Proteins in the urine associated with Duchenne muscular dystrophy and other neuromuscular diseases

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Summary

1. Up to 200 protein staining spots could be detected on two-dimensional electrophoresis of urine from healthy persons. Other minor spots were occasionally present.

2. Although the electropherograms exhibited constant characteristic features some variation in protein pattern was observed between individuals and with a given individual at different times.

3. Two additional proteins, spots C and D, were consistently present in urine from boys with Duchenne muscular dystrophy. Spot C was also present in the urine of about 60% of obligatory carriers of this dystrophy.

4. The protein responsible for spot C had a molecular weight of 26000 and an isoelectric point of 5.3.

5. Spot C was also detected in the urine of patients with other neuromuscular conditions. Neither spot C nor spot D could be detected in the urine of patients with physical disabilities other than those of neuromuscular origin.

6. It is concluded that the urinary excretion of spot C, and probably of spot D, is a consequence of muscle damage and that their detection has potential as a diagnostic tool.

Key words: Duchenne muscular dystrophy, human urinary protein, spot C, two-dimensional electrophoresis.

Introduction

The detection of abnormal amounts of protein in urine has long been common clinical practice as an index of disease. Owing to analytical difficulties less attention has been paid to the composition of the protein fraction that is normally present in human urine (about 200 mg/day) for this is a complex mixture and most components are present in very small amounts.

The composition of the complex mixture of proteins that is excreted daily in the urine reflects the nature of the biochemical and physiological processes occurring in the organism and if it can be analysed in detail it could provide information of potential diagnostic importance. Methods exist for determining individually the amount of a number of proteins normally found in urine [1], but high resolution two-dimensional electrophoresis [2] offers many advantages for the analysis of the whole complex mixture in one operation. Anderson & Anderson [3, 4] have developed this technique to carry out the simultaneous and reproducible analysis of large numbers of samples. By this means urinary proteins have been separated into several hundred components [5], some of which have been identified as derived from plasma, kidney, urinary tract and other normal or diseased tissues [6].

As part of a wider study of the protein composition of body fluids and tissues in patients with neuromuscular disease, we have investigated the proteins of urine using two-dimensional electrophoresis. Particular attention has been paid to boys suffering from Duchenne muscular dystrophy, an X-linked recessive myopathy characterized by progressive and severe muscular weakness leading to death often in the second decade. The urinary proteins of carriers of Duchenne muscular dystrophy have also been examined because a significant proportion of them exhibit some manifestation of the affected
gene; for example, elevated serum levels of muscle enzymes \[7, 8\] and abnormalities in sections of muscle-biopsy samples studied by histochemical procedures \[9\]. Evidence is presented for the presence of small amounts of proteins (spots C and D) in the urine of all boys with Duchenne muscular dystrophy examined. These proteins were present in the urine of some of the carriers of Duchenne muscular dystrophy and in patients with other myopathies. The proteins were not usually detected in the urine of normal individuals and it is considered likely that their presence in urine is a consequence of muscle damage.

Materials and methods

Urine samples

Samples were collected from healthy individuals, from patients with confirmed diagnosis of muscle disease and from their relatives. Samples were not taken from incontinent catheterized patients.

(a) Normal healthy males. See the Results section for details.

(b) Normal adult females. This group included women aged 18–55 years (mean 27.5 years), some married, with and without children, and some regularly taking oral contraceptives. No specific stage of the menstrual cycle was chosen for sampling.

(c) Pregnant females. Samples were obtained from normally healthy women at different stages of pregnancy.

(d) Normal boys. This group included boys aged 6–18 years (mean 9.8 years). All except one, being treated for hay fever, were in good health.

(e) Males with physical disabilities. This group consisted of boys from local schools for the disabled and young adults from a local college. The subjects (aged 6–39, mean 18.6 years) were chosen with disabilities that were not primarily of muscular origin.

(f) Patients with neuromuscular diseases. Urine samples from patients with myopathies were obtained from a number of special schools for disabled boys in the Birmingham area and from individuals who volunteered samples. All diagnoses of Duchenne muscular dystrophy in the West Midlands area were confirmed by Dr S. Bundey, Department of Clinical Genetics, Queen Elizabeth Medical Centre, Birmingham, U.K. The diagnoses of the clinical states of residents at Southwood House, Northampton, U.K., were as given in medical records.

