Biochem. J. (1973) **136**, 1017–1028
Printed in Great Britain

Comparison of the Turnover Patterns of Total and Individual Muscle Proteins in Normal Mice and those with Hereditary Muscular Dystrophy

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(*Received* 4 *May* 1973)

1. The incorporation of amino acids into hindleg muscle proteins of normal and dystrophic mice was measured ½h to 16 days after administration of the radioactive pulse. 2. Dystrophic animals showed a faster initial rate of incorporation into total and soluble proteins in the first few hours after injection, but the extent of incorporation relative to the size of the amino acid pool was similar in both. There was little difference between the overall degradation rates although this started later in the dystrophic proteins. An initial fast phase of degradation reached a plateau after 3 days whereupon the residual label in the protein remained constant up to 16 days after injection. 3. Analyses of individual radioactive proteins fractionated by polyacrylamide-gel electrophoresis showed that the distribution of label was similar in all the soluble proteins from normal and dystrophic muscle. Time-course experiments revealed that in dystrophic mice the two major soluble proteins of the muscle, creatine kinase and adenylate kinase, initially incorporated 2–3 times more label relative to the initial size of the precursor pool. This label was then lost equally rapidly and the final plateau value was much less than that in normal mice. This initial peak of activity was not observed in normal mice. 4. A group of dehydrogenases showed similar initial turnover patterns in both dystrophic and normal mice but the final plateau value was much higher in the former. 5. The results provide support for the hypothesis that there is no obvious defect in the protein synthetic machinery of dystrophic muscle. However, certain proteins do show anomalous turnover patterns relative to those in normal animals. A single structural gene mutation giving rise to one particularly unstable and readily degradable muscle protein is excluded as the cause of the dystrophy.

Several studies of normal and genetically dystrophic mice have demonstrated an increased rate of incorporation *in vivo* of labelled amino acids into muscle protein of the dystrophic animals (Coleman & Ashworth, 1959; Kruh *et al.*, 1960; Simon *et al.*, 1962; Srivastava, 1968). It was suggested that an increase in the degradation rate of such proteins resulted in a faster overall protein turnover and a decreased protein content seen in the atrophied muscles. The increased turnover rate was more evident with the older, more diseased animals (Coleman & Ashworth, 1959) and was demonstrated in both the myosin and water-extractable (sarcoplasmic) proteins (Kruh *et al.*, 1960), although Srivastava (1968) showed that the increased degradation of proteins was more striking in the microsomal and supernatant, than in the nuclear and myofibrillar fractions.

A limited number of experiments have been concerned with the turnover of specific proteins. As previously mentioned, Kruh *et al.* (1960) demonstrated faster myosin turnover and the recently published work of Srivastava (1972) suggested that the increased incorporation of amino acids into the structural protein of dystrophic muscle measured both *in vivo* and *in vitro* was associated more with tropomyosin, there being no change in the incorporation into actin and a decreased incorporation into myosin.

The present investigations were carried out to see whether the increased turnover of sarcoplasmic proteins in dystrophic mouse muscle was confined to specific proteins or was a more general phenomenon.

Experimental

Materials

Chemicals. [U-^{14}C]Protein hydrolysate from *Chlorella* (>45mCi/mg-atom of carbon) and *n*-[1-^{14}C]hexadecane (1.1 μCi/g) were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. NCS, a solubilizing agent for biological materials, was obtained as a 0.6M solution in toluene from the Amersham–Searle Corporation, High Wycombe, Bucks., U.K.

Other chemicals were supplied by the Sigma (London) Chemical Co. Ltd., London S.W.6, U.K., or BDH Chemicals Ltd., Poole, Dorset, U.K.

Animals

Mice were the Bar Harbor 129 strain, carrying the autosomally inherited recessive dystrophy gene, *dy*, that had been maintained for many years in our own laboratory. Families of mice that had shown no dystrophy for at least three generations were used as normals.

Methods

Amino acid incorporation experiments. Mice were fed *ad libitum* on Laboratory Chow supplemented every 2 weeks with wheat germ. They were killed by chloroform anaesthesia from ½h to 16 days after the intraperitoneal injection of ^{14}C-labelled protein hydrolysate (9μCi/10g body wt.). This mixture of amino acids was used to obtain sufficient radioactive labelling of the protein. With normal mice, 5-week-old litter mates were used wherever possible. However, owing to the limited availability of the dystrophic animals, litter mates were rarely obtainable and a wider range of ages was used. In practice this was less of a problem than the fact that the extent of dystrophy varied considerably irrespective of age. No attempt was made to match animals with respect to the extent of dystrophy.

The hindleg muscle was quickly dissected, weighed and homogenized in 10vol. of sucrose (0.25M), pH7.4, containing 1mM-EDTA and 1mM-mercaptoethanol, in a Polytron ST 10 homogenizer. In some experiments the red, type I, (soleus and medial gastrocnemius) and the white, type II, (lateral gastrocnemius) muscles were treated separately. A sample of the total homogenate was analysed for radioactivity (see below) and the remainder was centrifuged in an MSE High Speed 18 centrifuge at 25000*g* (14000rev./min.) at 4°C for 40min. The supernatant, excluding the top fatty layer, was pipetted off and concentrated by vacuum dialysis (Watts & Moreland, 1970) overnight at 4°C against fresh homogenizing buffer.

The protein content of the concentrated solution was determined by measurement of its absorption at 260nm and 280nm (Warburg & Christian, 1941) or by the method of Lowry *et al.* (1951), with bovine crystalline albumin as standard. Samples of the soluble fraction and diffusate were assayed for radioactivity.

