. R. Thomson, unpublished work). The system was modified to allow the enzyme and its substrate to react first in the mixer (Kuby *et al.* 1954). The phosphate liberated was then measured colorimetrically at 660 nm. Column fractions were scanned for enzyme activity at a rate of either 20 ml/h or 40 ml/h.

(c) pH-stat method. To compare Michaelis constants, K_m , and initial velocities, V , and maximum velocities, V_{max} , the pH-stat method of enzyme assay was also used (Mahowald, Noltman & Kuby, 1962). In this method the conversion of creatine into creatine phosphate is followed by the liberation of H^+ ions, which are titrated with NaOH at pH 8.8. The standard reaction mixture contained (final concentrations), ATP (4 mM), $MgSO_4$ (4 mM) and creatine (40 mM). For the titrations, which were carried out in a Radiometer pH-stat and Titragraph TTT1, 0.02–0.01 M-NaOH was used. N_2 was bubbled through the reaction mixture throughout the experiment to avoid alteration of pH by the absorption of CO_2 . Initially we had difficulty in obtaining reproducible titration curves, possibly owing to inactivation of the dilute enzyme solution, but this was overcome by incorporating 2-mercaptoethanol (1 mM) in the assay mixture.

Determination of the thiol groups. Thiol groups were determined by reaction with 5,5'-dithiobis(2-nitrobenzoic acid) in 10 mM-tris-HCl buffer, pH 8.0 (Ellman, 1959), both in the presence and in the absence of guanidine hydrochloride (6 M). To prevent oxidation of thiol groups during dialysis enzyme solutions were dialysed against 10 mM-tris-HCl buffer, pH 8.0, containing dithioerythritol (1 mg/l), the diffusate being used as the reference solution in the spectrophotometer.

Protein determination. Protein concentrations were determined either with the biuret reagent (Gornall, Bardawill & David, 1949), with crystalline bovine serum

albumin as standard, or from E_{280} values by using $E_{280}^{1\%}$ 8.7 (Kuby *et al.* 1954).

Electrophoresis. High-voltage electrophoresis was carried out in an apparatus similar to that described by Michl (1951). The coolant and pH 6.5 and 2.1 buffers were as described by Ambler (1963).

Proteins (approx. 25 μ g) were subjected to electrophoresis on Oxoid cellulose acetate sheets (12 cm $\times$ 12 cm) (Medical Division, Oxo Ltd., London E.C.4, U.K.) at various pH values between 5 and 9.6 at 15–20 V/cm and 10 mA for approx. 1 h. The sheets were then stained with Ponceau Red S [2% (w/v) in 3% (v/v) acetic acid].

Amino acid analysis. Enzyme solutions were thoroughly dialysed against distilled water, pH 9.0, and portions were then frozen, dried and hydrolysed with 6.5 M-HCl (redistilled) for 16, 40 and 64 h *in vacuo* at 105°C. After rapid removal of HCl *in vacuo* at approx. 30°C, samples for analysis were dissolved in 0.2 M-sodium citrate buffer, pH 2.0. Amino acid compositions were determined with a Technicon TSMI automatic amino acid analyser and a molecular weight of 81 000 was assumed in the calculations.

Peptide 'maps'. These were prepared by electrophoresis as above for 1 h at 50 V/cm followed by chromatography at right-angles in butan-1-ol-pyridine-acetic acid-water (15:10:3:12, by vol.) for 16 h at room temperature in an insulated chromatography tank.

RESULTS

Details of typical preparations of creatine kinase from normal and dystrophic breast muscle are given in Table 1 and Fig. 1. It is notable that, in contrast with the results of Hooton & Watts (1966a) obtained with mice, there was little difference in the specific activities of the enzyme from both normal and dystrophic tissue (84 and 70 units/mg respectively); however, the yield of enzyme from dystrophic tissue was much lower, 287 mg/kg, as compared with 989 mg/kg for normal muscle. Further, there was less enzyme in the potassium chloride and

Table 1. *Purification of creatine kinases from normal and dystrophic chicken breast muscle*

The values are corrected to 1 kg wet wt. of muscle.