(g) Potential heterozygotes of Duchenne muscular dystrophy. Samples from mothers and sisters of boys with Duchenne muscular dystrophy were obtained by contacts through the special schools in Birmingham attended by their sons or by courtesy of Dr Bundey in the West Midlands area and Dr D. Gardner-Medwin, Regional Neurological Centre, Newcastle General Hospital in the Newcastle upon Tyne area (U.K.). Carrier status was confirmed by Dr Bundey and Dr Gardner-Medwin. Where levels of serum creatine kinase are mentioned in the text, these values were made available by Dr Bundey from her records.

Urine sample collection

Samples that were processed and analysed in the absence of added preservative frequently showed evidence of degradation in that many protein spots on the electropherograms were missing or modified. For this reason, thymol was included in the sample at collection and in subsequent dialysis.

Early morning mid-stream urine samples were delivered into plastic bottles (300 ml) containing 0.5 ml of 10% thymol (w/v in propan-2-ol) and, where possible, immediately chilled or frozen and stored until collection. The presence of thymol caused some protein precipitation, particularly in smaller samples where the thymol concentration was high. This precipitate was not discarded as the whole sample was processed for electrophoresis. Samples were usually collected within 3 h of delivery and processed on arrival at the laboratory. If samples could not be processed immediately they were frozen and stored below −30°C until use.

Urine sample preparations for electrophoresis

After the volume (typically 50–250 ml) was measured the sample was dialysed overnight against 2 litres of water containing 1 ml of 10% thymol (w/v in propan-2-ol) at 4°C. All dialyses were carried out against the latter solution. The dialysed urine was freeze-dried, dissolved in 10 ml of water and redialysed for 24 h against two changes of 2 litres of water. The sample was again freeze-dried, redissolved in 2 ml of water and redialysed overnight. This procedure was found to be essential for complete removal of salts and other low-molecular-weight substances. Dialysis was carried out by the tall-cylinder method described by Anderson, Anderson & Tollaksen [10]. After the final dialysis the urine was freeze-dried and stored below −30°C as a dry powder until use.
Electrophoresis

Two-dimensional electrophoresis was performed in the IsoDalt apparatus [3, 4] under conditions similar to those described by these authors. The composition of the isoelectric focusing gels (14 cm x 0.16 cm diam.) was 4% (w/v) acrylamide, 0-24% N,N'-methylene bisacrylamide, urea (9 mol/l), 0-66% Nonidet P-40 (BDH Chemicals Ltd, Poole, Dorset, U.K.), 2% (w/v) pH 3.5-10 Ampholines and 0.8% pH 9-11 Ampholines (LKB, Croydon, Surrey, U.K.). The whole freeze-dried urine protein sample was dissolved in water (0.2% of the original urine sample volume), 30 µl applied to the gel and focused for 16-18 h at 400 V, then for 1.5-2 h at 740 V.

The first-dimension gel was equilibrated with Tris/HCl (125 mmol/l, pH 8.6) containing 4% (w/v) sodium dodecyl sulphate (SDS), 10% (v/v) glycerol and 0.5% 2-mercaptoethanol for 20 min before applying it to the second-dimension gel (15 cm x 16 cm x 0.2 cm) which consisted of a linear gradient of 10-20% (w/v) polyacrylamide in Tris/HCl (375 mmol/l, pH 8.6) containing 0.1% SDS. The gel was overlaid with 0.7% agarose in Tris/HCl (125 mmol/l, pH 6.8) containing 1% (w/v) SDS. The running buffer was Tris (25 mmol/l) containing glycine (0.2 mol/l) and 0.1% SDS.

Samples of serum were prepared for electrophoresis by mixing 1 vol. with 3 vol. of urea (6 mol/l) containing 0.6% SDS and 1.6% (v/v) 2-mercaptopethanol. The diluted serum (22.5 µl) was applied to isoelectric focusing gels with a composition similar to that described for the electrophoresis of urine proteins, except that the pH 9.11 Ampholines were omitted and the Nonidet P-40 concentration was increased to 2%.