Electrophoresis. The soluble protein was fractionated by polyacrylamide-gel electrophoresis by the technique of Ornstein (1964) and Davis (1964) as described by Smith (1968*b*). The 7% (w/v) polyacrylamide gels were pre-run for 30min at 1–2mA/gel to remove the ammonium persulphate used as a catalyst for gel formation. The protein sample (100–1000μg) was applied in 10% (w/v) sucrose and the electrophoresis started at 1mA/gel (250V). After 15min, the current was increased to 2–2.5mA/gel (350–450V) and the electrophoresis was continued until the Bromophenol Blue–albumin marker had migrated to within 1.5cms of the anodic end. All runs were performed at 4–6°C in fresh Tris–glycine buffer (pH8.3). After electrophoresis the gels were treated in one of two ways. (1) They were stained for protein in 0.2% (w/v) naphthalene Amido Black in 7% (w/v) acetic acid for a minimum of 2h. Excess of stain was removed either by repeated washings in 7% acetic acid or electrophoretically in 15% acetic acid (Richards *et al.*, 1965). (2) They were divided into two longitudinally by gently forcing the gel through a fine wire across the bottom of a hole in a rubber bung. One half was stained for protein as described in (1) and the other was stained for enzyme activity. Creatine kinase was assayed by the agar overlay technique of Eppenberger *et al.* (1964); adenylate kinase as described by Scopes (1968) and myoglobin by the method of Work & Work (1969). Dehydrogenases and esterases were stained by modification of the methods described by Smith (1968*a*).

Radioactivity determinations in polyacrylamide gels. Densitometer traces of gels whose radioactivity was to be estimated were obtained with a Joyce–Loebl chromatogram scanner. The gels were then placed in individual aluminium foil troughs of the same dimensions as the gel and frozen in powdered CO_2. The gel was allowed to thaw slightly before slicing into about 46×1.6mm slices by using a row of stainless steel razor blades each separated by 2×0.8mm washers. Each slice was incubated in 0.4ml of NCS in a covered tube for 2h at 65°C. The NCS and gel were decanted into scintillation vials containing 9ml of toluene scintillant [AnalaR toluene containing 0.4g/100ml of 2,5-diphenyloxazole (PPO) and 0.01g/100ml of 1,4-bis-(5-phenyloxazol-2-yl)-benzene (POPOP) to which a few drops of methanol were added]. The tubes were rinsed with 2×3ml of scintillation fluid and the vials were stored overnight at 4–6°C before counting (Basch, 1968).

Radioactive determinations in protein and amino acid samples. Protein was precipitated from homogenates, sedimented protein and dialysed soluble protein samples by the addition of trichloroacetic acid to a final concentration of 12% (w/v). The samples were heated at 90°C for 10min, cooled and centrifuged. The precipitate was washed by centrifugation with two successive 4ml portions of 12% (w/v) trichloroacetic acid. All trichloroacetic acid supernatants were combined and stored at 4–6°C. Addition of ^{14}C-labelled protein hydrolysate to unlabelled homogenates showed that, for the small

amounts of protein precipitated in the present experiments, all radioactive amino acids were removed by this method. The protein precipitate was extracted for a minimum of 20min in 4ml of chloroform–methanol (1:1, v/v) mixture containing 0.25% (v/v) conc. HCl. After centrifugation, the precipitate was extracted in the same manner with the acidified chloroform–methanol (2:1, v/v). The protein was washed with 2×5ml portions of sodium-dried ether and then dried in air. Up to 5mg of protein was dissolved in 0.5ml of NCS as described above and 10ml of toluene scintillant added. Vials were stored for 48h in the dark at 4–6°C by which time chemiluminescence had decreased to zero.

For measurement of radioactivity in non-protein N 0.5ml of trichloroacetic acid-treated supernatants or of the diffusate was added to 6ml of Bray's scintillant (Bray, 1960).

Vials were counted in a Beckman Automatic Scintillation Spectrometer model 1650. The efficiency of counting was estimated by the addition of a known amount of [1-^{14}C]hexadecane and standard quench curves were drawn. The efficiencies of counting were as follows: protein, 85–90%; polyacrylamide-gel slices, 80–90%; aqueous samples, 76–80%.

The recovery of label from the gels varied between 65 and 90% and was probably due to the exclusion of some of the 1000μg of protein from the gel in the electrophoretic separation.

Results

Spontaneous degradation of sarcoplasmic extracts

To check that sarcoplasmic protein awaiting analysis was not significantly degraded during the overnight dialysis to remove free amino acids and during the subsequent storage at 4°C, analyses were made of the ratio (c.p.m. in protein)/(c.p.m. in protein plus amino acid) before and after dialysis of samples taken 4h after the injection of labelled protein hydrolysate into normal and dystrophic mice. In one experiment, for example, dialysis increased this ratio from 0.49 to 0.85 for normal and from 0.63 to 0.90 for dystrophic extracts. (Undialysed preparations showed no change from the original ratio after overnight storage at 4°C.) These ratios remained constant for 24h, and after 3 days had only fallen to 0.84 and 0.88 respectively.

Dystrophic mouse muscle has been reported to contain higher amounts of alkaline cathepsin activity than normal muscle (Pennington, 1970). The thiol compound, dithiothreitol, at a concentration of 0.5–10.0mM has been shown to activate proteolytic enzymes (Kohn, 1969). Mercaptoethanol, 10mM, and soya-bean trypsin inhibitor, a non-specific protease inhibitor (Goldspink *et al.*, 1971), at a final concentration of 3.3μg/ml were added separately to homogenates to investigate whether dystrophic protein extracts became more susceptible to degradation than normal extracts. For both normal and dystrophic preparations, the trypsin inhibitor was found to maintain the ratio for about a day longer, whereas mercaptoethanol caused a very slight fall to 0.87 in dystrophic extracts but had no effect on the normal extracts. However, since these effects were so small, it was concluded that spontaneous degradation of the extracts by proteolysis was not a problem in the present work.

