Comparison of the Protein-Synthesizing Machinery in the Skeletal Muscle of Normal and Dystrophic Bar Harbor Mice

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1. Although the total weight of leg muscle increased with the age of a normal mouse the DNA and RNA content per leg did not change significantly. 2. The weight of leg muscle from a dystrophic mouse was only about 45% of that from a normal mouse but the DNA and RNA contents were the same and hence similar DNA/RNA ratios were obtained. 3. The total ribosome contents of normal and dystrophic mice were the same on a whole-leg basis, and for both the free ribosomes were about 60% of the total. However, comparison with similar data from liver suggested that some loss of ribosomes occurred during the isolation procedure. 4. The polyribosome patterns obtained by density-gradient centrifugation were the same for normal and dystrophic muscle, and comparable polyribosome fractions of different sizes obtained from such gradients had similar capacities for the incorporation of radioactive amino acids in a standard protein-synthesizing system. 5. By using a standard protein-synthesizing system with normal polyribosomes similar extents of incorporation were found with normal- or dystrophic-muscle pH 5 fraction or partially purified transfer RNA preparation. 6. It is concluded that there is no absolute difference between the protein-synthesizing systems of normal and dystrophic mouse muscle and that the observed apparent differences result from concentration differences caused by changes in muscle volume. 7. A possible cause of the failure of dystrophic muscle to resynthesize myofibrils is also suggested.

A major problem in elucidating the causes of muscular dystrophies is distinguishing between primary and secondary effects. Numerous changes in dystrophic muscle have been reported that seem to implicate the protein-synthesizing machinery as a possible primary cause. For example, in mice dystrophy is accompanied by a pronounced increase in the muscle content of RNA and DNA (Coleman & Ashworth, 1959; Girkin, Fitch & Dinning, 1962; Krupnick, Casa & Rosenkrantz, 1964; Srivastava, 1968) and an elevated rate of synthesis and turnover of proteins and nucleic acids (Simon, Lessell, Gross & Milhorat, 1958; Coleman & Ashworth, 1959; Kruh, Dreyfus, Schapira & Gey, 1960; Srivastava, 1968). However, these conclusions were derived from data expressed on the basis of g. wet weight or soluble non-collagen nitrogen, both of which demonstrably changed in the course of the disease (Nichol, 1964; Srivastava & Berlinguet, 1964; Berlinguet & Srivastava, 1966; Srivastava, 1968). The possibility that many of the observed effects could result from a decrease in the volume of the dystrophic muscle appears not to have been considered. West & Murphy (1960), using histological methods, thought that there was an increase in the

number of nuclei in dystrophic mouse muscle, but commented on the absence of mitotic figures to support this view.

Apart from an investigation of the amino acid-activating enzymes by Pennington (1960) a detailed comparison of the protein-synthesizing components of normal and dystrophic muscle has not been made. This is perhaps partly because of the inherent difficulties in working with muscle and the small size of the samples available from dystrophic mice. Valid comparisons are rendered more difficult by the greatly increased ribonuclease activity, that occurs in dystrophic muscle.

In the work presented here most of these problems have been overcome, in particular by scaling down the techniques used for large muscle samples (Florini & Breuer, 1966), by inhibiting the endogenous tissue ribonuclease with bentonite (Watts & Mathias, 1967) and by using a postmicrosomal supernatant as a resuspending medium (Blobel & Potter, 1967*a*). The absolute changes in the various components of the protein-synthesizing machinery were determined by reference to the content of a whole limb in addition to the more traditional methods.

MATERIALS AND METHODS

Materials

L-[U-¹⁴C]Lysine, DL-[1-¹⁴C]leucine and U-¹⁴C-labelled protein hydrolysate were obtained from The Radiochemical Centre, Amersham, Bucks.

MgCl₂, KCl, sucrose (A.R. grade), sodium deoxycholate, tris (A.R. grade) and bentonite (technical grade) were obtained from British Drug Houses Ltd., Poole, Dorset. All chemicals were recrystallized from 1mM-EDTA solution and distilled water. The magnesium content of the resultant hydrated MgCl₂ was determined by using an EDTA compleximetric titration method. Bentonite was prepared as a graded suspension by the method of Watts & Mathias (1967) and stored as a stiff paste. Lubrol W was obtained from Imperial Chemical Industries Ltd., London, S.W.1. All sucrose solutions used were treated with graded bentonite at a final concentration of between 0.5 and 1.0 mg./ml., the bentonite being removed by centrifugation.