Results

Adult male urine

To minimize possible variations in the appearance of the two-dimensional gels of the urinary proteins due to artifacts arising from the collection and handling of samples, the procedure described in the Materials and methods section was adhered to as rigorously as possible in all cases. As a basis for comparison the electrophotographic pattern of the urinary proteins of male laboratory workers of sedentary habit, and aged between 20 and 35 years (mean 28.2 years) to minimize possible effects of age and sustained physical effort, was studied in detail.

The electropherogram shown in Fig. 1 is a typical example of the pattern of proteins in the urine of this group of 23 males. The major components are the Tamm–Horsfall protein and serum albumin which together constitute more than 90% of the local protein of urine. Within this group of males, the urinary proteins from different individuals varied in the number and relative proportions of components present in a given sample. Nevertheless, careful examination showed that many of the spots were in fact present in most samples.

Fig. 2 is a diagram that indicates the spots which were consistently observed in two-dimensional electropherograms of male urine (Fig. 1). Most of the spots indicated were present in most of the samples obtained from this group of males, but all of them could not be detected in all samples. In a given individual there was variation in the relative amounts of some components with time. This is shown in Fig. 3 which illustrates the bottom right-hand quarter of electropherograms of samples taken from one individual on two different occasions in a single day. This region contains proteins with isoelectric points above pH 6.5 and molecular weights below 35 000. In any given sample minor spots, in addition to those mapped in Fig. 1, were also visible. As they were not present in most samples they have not been included in Fig. 1.

Two other factors affected the distribution of spots on the electropherograms. Urinary protein samples prepared as described in the Materials and methods section contain some component(s) that affects the pH gradient in the isoelectric focusing step in two ways. The first of these is a shift of the pH gradient towards the basic end. The extent of this shift differed with different samples and also depended on loading; as increasing amounts of a given sample were electrofocused there was an increasing shift of the whole pattern to the negatively charged electrode.

Staining and photography

Gels were stained overnight in a rocking bath containing 0.15% Coomassie brilliant blue R-250 (Serva Blau R, Serva, Heidelberg, Germany) in 50% (v/v) ethanol/12.5% (v/v) acetic acid (100 ml per gel) and destained by diffusion into repeated changes of 20% (v/v) ethanol/10% (v/v) acetic acid. Surface deposits of stain were removed by brief immersion in acetone.

Destained gels were washed in water for about 30 min before photography. Gels were photographed with a 535 nm interference filter and Ilford Technical Ortho Film, with exposures giving maximum contrast to fainter spots (i.e. the centres of the more densely stained spots were at the minimum of the Hurter–Driffield curve).
FIG. 1. Electropherogram of the proteins in the urine of a 28-year-old male. An early morning midstream sample of urine was prepared and examined by two-dimensional electrophoresis as described in the Materials and methods section.

This may be caused by the presence of the very large amount of the Tamm–Horsfall protein which, as a large acidic glycoprotein, would be expected to bind basic ampholytes and thus effectively remove the basic end of the pH gradient. Inclusion of isoelectric focusing stan-

FIG. 2. Diagram illustrating the position of the protein spots commonly observed on two-dimensional electrophoresis of normal adult male urine. The boxed area is the region of the electropherogram in which spots C and D (see the text and Fig. 5) were located. The vertical arrows indicate the variable part of the pH gradient referred to in the text and two protein spots have been numbered I and II as markers to help in the orientation of Figs. 3 and 5. The horizontal arrows indicate the regions in which the spots due to creatine kinase are located.

FIG. 3. Variation in the electrophoretic pattern of proteins in the urine of a given individual. The Figures are the bottom right-hand quarters of electropherograms of the proteins present in urine samples obtained from a given individual at (a) 08.30 hours and (b) 18.00 hours on the same day. Spots marked I and II correspond to those similarly marked in Fig. 2. Arrows indicate the positions of spots due to proteins, the amounts of which vary markedly.
Urinary proteins and Duchenne muscular dystrophy

These proteins (charge-modified train of carbonic anhydrase spots as described by Anderson & Hickman [11]) showed the gradient was moved as a whole relative to the gel matrix, with little effect on the relative position of the spots.