Amino acid pool size in muscle

The mean free concentration of those amino acids normally found in proteins in the tissue extracts as determined from amino acid analyses (S. E. Kitchin & D. C. Watts, unpublished work) was 9.5μmol/g wet wt. of muscle (range 8.7–10.8) in three normal 5-week-old mice and 9.8μmol/g wet wt. of muscle (range 7.8–12.5) in three dystrophic 4–8-week-old mice.

For normal mice, the weight of hindleg muscle increases in relation to the body weight as the animals develop. For example, there were, on average, 22mg of muscle/g body wt. in seven normal 3–5-week-old mice and 28mg of muscle/g body wt. in four 7–9-week-old animals. For dystrophic animals, this value falls, as the disease progresses, from 22mg of muscle/g body wt. in six 3–5-week-old mice to 18mg of muscle/g body wt. in nine 7–9-week-old animals. Since, in the present experiments, the volume of radioactive amino acid injected is related to body weight, the label will be diluted less in dystrophic than in normal muscle. The difference was corrected for by the calculation of relative specific radioactivity described below.

Incorporation of radioactive amino acids into muscle proteins

This was determined by using a series of 37 animals (19 normal and 18 dystrophic). The results obtained are summarized in Table 1 and analysed in the sections below.

Amino acid uptake into muscle and calculation of relative specific radioactivity. The total c.p.m./g of muscle found in normal animals killed at different times after injection was found to be initially high and decrease to a plateau representing about 10% of the initial value (Table 1). This trend was much less apparent in the dystrophic animals for which the results showed more scatter indicating that the concentration of labelled amino acids in the muscle available for protein synthesis varied from individual to individual. This would cause the specific radioactivity of the protein to vary in

Table 1. *Time-course of the incorporation of amino acids into hindleg muscle of normal and dystrophic mice*

Each value represents the results for both hindleg muscles of one mouse after the intraperitoneal injection of ^{14}C-labelled hydrolysate (9 μCi/10 g body wt.). Summary of the amino acid incorporation results for normal and dystrophic mice at h (Expt. A) and days (Expt. B) after injection of the isotope. The c.p.m. in free amino acids is obtained by subtracting the total counts in protein/g wet wt. of muscle from the total c.p.m./g wet wt. of muscle.

Time after injection	Age at injection (weeks)		Total c.p.m./g wet wt. of muscle		Percentage incorporation into protein $\left(\frac{\text{total c.p.m. in protein}}{\text{total c.p.m. in muscle}} \times 100\right)$		Total c.p.m. in protein/g wet wt. of muscle		$10^3 \times \frac{\text{c.p.m./mg of protein}}{\text{total c.p.m./g wet wt. of muscle}}$			
									Total protein		Soluble Protein	
Expt. A (h)	Normal	Dystrophic	Normal	Dystrophic	Normal	Dystrophic	Normal	Dystrophic	Normal	Dystrophic	Normal	Dystrophic
½	9	9	1285000	186100	18	68	200000	110000	1.4	6.4	2.2	8.2
1	9	9	226220	151000	50	76	113000	100000	4.0	7.4	4.8	7.9
2	5	4	278760	220900	70	81.5	195000	180000	5.3	16.5	4.7	14.2
2	9	9	313670	291700	73	72.7	229000	212000	5.4	14.6		8.5
4		8		218800		84		191000		19.0		22.4
10	5	4	174100	319700	94	88	163700	261300	3.9	16.3	9.8	15.1
10		4		351600		82		288000		9.5		10.3
10		8		358000		87		313000		9.7		10.0
12	6	8	147500	201000	77	83	111290	164400	24.5	13.8	24.5	13.9
Expt. B (days)												
1	5		125000		90							
1	6	8	196000	146000	87	77	188000	113000	21.8	25.9	20.8	25.9
2	5	6	137500	100730	87	86	101600	86900	18.0		20.0	
2	5	8	176500	120300	85	83	150000	99500	5.9	18.9	8.5	30.0
3	5	8	217200	263000	84	90	209300	237000	6·7	10.0	6.8	11.0
5	5	8	113900	287500	94	78	107200	234100	5.6	13.0	8.9	12.0
7	5	9	170200	240670	84	90	143100	217000	9.8	10.0	9.7	9.0
8	5		87400		98		85700		5.5		16.4	
12	5		126000		94		118160		4.4		11.5	
16	5		118500		97		114500		7.2		8.7	

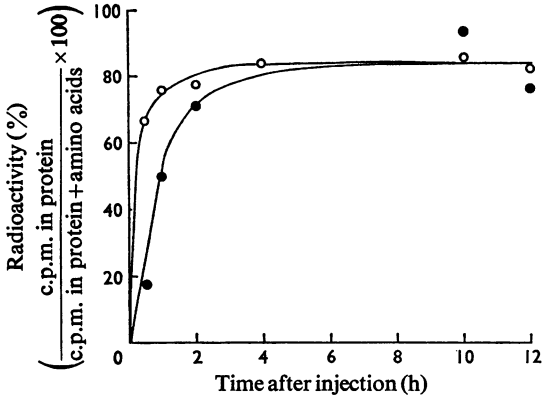

Fig. 1. *Time-course of the incorporation of labelled amino acids from muscle into protein of normal and dystrophic mice*

The results are expressed as a percentage of the radioactive counts in the muscle that are present in protein and are taken from the data in Table 1. ●, Normal mice; o, dystrophic mice.