Coenzymes and biochemicals were obtained from the following suppliers: ATP and creatine phosphate from Sigma (London) Chemical Co. Ltd., London S.W.6; GSH from British Drug Houses Ltd.; GTP (sodium salt) from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Creatine phosphokinase was prepared by method B of Kuby, Noda & Lardy (1954).

Mice, strain 129, obtained from Bar Harbor, Maine, U.S.A., carrying the autosomally inherited recessive dystrophy gene (*dy*) were used to establish a colony. Normal mice (*DyDy*) were obtained by breeding from families that had shown no expression of the dystrophic gene over many generations.

Methods

Preparation of mouse muscle polyribosomes. This was based on the procedure of Florini & Breuer (1966). Preparations were always made simultaneously from normal and dystrophic animals of the same age. This varied between 8 and 16 weeks.

Muscle extraction. Mice were killed by cervical dislocation and the hind-leg muscle was removed and rapidly frozen by compression between two metal blocks covered with polythene film at -20°. For pooled muscle samples each mouse was processed separately and the pooled muscle stored in a closed container at -20° in which it was weighed before homogenization.

Homogenization. The frozen muscle was shredded into homogenizing medium (2 ml./g. of muscle) of composition 0.25M-sucrose-10mM-MgCl₂-80mM-KCl-10mM-GSH-50mM-tris-HCl buffer, pH 7.8 at 4°, contained in the glass pot of a Potter-type homogenizer (2 cm. diameter, operated at about 500 r.p.m.), and graded bentonite (0.125 g./10 ml. of homogenizing medium) was added. Homogenization was conducted in two stages, first with a loose-fitting Teflon pestle (clearance 0.025 in.), the second a finer homogenization with a tighter-fitting pestle (clearance 0.005 in.). At each stage the pestle was passed up and down three times with minimum pressure.

Centrifugation procedure. All centrifugation was carried out at 4°, low speeds in an MSE High-Speed 18 and high speeds in a Christ Omega II ultracentrifuge. The homogenate was transferred to an ice-cold 15 ml. polypropylene centrifuge tube and centrifuged sequentially at 400g for

2 min., 1500g for 2 min. and 13000g for 14 min. The step-wise centrifugation minimizes sedimentation of supernatant RNA along with bentonite (Murthy & Rappoport, 1965).

The post-13000g supernatant was filtered through acid-washed glass wool and then centrifuged for 2 hr. at 78000g to obtain the microsomal pellet. The post-78000g supernatant was removed and after centrifugation at 100000g for 1 hr. was used for preparation of pH 5 fraction and, where needed, for resuspension of microsomes.

Microsomes corresponding to not more than 10g. of muscle were gently resuspended in 3.25 ml. of post-100000g supernatant plus 1 ml. of medium of composition 2.5M-KCl-10mM-MgCl₂-50mM-tris-HCl buffer, pH 7.8 at 4°, in a loosely fitting all-glass homogenizer (approx. 0.025 in. clearance). In some early experiments liver postmicrosomal supernatant, known to contain ribonuclease inhibition (Blobel & Potter, 1967a) was used. Subsequently, the post-microsomal supernatant from normal muscle was found to be equally effective. To the milky suspension in the homogenizer 0.25 ml. of 10% (w/v) Lubrol W was added and mixed by gentle homogenization, and then 0.5 ml. of 10% (w/v) sodium deoxycholate was added and mixed in the same way.

The detergent-treated microsome suspension was spun at 8000g for 10 min. and the supernatant, layered over 7 ml. of 1.0M-sucrose with the same ionic composition as the homogenizing medium, was centrifuged at 105000g for 2 hr. in the angle head.

The supernatants were removed, care being taken not to contaminate the ribosome pellet with the upper layer of solution. The pellet was transferred on the tip of a thin glass spatula to a clean centrifuge tube and gently resuspended in the same medium by using a glass rod, and the ribosomes were solubilized by placing the capped tube on a slowly rotating turntable overnight at 4°. The suspension was then centrifuged at 3000g to sediment material that had not dissolved. The supernatant was removed and used for all subsequent analyses. The RNA content of the polyribosome suspension was determined from the extinction at 260 nm. by using an extinction coefficient of 20 cm.²/mg. The E_{260}/E_{280} ratio for the polyribosome suspension was in the range 1.75-1.85 for all preparations.