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Purification	Recovery (%)
Normal-muscle fractions						
I Crude extract	2666.6	56 000	327 500	5.86	1.0	100
II Magnesium acetate extract	389.1	3 090	120 700	39	6.6	38.8
III Pooled column fractions	30.01	989	83 600	84.5	14.4	25.5
Dystrophic-muscle fractions						
I Crude extract	1921.4	37 600	263 500	7.04	1.0	100
II Magnesium acetate extract	505.5	1 552	53 700	34.6	4.91	20.3
III Pooled column fractions	7.2	287	20 050	70.0	9.9	7.6

magnesium acetate extracts of dystrophic tissue than from normal tissue, perhaps the result of less efficient extraction from the former, which was noticeably different both in appearance and consistency from normal tissue. In both cases the enzyme activity was eluted as a single major peak from DEAE-cellulose (Fig. 1). However, the enzyme from normal tissue was eluted at a higher salt concentration, and there was an additional inactive protein band. This different behaviour is possibly due to a difference in charge (though this is not supported by electrophoresis results; see below) or to the presence of lower protein concentrations and an altered spectrum of proteins in the dystrophic-tissue extracts.

Electrophoretic mobility. The enzymes from normal and dystrophic muscle showed identical electrophoretic mobilities at all pH values from 5 to 9.6. Fig. 2 shows a comparison of the mobilities of enzymes obtained from different species at pH 8.6. All are very similar apart from the brain enzyme, which moves faster than any of the other isoenzymes. These results agree with those of Hooton & Watts (1966a) and Jacobs *et al.* (1969),

both of whom were unable to detect any difference in mobility between creatine kinases isolated from normal and dystrophic mouse and human skeletal muscle respectively.

Thiol groups. In enzymes from both normal and dystrophic muscle approx. 2 thiol groups/molecule reacted in the absence of denaturant (Table 2). This value agrees with that for the enzymes from human dystrophic muscle determined by Jacobs *et al.* (1969). Similarly, in the presence of 6M-guanidine hydrochloride no difference in thiol groups could be detected (Table 2).

Iodoacetate inhibition. Purified enzymes from normal and dystrophic chicken muscle at concentrations of approx. 50 mg/ml were incubated for 30 min with iodoacetic acid, freshly recrystallized from light petroleum (b.p. 60–80°C) dissolved in tris-HCl and the pH was adjusted to 9.0. The molar ratios of iodoacetic acid to protein ranged from 0.5 to 3.0. Reaction with iodoacetate was stopped by dilution, and the residual activity was assayed as described in the Materials and Methods section. The inhibition curves of both the enzymes were very similar initially, both exhibiting a

Fig. 1. Chromatography of creatine kinases from (a) normal and (b) dystrophic chicken muscle on DEAE-cellulose. Experimental details are given in the text; 10ml fractions were collected. O, E_{280} ; ●, enzyme activity; (----), NaCl concentration.

Fig. 2. Cellulose acetate electrophoresis of creatine kinases from rabbit muscle (i), normal chicken muscle (ii), dystrophic chicken muscle (iii) and ox brain (iv). Electrophoresis was performed in 0.1 M-sodium barbitone buffer, pH 8.6, at 4°C on cellulose acetate sheets (12 cm × 12 cm). The sheets were then stained with Ponceau Red S.

steady loss of activity, but whereas the normal enzyme was completely inhibited the dystrophic enzyme retained some activity (Fig. 3). Extrapolation of the straight-line portion of the curves to zero enzymic activity in each case gave values of

approx. 2 mol of thiol groups/mol of creatine kinase. The lower final degree of inhibition reached with the enzyme from dystrophic muscle may be due to non-identity of the thiol groups or to co-operative effects in the two subunits whereby modification of one subunit affects the reactivity of the thiol group in the other subunit (Watts & Kumudavalli, 1970). Further work will be necessary to clarify this point in view of suggestions that the thiol groups are directly involved in the catalysis and are close to the substrate binding sites (Watts *et al.* 1961; Watts & Rabin, 1962; Watts, 1963). Recent investigation suggests that the thiol groups may be involved in conformational changes (Kumudavalli *et al.* 1970).

'Fingerprinting'. Enzymes from both normal and dystrophic muscle were allowed to react with iodo[^{14}C]acetate (Harris, 1964). (1) The reactive thiol groups were labelled with 2 mol of iodo[^{14}C]acetate/mol of enzyme and the buried thiol groups were then alkylated with unlabelled iodoacetate in the presence of 8 M-urea. (2) The labelling of the buried thiol groups was done by allowing the free

Table 2. *Thiol groups in creatine kinases from normal and dystrophic muscle*

Experimental details are given in the text. Numbers of determinations are given in parentheses.