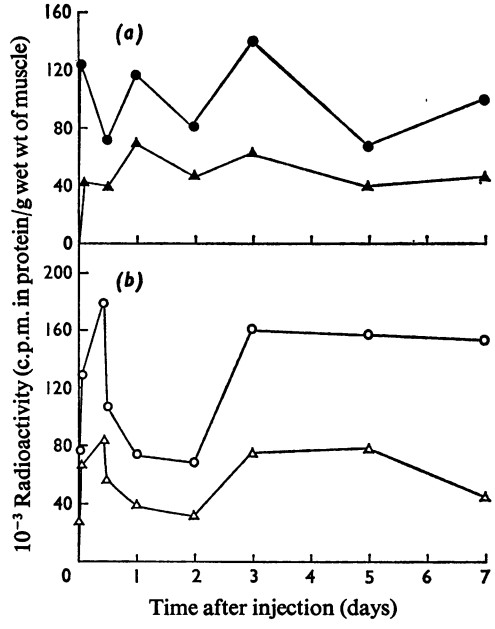

Fig. 2. *Time-course of the radioactivity in protein per unit weight of muscle*

The results show the total radioactivity in soluble and sedimented protein/g wet wt. of hindleg muscle from normal and dystrophic 5–9-week-old mice up to 7 days after injection. ●, Normal mice, sedimented protein; ▲, normal mice, soluble protein; o, dystrophic mice, sedimented protein; △, dystrophic mice, soluble protein.

proportion. While the radioactivity counts in the free amino acid pool are high relative to those in the protein the best way to correct for variation in pool size is to divide the specific radioactivity of the protein by the c.p.m. in free amino acids/g of muscle as shown for Fig. 3. The disadvantage of this procedure is that when the radioactivity of the pool becomes low relative to the radioactive counts in the muscle, small fluctuations in the size of the pool cause large variations in the derived value which have no relationship to the original rate of protein synthesis since most of the label was incorporated in the first few hours of the experiment. To allow for this in experiments showing time-courses lasting more than a few hours an alternative procedure was adopted in which values have been expressed as relative specific radioactivity, obtained by dividing the specific radioactivity by the total c.p.m./g of muscle.

Fraction of labelled amino acids incorporated into muscle protein. Fig. 1 shows that the total c.p.m. present in protein, expressed as a percentage of the total c.p.m. in the muscle, increased more rapidly in dystrophic than in normal muscle. Maximum incorporation was attained after about 2h in the dystrophic animals and about 5h in the normal animals. The plateau values obtained showed that similar amounts, 80–95% of the total c.p.m., were incorporated into the protein of both normal and dystrophic samples. This remained constant for the duration of the experiments.

The distribution of radioactivity between the soluble and sedimentable protein/unit weight of muscle is shown in Fig. 2. Between 25 and 40% of the incorporated amino acids were associated with the soluble fraction in both normal and dystrophic muscle. For dystrophic extracts, the initial rapid uptake of label was followed by a decrease which after 2 days returned to the initial high value (Fig. 2). The reason for this, unless it simply reflects the variation in size and protein content of the dystrophic muscle and pool of labelled amino acids, is not known.

For both soluble and sedimentable protein, the counts in protein/unit wet wt. of muscle were greater in the dystrophic than in the normal animals. However, in two groups of three animals, 3 weeks old, the counts in the protein were greater in the normal mice.

Time-course of the change in relative specific radioactivity of muscle proteins. The change in relative specific radioactivity of the total and soluble protein over short periods of time (up to 5h after injection) are shown for the total and soluble protein in Figs. 3(*a*) and 3(*b*). For both, the initial rise in relative specific radioactivity is much faster in the dystrophic than in the normal mice. Following the time-course for longer periods shows that the maximum relative specific radioactivity achieved is about the same for both soluble and total protein in dystrophic and normal muscle (Figs. 4*a* and 4*b*). However, the plateau region for the maximum lasts longer in the dystrophic samples (Figs. 4*a* and 4*b*) so that the decay part of the relative specific radioactivity curve commences sooner in the normal animal. In all the samples the decay rate was biphasic; a fast phase lasting 2–3 days which reached a plateau at 25–35% of the maximum value. The half-lives for this initial decay from the maximum value were similar for normal and dystrophic animals and were about 30h for the total and 50h for the soluble protein. The plateau values remained too constant for half-lives to be calculated.

Protein fractionation on polyacrylic gels

Samples of soluble protein from all the animals listed in the experiments reported in Table 1 were subjected to polyacrylamide-gel electrophoresis. Typical patterns for normal and dystrophic mixed (undifferentiated) muscle proteins are compared in Fig. 5. For identification purposes, the major protein bands have been labelled 1–11, band 1 being the slowest anodic migrating species at pH8.3 and band 11 the fastest migrating species. From visual observations and quantitation of chromoscan traces (Fig. 5), the abundance of the individual proteins was assessed.

In some normal and most dystrophic extracts, a small amount of a very slowly migrating species, labelled band A, was also present. Specific staining showed the presence of a number of dehydrogenases in the region of bands 1–4. These included the slower

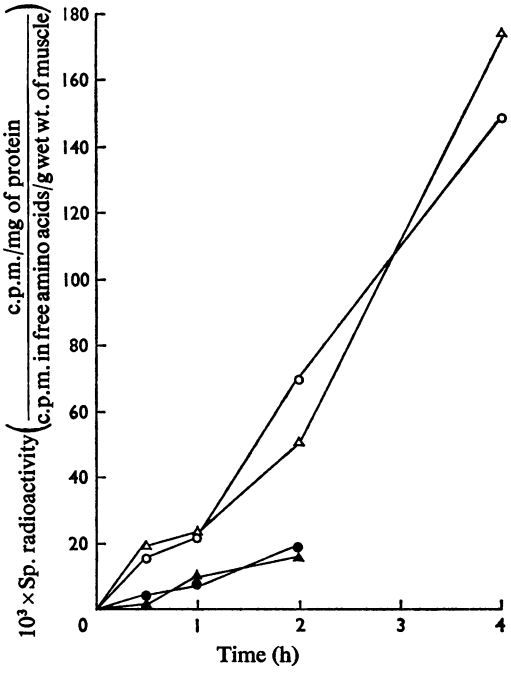

Fig. 3. *Time-course of the specific radioactivity of hindleg muscle protein in relation to the c.p.m. in the amino acid pool in normal and dystrophic mice after injection of a pulse of radioactive protein hydrolysate*

●, Normal mice, total protein; ▲, normal mice, soluble protein; ○, dystrophic mice, total protein; △, dystrophic mice, soluble protein.