The particle size distribution of the ribosome preparations was determined by sucrose-density-gradient centrifugation in 5 ml. linear 15-30% (w/v) sucrose gradients in 10mM-MgCl₂-80mM-KCl-50mM-tris-HCl buffer, pH 7.8 at 4°. The ribosome suspension was layered on to the top of the gradient and centrifuged at 35000 rev./min. for 1 hr. in the 5 ml. swing-out head. The contents of the tube were analysed by extrusion through a 0.2 mm.-path-length flow cell fitted at the second sample position of a Unicam SP.800 spectrophotometer (Reid & Watts, 1968). Fractions for incorporation experiments were collected to correspond with particular peaks of the extinction profile measured at 260 nm.

The concentration of sucrose in these fractions was determined, with an Abbé refractometer, and adjusted to 0.5M with a sucrose solution in 10mM-MgCl₂-80mM-KCl-50mM-tris-HCl buffer, pH 7.8 at 4°. For experiments with the total ribosome sample the sucrose concentration was raised to 0.5M in the same way.

Protein-synthesizing system. This differed slightly from the general procedure of Florini & Breuer (1965).

(a) Solution I. A pH5 fraction was prepared by the method of Decken & Campbell (1962). The pH5 fraction collected by centrifugation was washed by resuspension in homogenizing medium adjusted to pH 5.2, recentrifuged and resuspended in 0.5M-sucrose solution (ionic composition and pH as the homogenizing medium) to give a final protein concentration of 4mg./ml. and an added concentration of creatine phosphokinase of 200 μ g./ml. The final pH of this solution was also adjusted to give 7.8 at 4°.

(b) Solution II. This contained the following (final concentrations): ATP, 4mM; creatine phosphate, 80mM; GTP, 2.0mM; $MgCl_2$, 40mM; KCl, 0.32M; tris-HCl buffer, 0.2M; sucrose, 2.0M; 20 unlabelled amino acids, each 0.1 μ M; DL-[^{14}C]leucine (0.875 μ C), L-[^{14}C]lysine (0.875 μ C) or protein hydrolysate (2.0 μ C); final pH 7.8 at 4°.

A 1:1 mixture of solution I and solution II equilibrated at 37° was made just before incubation, and 0.5ml. of this was added/0.5ml. of ribosome preparation to start the incubation, which was for 30 min. The reaction was stopped by addition of 5ml. of 10% (w/v) trichloroacetic acid containing approx. 1mg. each of unlabelled leucine and lysine/ml. The resultant precipitate was collected by centrifugation, washed three times with 5ml. of cold 5% (w/v) trichloroacetic acid, extracted with 5ml. of 5% trichloroacetic acid at 90°, washed with 5ml. of ethanol-ether (1:1, v/v) and extracted overnight with chloroform-methanol (3:2, v/v). The precipitates were redissolved in 0.3M-KOH and, after precipitation with 10ml. of 5% trichloroacetic acid, were collected on glass filter pads. The discs were twice washed with cold 5% trichloroacetic acid and then with ethanol-water (1:1, v/v), ethanol, ethanol-ether (1:1, v/v) and finally ether, before being dried at 105° for 30 min. The dry discs were placed in 1ml. of scintillation mixture consisting of 0.4% (w/v) 2,5-diphenyloxazole and 0.01% (w/v) 1,4-bis-(5-phenyloxazol-2-yl)benzene in dry sulphur-free toluene, and counted for 20 min. in a Beckman type CPM 200 liquid-scintillation counter.

Determination of RNA and DNA in normal and dystrophic muscle. Total RNA was determined with a modified Schmidt-Tannhauser procedure (Fleck & Munro, 1962) by using $E_{260}^{1\%}$ 312 at 260nm., from which 32 μ g./ml. is equivalent to 1.000 E_{260} units (Munro & Fleck, 1966). The total RNA was determined as soon as possible after the killing of the animals. Total DNA was determined by the method of Ceriotti (1952), with 2.5M-HCl used instead of conc. HCl (Keck, 1956) and with heating for 20 min. instead of 10 min., with calf thymus DNA as standard. For these

experiments muscle from the hind legs of each animal was removed and treated as described above.