The second of these anomalies was a discontinuity in the pH gradient possibly due to deficiencies in the Ampholine mixture (shown arrowed in Fig. 1). This region of the two-dimensional electropherogram usually contained little protein, but with increasing amounts of sample it became wider without apparently affecting the relative spacing of spots on either side.

**Adult female urine**

Electropherograms of female urinary protein gave much more variable patterns than did samples from males. In many cases the pattern was essentially similar to that illustrated in the map of male urinary protein (Fig. 2) with a few additional minor components. These additional components were acidic proteins with molecular weights below 40 000.

Occasionally samples showed major differences from the normal patterns in that the majority of the proteins identified in Fig. 1 were absent or very much reduced in amount. In these cases the pattern was dominated by small acidic proteins that increased in number with decreasing molecular weight. These proteins (collectively designated ‘female proteins’) have been shown to be the dominant feature of female urinary protein throughout a period of several days between episodes of menstrual bleeding and during weeks 14–30 of pregnancy (R. D. Taylor & N. Frearson, unpublished work) and are illustrated in Fig. 4. The pattern of these ‘female proteins’ observed during pregnancy was consistent from one individual to another, as was the pattern obtained during the midpoint of the menstrual cycle. Although the changes occurring in the urinary proteins during pregnancy and in the midpoint of the menstrual cycle were similar, in that they are both characterized by a decrease in the usual urinary protein components and by an increase in the small acidic ‘female proteins’, the two patterns were distinctly different.

A third difference was noted in samples from women with urinary tract infections. The electropherograms of these urine samples exhibited a large number of additional low-molecular-weight proteins in both the acidic and the basic half of the pattern.

**Duchenne muscular dystrophy**

Electropherograms of the urine of boys with Duchenne muscular dystrophy exhibited three features that appeared to be associated with the disease. These features designated streak A, spot C and spot D were therefore further investigated.

**Streak A.** The material responsible for streak A had a molecular weight similar to that of transferrin (77 000) and streaked across the part of the gradient ranging from pH 7 to 9. Although streak A was initially seen in urine samples from obligatory carriers of Duchenne muscular dystrophy and from boys with the disease, the study of a larger number of samples showed it to be present in some control samples of urine. This was often the case when electropherograms were overloaded. Studies with isolated transferrin showed that its position on electropherograms was influenced by other components of urine, causing it to appear at different regions of the gradient between pH 7 and the basic end of the gel.

For these reasons, and because the detection of a streak on the electropherograms is more subjective than is that of a clearly defined spot, this observation has not been pursued further as it was considered to be of limited value for diagnostic purposes.

**Spot C.** A clear discrete spot corresponding to a molecular weight of 26 000 and an isoelectric point of 5.3 was seen in every urine sample taken from boys with Duchenne muscular dystrophy (37 electrophoretic analyses involving 28 patients). The position of spot C is shown in Fig.
5. Apart from the two major proteins, serum albumin and the Tamm-Horsfall protein, spot C was one of the more intensely staining features seen in electropherograms of samples from affected boys. When urine samples were obtained from a single boy for 7 successive days, spot C was present in all samples although the relative amount of this and other proteins varied as far as could be judged from the intensity of staining.

Although spot C was clearly consistently present in all samples of urine from boys with Duchenne muscular dystrophy, this was not the case with urine from their mothers (Table 1). When spot C was present, its intensity was less in electropherograms of the mother's urine than in those of their sons. Amounts were more variable in the mother's urine and in several cases spot C was present in a sample from a given carrier on one day, but not on another.

Spot C was present in the urine of some of the sisters of boys with Duchenne muscular dystrophy. All of the sisters studied in the group were daughters of obligatory carriers and ranged in age from 11 to 25 years (mean 17.1 years). Spot C was detected in the urine of five of the nine sisters studied in this group.

Although there were two affected boys in family A, the mother had a lower than normal level of serum creatine kinase and it was not possible to detect spot C in three urine samples from this woman. Spot C was detected in urine samples from two of the three daughters, all of whom had moderately elevated serum creatine kinase levels.

In family B, also with affected boys, the mother had an elevated serum creatine kinase level when tested in 1974, but not when measured in 1977. Spot C was clearly present in one urine sample...
Urinary proteins and Duchenne muscular dystrophy

TABLE 1. Occurrence of abnormal proteins in the urine of patients with muscular dystrophies

Numbers of patients are shown in parentheses.