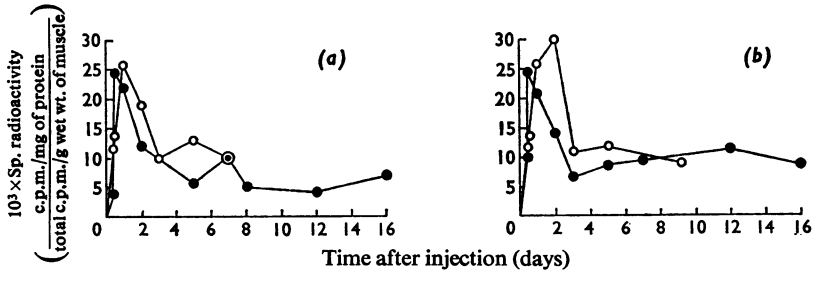

Fig. 4. *Time-course over* 16 *days of the relative specific radioactivity of hindleg muscle protein in normal and dystrophic mice after injection of a pulse of radioactive protein hydrolysate*

(*a*) Total protein, (*b*) soluble protein. Symbols have the same meaning as in Fig. 3. The point for the soluble protein of the normal animal at 8 days appeared to be abnormally high (cf. Fig. 8) and has been omitted from the curve as indicated by the broken line.

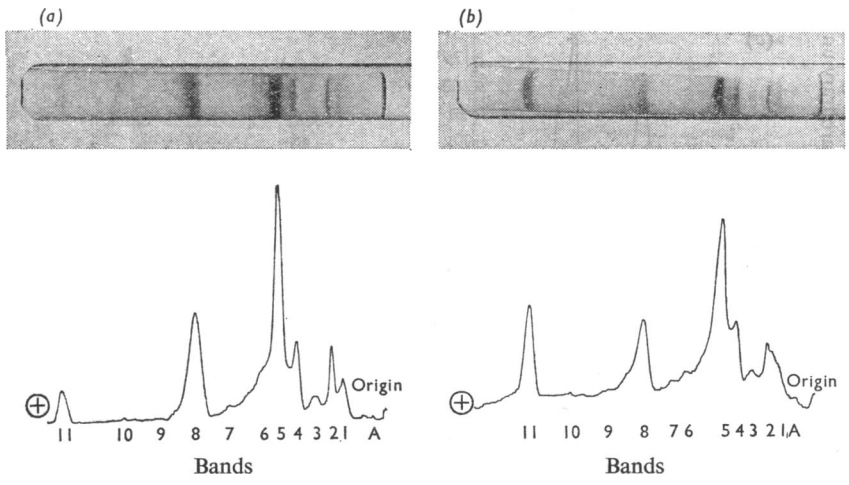

Fig. 5. *Polyacrylamide-gel electrophoresis of soluble protein from (a) normal and (b) dystrophic hindleg muscle*

Mixed soluble protein (100μg) from the hindleg muscles of normal (8 weeks old) and dystrophic (9 weeks old) mice was applied to 7% polyacrylamide gels, pH 8.9, and subjected to electrophoresis in Tris–glycine buffer, pH 8.3, at 4–6°C. Gels were stained in 0.2% (w/v) Naphthalene Amido Black and densitometer traces obtained with a Joyce–Loebl Chromoscan.

migrating isoenzymes of lactate, malate, glutamate, α-glycerophosphate, succinate and xanthine dehydrogenases. General esterase activity was also present in this region. Peak 5, creatine kinase, was consistently the strongest band, but in dystrophic samples the relative amount was significantly less. On average, in normal mice 28% of the soluble protein was creatine kinase; in dystrophic mice only 20%. The shoulder on the anodic side of peak 5 was present in both normal and dystrophic samples. Peaks 6 and 7, which contained some of the faster dehydrogenase isoenzymes, were present in very small amounts and were not always detectable.

The protein at peak 8 was identified, by its resistance to acid treatment (Fildes & Harris, 1966), as the slowest migrating and most abundant of the three isoenzymes of adenylate kinase. Full enzyme activity was detected in peak 8 after the muscle had been acidified to pH 2.0 with 2M-HCl for 10min at room temperature (20°C) and then neutralized with 2M-NaOH to pH 7.0 before electrophoresis. Staining for protein showed that this treatment had no effect on peak 8, it destroyed bands 1–5 and slightly decreased the intensity of band 11, the albumin component (see below). The amount of adenylate kinase showed considerable variation but there was generally less present in the dystrophic animals.

Myoglobin stained in the region between bands 8 and 11. Specific staining with benzidine showed a close correspondence to peak 9. Both the myoglobin peak and the protein peak 9 decreased in the dystrophic condition.

The fastest moving band in the extracts was albumin; relatively more was found in the dystrophic samples but the amounts varied considerably owing to contamination of the excised muscle by blood.

Radioactivity in gel-fractionated proteins

To obtain sufficient radioactivity in the protein bands, up to 1000μg of soluble protein was applied to each gel. Under these conditions some protein, as judged from the intensity of staining and from the radioactive counts, remained at the origin and the individual peaks were not always as well resolved as when lower loadings were applied.