Attempts were made to separate ribosomal RNA (free and bound) by using the method of Blobel & Potter (1967b) for liver. The total recovery of RNA was very low. Dystrophic muscle was compared with muscle from normal animals of the same age.

RESULTS AND DISCUSSION

RNA and DNA content of muscle. Since the research would inevitably involve experiments on and comparisons between animals of different weights and also since animals of the same age varied in weight, the effect of weight variation on the total RNA and DNA content of normal mice was first investigated. Table 1 shows results for groups of four mice chosen primarily for weight and only secondarily for age matching. The total weight of leg muscle increased with the weight of the animal, but the total RNA and DNA content did not alter. This meant that the RNA and DNA content would fall with weight if expressed on a wet-weight basis but remain constant if expressed on a 'per-leg' basis. Also the DNA/RNA ratio remained approximately constant, so that this could also be used as a meaningful way of expressing the results. Florini (1964), using rats, also observed that the DNA per leg was a very constant value irrespective of the age of the animal.

Table 2 compares the RNA and DNA contents of non-dystrophic and dystrophic litter-mates of the same age (12 weeks). The non-dystrophic animals were assumed to be normal although some of them might have been heterozygous (*Dydy*) since these are morphologically indistinguishable from the normal animals. Separate determinations were made on each of two groups of six animals. Although the weight of muscle obtained from the leg of a dystrophic was only about 45% of that obtained from a non-dystrophic mouse, both the DNA and RNA contents per leg were found to be similar and the same RNA/DNA ratio was obtained. However,

Table 1. *Effect of increasing body weight on the RNA and DNA contents of hind-leg muscle from normal male Bar Harbor mice*

In each weight group, four mice of about the same age and closely similar weight were used for analysis as described in the text.

	Group no. ...	1	2	3	4	5	6
Average body wt. (g.)		11	14	16	18	23	29.5
Total wt. of muscle from both legs of four mice (g.)		2.8	3.1	3.9	4.9	6.5	8.8
Total RNA from four mice (mg.)		6.45	6.35	5.5	7.0	6.25	6.05
Total DNA from four mice (mg.)		6.4	6.45	6.4	6.9	6.4	6.9
RNA (mg./g. wet wt. of muscle)		2.28	2.06	1.41	1.43	0.96	0.69
DNA (mg./g. wet wt. of muscle)		2.28	2.08	1.64	1.41	0.99	0.78
RNA (mg./leg)		0.81	0.79	0.69	0.88	0.78	0.76
DNA (mg./leg)		0.80	0.81	0.80	0.86	0.80	0.86
DNA/RNA ratio		1.0	1.0	0.82	1.0	0.98	0.88

Table 2. Comparison of the total RNA and DNA contents of leg muscle from non-dystrophic and dystrophic individual Bar Harbor mice of the same age

Experimental details are given in the text.

Animal no.	Total body wt. (g.)	Total wt. of muscle from both rear legs (g.)	Total RNA (mg.)	Total DNA (mg.)	RNA (mg./g. wet wt. of muscle)	DNA (mg./g. wet wt. of muscle)	RNA (mg./leg)	DNA (mg./leg)	RNA/DNA ratio
Non-dystrophic									
1	21.5	1.65	1.72	1.70	1.04	1.03	0.86	0.85	1.01
2	22.0	1.73	1.80	1.80	1.04	1.04	0.90	0.90	1.00
3	23.7	1.82	1.80	1.78	0.98	0.98	0.90	0.89	1.01
4	22.6	1.68	1.76	1.74	1.05	1.04	0.88	0.87	1.01
5	22.3	1.72	1.78	1.70	1.03	1.00	0.89	0.85	1.05
6	24.0	1.85	1.74	1.80	0.97	0.97	0.88	0.90	0.98
Mean			1.77	1.75	1.02	1.01	0.89	0.87	1.01
Dystrophic									
1	16.5	0.74	1.77	1.76	2.40	2.40	0.89	0.88	1.00
2	15.0	0.65	1.76	1.86	2.71	2.86	0.88	0.93	0.96
3	14.7	0.70	1.72	1.72	2.46	2.46	0.86	0.86	1.00
4	15.9	0.85	1.64	1.76	1.93	2.07	0.82	0.88	0.93
5	16.4	0.79	1.68	1.84	2.13	2.33	0.84	0.92	1.09
6	17.3	0.84	1.75	1.84	2.08	2.19	0.87	0.91	1.04
Mean			1.72	1.80	2.29	2.39	0.86	0.90	1.00