<table>
<thead>
<tr>
<th>Abnormal proteins present</th>
<th>Spot C (no.)</th>
<th>Spot D (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult females (32)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Pregnant females (24)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adult males (23)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Boys (19)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Duchenne muscular dystrophy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boys (28)</td>
<td>28</td>
<td>24</td>
</tr>
<tr>
<td>Mothers: Obligatory carriers (21)</td>
<td>12</td>
<td>3–4</td>
</tr>
<tr>
<td>Isolated cases (8)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Sisters (10)</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Disabled males* (14)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Other myopathies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conditions: Limb girdle (2)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Werdnig–Hoffman (3)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Spinal muscular atrophy (2)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Charcot–Marie–Tooth (3)</td>
<td>2–3</td>
<td>0</td>
</tr>
</tbody>
</table>

*These included spina bifida (3), cerebral palsy (6, one with epilepsy), a congenital deformity, osteogenesis imperfecta, Friedrich's ataxia, an unspecified neurological disorder and one with spasticity after meningitis.

from this woman and barely detectable in another. One of the two daughters had both an elevated serum creatine kinase level and spot C in a urine sample, whereas the other with a near normal creatine kinase level had no detectable spot C.

Spot C was detected in the urine of a pregnant, sister of an affected boy. This woman had two normal healthy sons although she had an elevated serum creatine kinase level. Although pregnancy can dramatically affect the electrophoretic pattern of urinary protein, in this case the pattern of the urinary proteins was essentially similar to Fig. 1.

Spot D. Over 80% of the samples obtained from boys with Duchenne muscular dystrophy contained, in addition to spot C, a component of molecular weight 29 000 and an isoelectric point of 5.8. This spot, designated spot D (position indicated in Fig. 5b) was much fainter than spot C. Spot D was faintly discernible in the urine of some of the mothers and sisters of affected boys that exhibited spot C.

Other myopathies

Urine samples from patients with a number of other diseases involving the musculature other than Duchenne muscular dystrophy were also examined. The results obtained are summarized in Table 1. The three cases of Charcot–Marie–Tooth disease were males in three successive generations of a single family. Spot C was clearly present in the two older cases but was not unequivocally detected in the youngest, even though the latter was the most severely affected. Neither spot C nor spot D was detected in samples obtained from the brothers and sisters of the two younger cases.

Several samples were obtained from patients whose condition was diagnosed to be within the general classification of spinal muscular atrophy. These included three patients diagnosed as arrested cases of Werdnig–Hoffman disease (aged 14, 20 and 21 years) in two of which spot C was detected, but not spot D. In the urine of two other less specifically diagnosed cases of spinal muscular atrophy (aged 11 and 19 years), spot C, but not spot D, was detected.

Two samples of urine were obtained from patients with limb girdle muscular dystrophy and a single sample from a case of myotonic muscular dystrophy. All three contained spot C. A 6-year-old boy with an unspecified neurogenic myopathy showed neither spot C nor spot D. Of patients disabled by conditions where neuromuscular disease was not the primary cause, only the one with cerebral palsy and epilepsy showed spot C. None showed spot D.

Creatine kinase

The position to which creatine kinase migrated (see Fig. 2) was located by electrophoresing a standard sample of urine protein to which 1 μg of pure canine muscle creatine kinase (Boehringer-Mannheim GbH, Mannheim, West Germany) had been added and by comparison with the known position of human creatine kinase on two-dimensional electrophoresis of extracts of human muscle made in urea (10 mol/l) ([12]; N. Frearson & R. D. Taylor, unpublished work).