	Specific activity (units/mg)	Thiol groups (mol/mol of enzyme)			
		Reactive		Total	
		Mean	Range	Mean	Range
Chicken					
Normal (Compton)	69.5	1.87		4.8	
Normal (line 200)	84.5 (3)	1.55 (3)	1.30–1.90	6.81 (3)	6.65–6.90
Dystrophic (line 304)	73 (3)	1.97 (3)	1.91–2.10	7.11 (3)	7.03–7.29
Mouse (Hooton & Watts, 1966a)					
Normal	204*	2.1		6.8	
Dystrophic	110*	1.2		5.5	
Rabbit (Mahowald <i>et al.</i> 1962)					
Normal	72	2		6.3†	
				6.5‡	
Human (Jacobs <i>et al.</i> 1969)					
Normal		2		6.66§	
Dystrophic		2		—	

* Specific activity expressed as equiv. of H⁺ released/min per mg of protein (Noltman *et al.* 1962).

† Noltman *et al.* (1962).

‡ Benesch, Lardy & Benesch (1955).

§ Kumudavalli *et al.* (1970).

Fig. 3. Inhibition of creatine kinases from normal (a) and dystrophic (b) chicken muscle by iodoacetate. Experimental details are given in the text.

thiol groups to react with unlabelled iodoacetate followed by alkylation of the buried thiol groups with iodo[¹⁴C]acetate in the presence of denaturant. They were then digested with 2% (w/w) trypsin for 2h at 30°C. Peptide 'maps' of the trypsin hydrolysates were then prepared as indicated in the Materials and Methods section. The 'maps' were radioautographed with Kodirex X-ray film for 1–5 days, and the peptides were detected by staining with 2% (w/v) ninhydrin solution. 'Fingerprints' of the enzymes from normal and dystrophic muscle were very much alike and the similarity of the radioautographs is shown in Plates 1(a) and 1(b). Since peptide mapping and radioautography are not exactly reproducible the R_F values of the two peptides varied in each chromatographic run. The amount of undigested protein in the sample possibly

affects the mobility of such peptides. In Plates 1(a) and 1(b) the R_F values of the peptides are not exactly the same but the small difference is possibly due to experimental error. In Plates 1(c) and 1(d) the general pattern of the buried thiol-labelled peptides in normal and dystrophic enzymes are similar but the intensities of the spots vary. Purified enzymes from normal and dystrophic muscle with either 'reactive' or 'buried' thiol groups labelled with iodo[¹⁴C]acetate were hydrolysed with concentrated hydrochloric acid for 2 days at 37°C. This procedure yields mainly small peptides and electrophoresis (Plate 2) of such hydrolysates would be expected to demonstrate differences in sequence around the labelled thiol groups.

Thermal stability. The stabilities of purified enzymes from normal and dystrophic chicken muscle were compared by incubating enzyme (1mg/ml) in 10mM-tris-HCl buffer, pH 8.0, containing dithioerythritol (80mg/l) at 50°C. Samples were taken at intervals and assayed for enzyme activity. The activities of the two enzymes did not appreciably decrease even after 2½h of incubation. The experiment was repeated with enzyme solutions that had been dialysed to remove the dithioerythritol present in the stock enzyme solutions, but again there was no marked difference in the stabilities of the two enzymes after prolonged incubation. Eppenberger *et al.* (1967) observed thermal instabilities of their chicken preparation. This difference may be due to the differences in the method of purification we used and particularly to

EXPLANATION OF PLATE I

(a) and (b) Radioautographs of peptide 'maps' of trypsin digests of creatine kinases from normal (a) and dystrophic (b) chicken muscle with reactive thiol groups alkylated with iodo[^{14}C]acetate. The directions of electrophoresis at pH 6.5 at 50 V/cm for 60 min and of paper chromatography in butan-1-ol-acetic acid-pyridine-water (15:3:10:12, by vol.) are indicated. X shows the position of the origin. (c) and (d) Radioautographs of peptide 'maps' of trypsin digests of creatine kinases from normal (c) and dystrophic (d) chicken muscle with 'buried' thiol groups alkylated with iodo[^{14}C]acetate. Conditions for electrophoresis and chromatography were as for (a) and (b).