on the basis of g. wet wt. of muscle the values for the dystrophic animals were about twice those of the non-dystrophic animals, and this is in accord with previous findings, which have been expressed on a wet-weight basis (Coleman & Ashworth, 1959; Simon *et al.* 1958; Srivastava, 1968).

Attempts were made to characterize the RNA into ribosomal and soluble fractions and the ribosomal RNA into the free and membrane-bound fractions by using the method that Blobel & Potter (1967*b*) used for liver. Total ribosomes were found to be 0.16–0.17 $\mu\text{g.}/\mu\text{g.}$ of DNA in both normal and dystrophic muscle, and this represented about 20% of the total RNA. The free ribosomes were about 60% of the total ribosomes. Comparison with the values found for liver (Blobel & Potter, 1967*b*), in which total ribosomes represented 80% of the total RNA, indicates that our values are probably low if it is assumed that the ribosomal RNA/total RNA ratio is approximately the same in different tissues. A possible explanation of this is that the myofibrillar fraction of a muscle homogenate shows a great avidity for RNA (S. V. Perry, personal communication) and this seems to occur most specifically with the ribosomal fraction. An attempt to improve the yield by extraction in the presence of detergents [sodium deoxycholate (1.2%, w/v) plus Lubrol W (0.7%, w/v)] was unsuccessful.

Comparison of polyribosome patterns. From the above results it is clear that, if selective removal of polyribosomes of a particular size occurred, then this could result in a spurious polyribosome pattern,

Fig. 1. Comparison of the polyribosome patterns from dystrophic (a) and normal (b) mouse muscle. Each preparation was obtained from the whole muscle of both legs of one mouse treated as described in the Materials and Methods section. The line drawn immediately below that for each polyribosome pattern is the instrument baseline obtained by running an unloaded gradient through the flow cell. The highest peak is the dimer, and the peaks numbered 1–3 do not contain an integral number of ribosomes (see Fig. 2).

which might be different between normal and dystrophic muscle. It was also found that, because of the inevitable slight variation in some parts of the experimental procedure, no two preparations of polyribosomes from the same type of muscle were ever exactly alike. The latter problem was over-

come by simultaneously isolating polyribosomes from normal and dystrophic muscle so that any slight variation in treatment affected both preparations equally. This led to a partial solution of the first problem in that, by careful inspection of preparations showing various degrees of degradation, it was possible to judge whether selective removal of particular polyribosome peaks had occurred. We concluded that any removal that occurred was non-selective, although it was difficult to be sure about the very heavy polyribosome region.

Fig. 2. Plot of the relative sedimentation distance, $D^{3/2}$, of the polyribosome peaks obtained in Fig. 1(a) as a function of the assumed number of ribosomes in the polyribosome. The peaks 1, 2 and 3 of Fig. 1, indicated by ●, do not form part of the integral series and probably represent low-molecular-weight RNA, monoribosomes containing an extra half-ribosome and diribosomes containing an extra half-ribosome respectively. Fig. 1(b) gave an identical plot.

Once the problem of the high ribonuclease activity in dystrophic tissue had been overcome by the use of bentonite and the 100 000g supernatant (see the Materials and Methods section), which contains ribonuclease inhibitor, as a resuspending medium, the striking fact emerged that the polyribosome patterns of normal and dystrophic muscle were essentially indistinguishable. Fig. 1 shows the best preparation we obtained where the patterns are identical even with the series of subsidiary peaks that lie between the main peaks and probably correspond to polyribosomes carrying an additional half subunit. This latter conclusion is inferred from the plot of assumed number of ribosomes in the polyribosome as a function of the sedimentation distance (Fig. 2).