Spots corresponding to creatine kinase could not be detected on electropherograms of urine samples from either control groups or patients with neuromuscular diseases. They could, however, be detected in two-dimensional electropherograms of serum from patients with elevated serum creatine kinase. At loadings of serum adequate to show the presence of creatine kinase no spot corresponding to spot C could be detected on the electropherogram, although it could be detected in the urine of the patient.
Discussion

In normal urine samples usually 100–200 components could be clearly resolved by two-dimensional electrophoresis and many of these proteins were present in most urine samples most of the time. The positions on the electropherograms are illustrated on the map (Fig. 2), which is in close agreement with that published by Anderson et al. [6]. The relative amounts of individual proteins varied greatly from one person to another and from time to time. Components other than those mapped appeared in individual urine samples, particularly during illness. Many of these additional spots were not consistently present and cannot therefore be designated as either 'normal' or as characteristic of any particular physiological status. For these reasons our analyses of urinary proteins included the study of diverse control groups to confirm that any unusual components seen in the affected individuals were consistent features.

The protein components responsible for spots C and D were consistently found in the urine of boys with Duchenne muscular dystrophy. Other minor unusual spots were seen in some of these samples, but only spots C and D were present in all samples from all of the affected boys studied. Neither of the latter two spots were normally present in urine samples from healthy boys of similar age range nor from boys who were disabled by conditions other than neuromuscular diseases.

The connection between muscle disease and spot C was reinforced by the finding that this component was frequently detected in the urine of both mothers and sisters of affected boys, whereas it was not usually present in urine from normal healthy women. In general only half of the mothers of affected boys are carriers [13], the remaining affected boys not accounted for being new mutations. If spot C is an indirect consequence of the abnormal gene in these cases, then samples from approximately half the mothers of affected boys, a fraction which would include all the obligatory carriers, would be expected to show spot C. This was not the case for, although about half of the mothers did show spot C, it was detectable only in 12 out of the 21 obligatory carriers.

The detection of spot C in urine is a potentially useful additional method for carrier diagnosis, since only about 8% of obligatory carriers of Duchenne muscular dystrophy show significant weakness of damage [14] whereas about 80% have elevated levels of serum creatine kinase. Detection of spot C in the urine of high-risk pregnancy cases may be of particular importance.

A decline in serum creatine kinase occurs during early pregnancy [15] and therefore casts some doubt on the usefulness of this test in diagnosis of carriers. Here detection of spot C could be of particular value in supporting diagnosis of carrier status, since spot C was not detected in any of the samples from normally healthy pregnant women.

Some caution must be exercised in drawing conclusions as to the significance of spot C. Firstly, spot C was detected in urine in cases of other muscle diseases (see Table 1) and is not therefore specific to the genetic lesion responsible for Duchenne muscular dystrophy. It is considered that spot C appears in urine as a consequence of damage to the muscle arising from muscle disease. Secondly, spot C was detected in a few of the urine samples from control groups. The presence of spot C in the urine sample from the patient with cerebral palsy and epilepsy could easily be accounted for by muscle damage: medical records showed that this patient had frequent falls and was therefore liable to suffer minor muscle damage.

The relative amount of spot C present in the urine samples, estimated by inspection of the stained electropherograms, is entirely consistent with this explanation. The most disabled of the patients with muscle disease were the boys with Duchenne muscular dystrophy and spot C was more intense in this group than in any other. The cases of limb-girdle muscular dystrophy and myotonic dystrophy were less severely affected and in these patients the amount of spot C was lower. The two cases of Charcot–Marie–Tooth syndrome were even less severely affected and here spot C was much less intense than was the case for Duchenne muscular dystrophy. Similarly the obligatory carriers of Duchenne muscular dystrophy showed amounts of spot C ranging from about half the amount present in the boys' urine to the lowest limit detectable. This would be expected of heterozygotes in whom only a proportion of cells would express an abnormal gene partly because of random X-chromosome inactivation [16] and partly because of the other regulatory mechanisms [17].

The presence of spot C in two healthy women and one healthy boy is less easily explained. Recent results, however, suggest that strenuous physical activity (e.g. weight-lifting) may result in the subsequent excretion of spot C (N. Frearson & R. D. Taylor, unpublished work). It is possible that all physical exertion above the normal level leads to the excretion of this protein, but usually in amounts below the level of detection of the method used here. In the few controls where spot C was detected the amount was very small.
Urinary proteins and Duchenne muscular dystrophy

compared with that present in the urine of the boys with Duchenne muscular dystrophy and therefore consistent with the suggestion that its presence was transient and was caused by the kind of minor muscle damage that might be expected from vigorous exercise or falls.

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References