The distribution of label in the soluble protein was basically similar in normal and dystrophic samples in that most of the label was incorporated into the three most abundant proteins, creatine kinase, adenylate kinase and albumin, and into the mixture of slowly migrating proteins labelled bands 1–4 of which peak 2 generally contained most label (Figs. 6*a* and 6*b*). Although slight differences can be seen, analysis of a number of samples showed that the presence or absence of any particular minor protein species was not a consistent feature of the disease.

Quantitative analyses were made of the three main muscle protein peaks, creatine kinase, adenylate kinase and protein bands 1–4 migrating behind

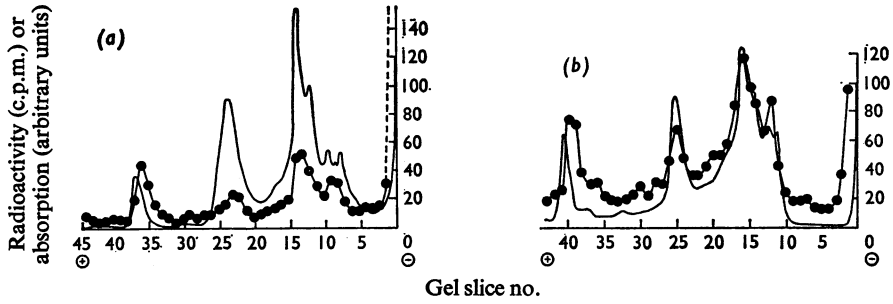

Fig. 6. *Radioactivity of proteins fractionated on polyacrylamide gels*

Mixed soluble protein (1000 μg), prepared from the hindleg muscles of a normal (5-week-old) and a dystrophic (4-week-old) mouse, 10h after administration of ^{14}C-labelled protein hydrolysate, was electrophoresed and stained as described in the legend for Fig. 5. The gels were transversely cut into 1.6mm slices and the radioactivity in each slice was estimated as described under 'Methods'. (*a*) Normal, (*b*) dystrophic muscle proteins. Radioactivity ●, (c.p.m.); ——, absorption (arbitrary units).

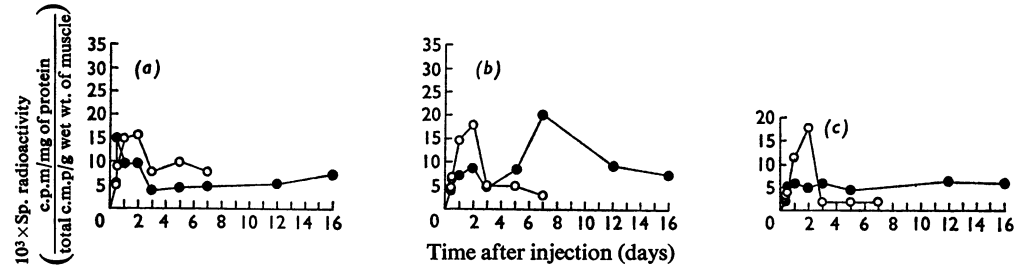

Fig. 7. *Time-course of the relative specific radioactivities of proteins fractionated on polyacrylamide gels* 0–16 *days after injection of a pulse of radioactive protein hydrolysate*

(*a*) Peaks 1–4; (*b*) peak 5; (*c*) peak 8. ●, Normal mice; o, dystrophic mice.

creatine kinase, at known time-intervals after the administration of the labelled amino acids. Calculations of the relative specific radioactivities of these proteins on the gels were made by measuring the ratio of the area associated with each protein to the total area on the chromoscan records for a known amount of protein subjected to electrophoresis. Over the first 2h of incubation the relative specific radioactivities of all these proteins increased faster in the dystrophic than in the normal animals.

Over the longer period of incorporation (Fig. 7), the normal and dystrophic patterns for protein bands 1–4 were similar to those for the total proteins (Figs. 4*a* and 4*b*) showing a sharp rise in relative specific radioactivity followed by a gradual decay. For creatine kinase and adenylate kinase, however, the pattern was somewhat different. In the normal animals, the relative specific radioactivity for both enzymes rose to reach a plateau value after a few hours; this plateau value for creatine kinase was about twice that for adenylate kinase suggesting a faster rate of synthesis for the former. In the dystrophic animals, the relative specific radioactivity of creatine kinase rose to a value about twice that of the normal animals and then rapidly decayed. For adenylate kinase, maximum relative specific radioactivity was three times greater in the dystrophic than in the normal animals. The turnover of this protein was significantly faster in the dystrophic muscle.

In other experiments, the results of which are not shown in the figures, two severely afflicted, 6-week-old dystrophic litter-mates were killed 2 and 6 days respectively after injection of the radioactive label. On subjecting the soluble protein to gel electrophoresis, although the pattern of protein bands obtained was similar to that shown in Fig. 5, only the albumin contained measurable radioactivity. Such results suggest that the enhancement of turnover rate is proportional to the severity of the disease, in agreement with Coleman & Ashworth (1959).

The specific radioactivity of serum albumin was

Table 2. *Protein analyses of red and white differentiated muscles*

The results are the means of three analyses of the red (soleus and medial gastrocnemius), white (lateral gastrocnemius) and total mixed muscles from both hindlegs of 9-week-old normal (male litter mates) and dystrophic (female litter mates) mice.