Comparison of protein-synthesizing ability. The ability of normal and dystrophic muscle polyribosomes to incorporate into protein either a mixture of [^{14}C]leucine and [^{14}C]lysine or ^{14}C -labelled amino acids from a protein hydrolysate was compared in the presence of normal pH 5 fraction and the standard incorporation mixture described in the Materials and Methods section. Polyribosomes were compared before fractionation on a density gradient and in fractions (from gradients similar to those shown in Fig. 1) containing about 1–2, 3–8 and 9+ ribosomes per polyribosome. Table 3 summarizes two such experiments. It was concluded that there was no difference between corresponding fractions from the two preparations. Other experiments showed that the use of shorter incubation times (10 or 20 min.) gave similar results.

Comparison of pH 5 fractions. The pH 5 fractions from normal and dystrophic mouse muscle were prepared as described in the Materials and Methods section and then each was further partially purified by passing the preparation, dissolved in homogenizing medium, down a column of Sephadex G-25

Table 3. *Protein-synthesizing ability of purified normal and dystrophic mouse muscle polyribosomes*

Each incubation contained normal pH 5 fraction and a complete mixture of amino acids including 0.9 μC each of [^{14}C]leucine and [^{14}C]lysine, together with an ATP-regenerating system and polyribosomes as indicated below. Details of the polyribosome fractions are given in the text. The incubation time was 30 min.

	Normal		Dystrophic	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Preparation of polyribosomes				
Ribosomal RNA (mg./g. of muscle)	0.150	0.168	0.270	0.320
Ribosomal RNA (mg./leg)	0.095	0.093	0.092	0.097
Amino acids incorporated into protein (c.p.m./mg. of ribosomal RNA)				
Total sample	3220	1130	2870	1190
Fraction 1	330	80	720	108
Fraction 2	2720	1200	2110	1140
Fraction 3	3440	1370	4450	1200

equilibrated with homogenizing medium. The first 25ml. of material with absorption at 260nm. was collected and concentrated to 1.5ml. by dialysis under reduced pressure against 3l. of homogenizing medium at 0°. The concentration of the purified pH5 fraction was adjusted to 1mg. of RNA/ml., and 0.1ml. of this used for each experiment in the standard incorporation mixture described in the Materials and Methods section, to which were added normal mouse polyribosomes equal to 1mg. of polyribosomal RNA. The standard incubation time of 30min. at 37° was used. No difference was found between the two preparations. For example, in one experiment the incorporations of labelled amino acids were 3080 and 3190 c.p.m./mg. of pH5 fraction RNA for the normal and dystrophic preparations respectively. Similar results were obtained when the unpurified pH5 fractions were used. These findings are in agreement with those of Pennington (1960) who, using the pyrophosphate-exchange method, was unable to find any marked differences between the amino acid-activating enzymes of normal and dystrophic mouse muscle.

CONCLUSIONS

The results presented here suggest that there is no significant difference in the properties of the components of the protein-synthesizing systems from normal and dystrophic mouse muscle. Hence dystrophy in these animals does not result from any general defect in the protein-synthesizing system.

In spite of the fact that the activities of many enzymes alter markedly in dystrophic muscle (Schapira & Dreyfus, 1963; McCaman, 1963) and a considerable loss of actomyosin occurs, this was not reflected by any significant change in the polyribosome pattern, in the ability of various polyribosome fractions to incorporate radioactive amino acids, or in the amino acid-activating properties of the pH5 fraction as inferred from the amino acid-incorporation experiments. Several workers, mainly on the basis of isoenzyme studies, have suggested that dystrophy is accompanied by a reversion to the foetal state. Breuer & Florini (1965) reported that young rats have more of the heavy polyribosomes than have old rats. We did not examine very young animals, but could not obtain any evidence for an increased heavy-polyribosome fraction in dystrophic animals. If dystrophy is characterized by an increased protein turnover, then failure to compensate by increasing the supply of messenger RNA for the synthesis of contractile elements could account for the observed progressive wasting.

As mentioned in the introduction, an increase in the protein-synthesizing ability of dystrophic muscle on a wet-weight basis is well documented.

Our results add further confirmation but also show that this results not from an increase in the total amount of protein-synthesizing machinery per cell but from a decrease in cell size and, concomitantly, total wet weight. The surprising fact emerges that the total DNA and the protein-synthesizing machinery per limb are virtually the same in normal and dystrophic mice in spite of the great disparity in size. This also seems to hold for normal mice of different age and size over the range in which we were able to make measurements.

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