EXPLANATION OF PLATE 2

Comparative radioautographs of acid hydrolysates (conc. HCl for 48 h at 37°C) of creatine kinases from normal and dystrophic chicken muscle. (i) and (ii) Enzymes from normal and dystrophic muscle respectively with reactive thiol groups only labelled with iodo[^{14}C]acetate; (iii) and (iv) enzymes from normal and dystrophic muscle respectively with 'buried' thiol groups only labelled with iodo[^{14}C]acetate. The digests were subjected to electrophoresis at pH 2.1 for 1 h at 50 V/cm.

the superiority of dithioerythritol over mercapto-ethanol as a stabilizing agent for thiol enzymes.

Ultracentrifugal studies. In the analytical ultracentrifuge the enzymes from both normal and dystrophic muscle migrated as symmetrical, sharp and well-defined peaks (Fig. 4). The calculated sedimentation coefficients were 4.30S for the normal enzyme and 4.35S for that from dystrophic muscle, an insignificant difference.

Kinetic properties. Michaelis constants for creatine were determined by measuring the initial velocities of the forward reaction at a constant ATP concentration of 4mM while varying the creatine concentration between 4 and 40mM. Similarly, for ATP the creatine concentration was held constant at 4mM and the ATP concentration was varied between 0.2 and 4mM and the Mg^{2+} concentration was kept constant at 4mM. The K_m values were obtained graphically from double-reciprocal plots (Lineweaver & Burk, 1934) and are listed in Table 3.

Fig. 4. Sedimentation-velocity analysis of creatine kinases from normal (upper) and dystrophic (lower) chicken muscle at 20°C at 59 780 rev./min in a Spinco model E analytical ultracentrifuge. The enzymes were dialysed against 10mM-tris-0.1 M-KCl buffer, pH 9.0. The protein concentrations were 5.1 and 5.5mg/ml for the enzymes from normal and dystrophic muscle respectively.

DISCUSSION

The purification procedure results in a highly pure and stable preparation with specific activities of 84 units/mg for the normal and 70 units/mg for the dystrophic enzyme, values similar to those of crystalline rabbit muscle enzyme (Kuby *et al.* 1954). The total protein concentration and the total activities in the extract indicate the presence of a high percentage of creatine phosphotransferase in the chicken breast muscle. The breast muscle, even a dystrophic one, although affected by the condition, is a reasonably good source of this enzyme. There was less enzyme in the potassium chloride extracts from dystrophic muscle compared with that of the normal, possibly due to the noticeably fibrous texture of the former, which made homogenization more difficult (Table 1). Lower yield on subsequent purification is puzzling in view of the stability of the purified enzyme from dystrophic muscle (see above). It could be due to inherent differences between the enzymes from normal and dystrophic tissue or to the preferential loss of a less stable isoenzyme during purification.

The amino acid compositions of the enzymes from normal and dystrophic muscle were very similar (Table 4), although the differences in threonine, glutamic acid and proline are just outside experimental error and may be significant. Further work will be necessary to confirm this. Our analyses of enzymes from normal chicken breast muscle (Table 4) agree well (apart from aspartic acid and glycine) with those of Eppenberger *et al.* (1967), and again emphasize the homology of creatine kinases from the same tissue in different species (Table 4).

The intent, in part, of this investigation was to determine whether the differences reported by Hooton & Watts (1966a) between creatine kinase isolated from normal and dystrophic mouse skeletal muscle were observed in other species bearing genetically linked muscular dystrophy, and to attempt to determine at the molecular level the details of the lesion. It is clear from the results reported here that the enzyme from dystrophic

Table 3. *Kinetic properties of creatine kinases from normal and dystrophic muscle*

Experimental details are given in the text. V_{max} . is expressed as μ equiv. of NaOH/min per ml of reaction mixture (Mahowald *et al.* 1962).

	Substrate	Rabbit (Kuby <i>et al.</i> 1954)	Chicken (this paper)		Mouse (Hooton & Watts, 1966a)	
			Normal	Dystrophic	Normal	Dystrophic
K_m (mM)	Creatine	16	8	9.4	10	17
K_m (mM)	ATP	0.5	1.1	1.39	0.9	0.8
V_{max} .	Creatine		0.05	0.066	—	—
V_{max} .	ATP		0.083	0.050	—	—

Table 4. *Amino acid compositions of creatine kinases from normal and dystrophic chicken muscle and comparison with that of other species*

Experimental details are given in the text. The numbers of residues/mol have been corrected to the nearest integer.