Expt. A	Wet wt. of muscle (g)		Dystrophic as a percentage of normal value
Muscle type ...	Normal	Dystrophic	
Red (soleus and medial gastrocnemius)	0.036	0.020	55%
White (lateral gastrocnemius)	0.120	0.023	19%
Mixed total	0.720	0.250	35%
Expt. B	Total protein content (mg)		
	Normal	Dystrophic	
Red (soleus and medial gastrocnemius)	3.4	2.2	65%
White (lateral gastrocnemius)	9.8	2.2	23%
Mixed total	89.4	26.7	30%

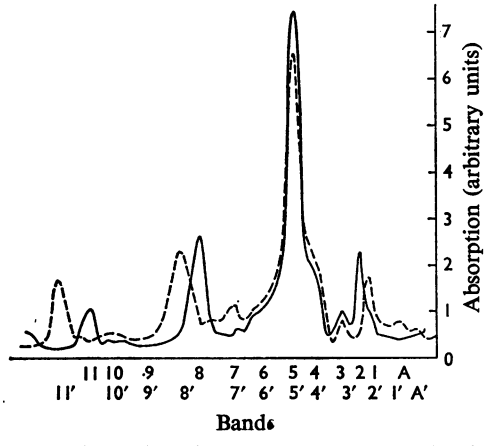

Fig. 8. *Polyacrylamide-gel electrophoresis of red and white soluble protein from the hindleg muscles of normal and dystrophic mice*

100μg of red soleus and medial gastrocnemius (----, unprimed numbers) and white lateral gastrocnemius (——, primed numbers) from a 5-week-old normal mouse was subjected to polyacrylamide-gel electrophoresis as described in Fig. 3. The time of electrophoresis for the red protein was longer than that for the white and therefore the protein bands are not quite superimposed.

calculated from polyacrylamide-gel traces (Fig. 6) but without any correction for the size of the free amino acid pool since this was not determined for liver. The rate of radioactively labelled albumin appearing in muscle measured up to 15h after administration of labelled amino acids was three times faster in normal than in dystrophic animals. The maximum specific radioactivity was also attained sooner in the normal mice but the decay rates were not significantly different and gave an approximate half-life of 2–3 days.

Protein synthesis in red and white muscles

A limited number of experiments were performed on the incorporation of labelled amino acids into red and white muscles, as distinct from the total mixed hindleg muscle. Older animals were used to obtain a sufficient amount of differentiated muscle types.

Comparison of the muscles of normal and dystrophic mice on a wet weight basis showed (Table 2, Expt. A) that the total hindleg muscle mass of dystrophic animals was one-third that of the normal mice; the red muscle was rather more than half the normal value whereas the white muscle was only one-fifth. The total protein content of the muscles followed the same pattern (Table 2, Expt. B).

With both normal and dystrophic mice the soluble protein patterns of red and white muscles were similar except that there was less creatine kinase and more myoglobin and albumin in the red muscle extracts (Fig. 8). Assay of creatine kinase on starch gels demonstrated that there was relatively more brain-type (fast-migrating) creatine kinase in the red muscle and more in the dystrophic than the normal extracts. However, even in the red muscle, this creatine kinase constituted a very small percentage of the muscle type enzyme and would not contribute to the protein-stained bands on polyacrylamide gels.

As with the mixed proteins, the relative proportions of creatine kinase and adenylate kinase decreased in the dystrophic samples and this was more significant in the white muscles.

The small amount of differentiated muscle available did not permit radioactive analyses of the proteins on the gels.

Discussion

Hereditary muscular dystrophy in the mouse, like that in man, is a condition particularly associated with the white striated muscles. However, the red striated muscles are also decreased in size (Table 2) indicating some degree of involvement in the disease. Ideally, therefore, red and white muscles should be investigated separately. In practice, this was not possible because the amount of dystrophic muscle available was too small for reliable quantitative measurement. The alternative adopted, of analysing mixed red and white muscles, means that the change in proportion of the components must be borne in mind when analysing the results.

One problem that arises in this connexion is the difficulty of establishing similar concentrations of labelled amino acids in the muscles at the beginning of the experiment since the dystrophic animals are much smaller than normal mice and there is no simple relationship between total body weight and muscle volume in the two groups of animals. This difficulty was circumvented by injecting the radioactive pulse in proportion to body weight, as done by others, and determining the radioactivity per unit weight of muscle and in the precursor amino acid pool separately. Fortunately, the pool of free amino acids is sufficiently similar in red and white normal and dystrophic muscle (S. E. Kitchin & D. C. Watts, unpublished work) for the extent of dilution of the label in the muscle not to be greatly affected between the two groups. When the radioactivity in the pool is high relative to that in protein an appropriate correction for the differences in pool size in different animals is to divide the specific radioactivity of the protein by the c.p.m. in the pool/g of muscle. This has been done for Fig. 3 where the time-course is short. However, when the time-course is followed over several days the pool radioactivity falls to a very low value relative to that in protein. Any variation in pool size now causes big differences in the value of protein specific radioactivity/c.p.m. in the pool, which have no relationship to the original rate of incorporation of radioactive label into protein. For this reason it was found preferable for the longer time-course experiments to divide the protein specific radioactivity by the total c.p.m./g of muscle. This correction is not ideal but to some extent meets the problem that the amount of free amino acids required to be known is that relating to the whole time-course of the incorporation part of the experiment rather than, as can only be measured, at the end of the experiment. Both methods of expressing the results give a qualitatively similar picture. Failure to allow for the size of the free amino acid pool results in a considerable overestimate of the amount of label incorporated by dystrophic muscle as discussed below.

Analysis of the experiments reported here show some similarities between the amino acid incorporating properties of whole extracts of normal and dystrophic muscle. The balance of distribution of radioactivity between free amino acids and that incorporated into protein is similar in normal and dystrophic muscle once the process of amino acid incorporation has reached equilibrium (Fig. 1). Similarly, the proportion of the label in the soluble fraction remains at 25–40% (Fig. 2). In accord with other workers (Coleman & Ashworth, 1959; Simon *et al.*, 1962; Srivastava, 1968), it was found that the initial rate of incorporation is much faster in the dystrophic animals (Figs. 3*a* and 3*b*) both in total and soluble protein and this correlates with the finding that the protein synthesizing system is more concentrated in dystrophic muscle, probably as a simple result of the decrease in muscle volume (Watts & Reid, 1969). However, contrary to previous findings, the extent of incorporation, expressed as relative specific radioactivity, is about the same in both tissues. If the results are expressed simply as specific radioactivity then, as is generally observed with this presentation, the extent of incorporation is found to be much greater in dystrophic muscle as well. This apparent difference clearly results from the higher initial concentration of radioactive amino acids in the dystrophic muscle and is not a fundamental feature of the dystrophy. Expressing the radioactivity incorporated into protein on a g wet wt. basis (Fig. 2) gives a similar enhancement.