Source of enzyme ...	Normal chicken breast muscle*	Normal chicken breast muscle†	Dystrophic chicken breast muscle†	Rabbit muscle‡	Rabbit brain*	Chicken brain*
Amino acid						
Aspartic acid	73	78	79	81	82	87
Threonine	29	32	37	30	34	35
Serine	32	38	38	35	32	35
Glutamic acid	83	84	80	79	74	75
Proline	41	45	41	39	40	31
Glycine	60	54	55	65	65	68
Alanine	33	34	34	38	35	36
Valine	49	49	52	54	47	48
Methionine	20	21	20	18	20	19
Isoleucine	24	26	24	30	30	28
Leucine	71	68	69	68	81	82
Tyrosine	16	18	17	16	21	21
Phenylalanine	33	36	35	32	41	30
Lysine	65	63	66	65	52	49
Histidine	34	34	32	29	30	21
Arginine	40	42	42	34	22	40

* Eppenberger *et al.* (1967).

† This paper. Averages of single analysis of 16, 40 and 64 h hydrolysis. Serine and threonine values were extrapolated to zero time.

‡ Noltman *et al.* (1962)

chicken muscle is very similar to that from normal tissue, in that it has the same number of reactive and total thiol groups, an identical electrophoretic mobility, yields peptide 'maps' indistinguishable from those of the normal enzyme, has a very similar amino acid composition and probably has very similar sequences around all its thiol groups. There were no significant differences in the sedimentation coefficients and K_m values for creatine and ATP of the two enzymes, although the normal enzyme had a somewhat higher V_{max} for ATP. In normal rabbit muscle creatine kinase the two reactive thiol groups are alkylated at precisely the same rate as for iodoacetate (Watts & Rabin, 1962). The results in Fig. 3 suggest that the two reactive thiol groups of the dystrophic chicken muscle enzyme may not be identical, or alternatively that the three-dimensional structure of the enzyme molecule makes one of them more sensitive to alkylation by iodoacetate.

These results agree in the main with those of Jacobs *et al.* (1969), who isolated creatine kinase from normal human muscle and from the muscle of a patient with Duchenne-type muscular dystrophy, and similarly found no difference in reactive thiol content, kinetic properties and molecular weight. It should be noted that the substitution of a glutamic acid residue for cysteine, postulated by

Hooton & Watts (1966a) on the basis of electrophoretic mobilities and peptide 'mapping', would involve at least two, but more likely, three base changes (Morgan, Wells & Khorana, 1966) and would therefore have a low probability. In their work with dystrophic mouse creatine kinase, Hooton & Watts (1966b) suggested the possibility of oxidation of mouse enzyme thiol groups as an alternative to a mutation in the structural gene.

It is appreciated, of course, that the term muscular dystrophy covers a group of diseases that differ considerably in detail, and that the results of experiments on one species can be extrapolated to another species or to man only with great caution. Nevertheless, it seems unlikely that a lesion involving the reactive thiol groups of creatine kinase is a primary cause of muscular dystrophy but the possibility that the mouse dystrophy may be a different kind of abnormality to that of chicken cannot be excluded (Hooton & Watts, 1966a). This investigation, together with that of Jacobs *et al.* (1969), does not, however, preclude the possibility that modification of other properties of the enzyme could be involved in the aetiology of hereditary muscular dystrophy. Results obtained by D. C. Watts (personal communication) on the reactivity towards iodoacetamide of the thiol groups of creatine kinase from dystrophic human skeletal muscle

suggest that this may be so, and determination of the amino acid sequences of the peptides derived from iodo[¹⁴C]acetate-labelled thiol groups of the enzymes from normal and dystrophic chicken muscle (B. P. Roy, unpublished work) may also help to clarify this question.

B.P.R. acknowledges support (personal grant and expenses) from the Muscular Dystrophy Group of Great Britain for this work and also thanks Dr J. L. Putman (Isotope Research Division, Wantage Research Laboratory) and Dr C. M. Nicholls (Chemical Engineering Division, Atomic Energy Research Establishment, Harwell) for providing laboratory facilities. We sincerely thank Dr D. W. Peterson (University of California, Davis) for the supply of dystrophic chickens and Dr P. Esnouf (Radcliffe Infirmary, Oxford) for ultracentrifugal analysis. The skilled technical assistance of Mr B. J. Miles, Mr S. Milsom, Miss J. Scott and Miss S. E. Dally is appreciated. Requests for reprints should be addressed to B.P.R.

REFERENCES

- Ambler, R. P. (1963). *Biochem. J.* **89**, 349.
- Asmundson, V. S. & Julian, L. M. (1956). *J. Hered.* **47**, 248.
- Benesch, R. E., Lardy, H. A. & Benesch, R. (1955). *J. biol. Chem.* **216**, 663.
- Dance, N. & Watts, D. C. (1962). *Biochem. J.* **84**, 114 P.
- Dawson, D. M. & Kaplan, N. O. (1965). *J. biol. Chem.* **240**, 3215.
- Ebashi, S., Toykura, Y., Momoi, H. & Sugita, H. (1959). *J. Biochem., Tokyo*, **46**, 103.
- Ellman, G. L. (1959). *Archs Biochem. Biophys.* **82**, 70.
- Eppenberger, H. M., Dawson, D. M. & Kaplan, N. O. (1967). *J. biol. Chem.* **242**, 204.
- Eppenberger, H. M., Eppenberger, M., Richterich, R. & Aebi, H. (1964). *Devl Biol.* **10**, 1.
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 752.
- Harris, J. I. (1964). *Nature, Lond.*, **203**, 30.
- Homburger, F., Baker, J. R., Nixon, G. W. & Whitney, R. (1963). *Medna exp.* **6**, 339.
- Hooton, B. T. & Watts, D. C. (1966a). *Biochem. J.* **100**, 637.
- Hooton, B. T. & Watts, D. C. (1966b). *Clinica chim. Acta*, **16**, 173.
- Jacobs, H., Okabe, K., Yue, R., Deutel, H., Ziter, F., Palmehi, R. & Kuby, S. A. (1969). *Fedn Proc. Fedn Am. Socs exp. Biol.* **28**, 495.
- Kendrick-Jones, J. & Perry, S. V. (1967). *Nature, Lond.*, **208**, 1068.
- Kuby, S. A., Noda, L. & Lardy, H. A. (1954). *J. biol. Chem.* **209**, 191.
- Kumudavalli, I., Moreland, B. H. & Watts, D. C. (1970). *Biochem. J.* **117**, 513.
- Lineweaver, H. & Burk, D. (1934). *J. Am. chem. Soc.* **56**, 658.
- Mahowald, T. A. & Kuby, S. A. (1960). *Fedn Proc. Fedn Am. Socs exp. Biol.* **19**, 46.
- Mahowald, T. A., Noltman, E. A. & Kuby, S. A. (1962). *J. biol. Chem.* **237**, 1935.
- Michelson, A. M., Russell, E. S. & Harman, P. J. (1955). *Proc. natn. Acad. Sci. U.S.A.* **41**, 1079.
- Michl, H. (1951). *Mh. Chem.* **85**, 1251.
- Miyazaki, K., Toyada, R., Tomino, H., Yoshimatsa, K. S., Katsunama, N. & Fujino, A. (1965). *Mod. Med., Osaka*, **20**, 202.
- Morgan, A. R., Wells, R. & Khorana, H. G. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56**, 1899.
- Noltman, E. A., Mahowald, J. A. & Kuby, S. A. (1962). *J. biol. Chem.* **239**, 460.
- Rigdon, R. H. (1966). *Ann. N.Y. Acad. Sci.* **138**, 28.
- Roy, B. P., Laws, J. F. & Thomson, A. R. (1969). *Abstr. FEBS 6th Meet., Madrid*, p. 849.
- Walton, J. N. & Gardner-Medwin, D. (1969). In *Disorders of Voluntary Muscles*, p. 458. Ed. by Walton, J. N. London: J. and A. Churchill Ltd.
- Watts, D. C. (1963). *Biochem. J.* **89**, 220.
- Watts, D. C. & Kumudavalli, I. (1970). *Biochem. J.* **118**, 22P.
- Watts, D. C. & Rabin, B. R. (1962). *Biochem. J.* **85**, 507.
- Watts, D. C., Rabin, B. R. & Crook, E. M. (1961). *Biochem. J.* **82**, 412.
- Wood, T. (1963). *Biochem. J.* **87**, 453.