It has been reported for mice that the uptake of the non-metabolizable amino acid, α-aminoisobutyrate, is much greater by dystrophic than by normal muscle (Baker, 1964), and in rats the uptake by striated red muscle is greater than by white muscle (Goldberg, 1967). The above findings generally agree with these observations although the total c.p.m./g of muscle recorded in our experiments showed considerable variability among both normal and dystrophic individuals (Table 1). This may partly reflect the use of a mixture of labelled amino acids in the present work, some of which would undergo general metabolism.

The apparent enhancement in the extent of amino acid incorporation in dystrophic muscle observed on the specific radioactivity basis also gives an apparent enhancement in the subsequent general rate of degradation. On a relative specific radioactivity basis (Figs. 4*a* and 4*b*) the rate of degradation is not significantly different between the two tissues. Indeed, the surprising finding emerges that in dystrophics although the rate of uptake of label is initially faster the onset of loss of label occurs later,

resulting in a rather broad maximum in the relative specific radioactivity of dystrophic protein. This could result from a higher rate of reutilization of labelled amino acids by the dystrophic muscle although the amount of radioactivity in free amino acids was much the same in both groups of animals by this time (Table 1). However, the plateau protein value found after 3–4 days might also be expected to be much higher in dystrophic mice but this appears not to be greatly elevated for either total or soluble protein (Figs. 4*a* and 4*b*). Thus the inference is that the dystrophic muscle proteins as a whole turn over no faster than those in normal mice. However, it should be stressed that in a few dystrophic individuals (results not shown) the loss of label was extremely rapid, particularly if the disease was well advanced (not necessarily related to age, even in this highly inbred population). This extreme variability in the biochemical pattern of dystrophic muscle is a common feature of the disease.

Differences in the extent as well as in the rate of amino acid incorporation become evident when the analysis is extended to individual proteins. The general protein profile of normal and dystrophic muscle was very similar when analysed by polyacrylamide-gel electrophoresis (Fig. 5) except that relatively less creatine kinase was present in the dystrophic extracts. However, the turnover pattern of the individually labelled proteins showed a number of differences. Reliable turnover information could only be obtained for the three major peaks resolved by electrophoresis; peaks 1–4 (containing several dehydrogenases), peak 5 (creatine kinase) and peak 8 (myokinase). The turnover of the dehydrogenase group gave much the same pattern in normal and dystrophic animals although the extent of incorporation was generally greater in the latter and the final plateau value was twice that of the normal value. If this plateau value represents reutilization of labelled amino acids released by the degradation of other proteins this is a very efficient process with the dystrophic dehydrogenases and is in sharp contrast with the kinases. With the two kinases the patterns were quite different. Whereas in the normal animals the relative specific radioactivity rose to an almost flat plateau (Figs. 8*b* and 8*c*) that in the dystrophic animals rose more rapidly to two to three times the specific radioactivity before, equally rapidly, decaying to less than the normal plateau value. It has been reported that loss of creatine kinase into the bloodstream is not a significant feature of mouse dystrophy (Nichol, 1964) making these findings even more significant.

Muscular dystrophy in the mouse follows simple Mendelian inheritance as an autosomal recessive. The occurrence of at least two proteins in dystrophic muscle with abnormal turnover patterns formally excludes the possibility of a single protein structural gene mutation giving rise to an unstable and readily degraded protein (Goldberg, 1972) as the cause of mouse dystrophy. The recent finding by Srivastava (1972) that myosin and tropomyosin, but not actin, also have abnormal patterns of amino acid uptake agrees with this view. We also find that the serum appearing in the muscle extracts is labelled at quite different rates and makes a significant contribution to the overall incorporation pattern of the muscle. However, the serum proteins have their origin in the liver which appears normal on the basis of its overall pattern of amino acid turnover (Simon *et al.*, 1962). The differences observed here could result from a less efficient blood transport system.

Previous investigations here failed to locate any abnormality in the protein synthesizing system that could explain the unusually rapid degradation of selected proteins (Watts & Reid, 1969) although some sort of transcriptional abnormality, perhaps comparable with the thallassaemias, remains a possibility. Alternatively, an explanation may be sought in terms of the adventitious binding of a deleterious molecule or oxidative destabilization of the proteins. Evidence for the latter has already been obtained in that a decrease has been reported in the concentration of free non-protein thiols (Nichol, 1964; Hooton & Watts, 1966*a*) and in the concentration of reactive thiols of the soluble muscle proteins (Hooton & Watts, 1966*a*). Modification of the essential thiol groups of dystrophic mouse creatine kinase (Hooton & Watts, 1966*b*) has also been reported and from the present results the possibility that a mutation away from cysteine in a structural gene for this enzyme might be the cause of the dystrophy can now be excluded. However, the myokinase from muscles of humans with Duchenne dystrophy has also been found to respond abnormally towards thiol reagents (Schirmer & Thuma, 1962) suggesting a possible biochemical connexion between the two conditions. Clearly this aspect of the disease deserves further investigation.

We are grateful to Mrs. R. L. Watts and Miss J. Eve for maintaining the colony of Bar Harbour mice. We are also indebted to the Medical Research Council for providing the scintillation spectrometer and the Chromoscan densitometer used in this work, and to the Muscular Dystrophy Group of Great Britain for their continued support and for providing personal grants for S. E. K. and J. E.

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