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Systematic distribution of muscle fibre types in the rat and rabbit diaphragm: a morphometric and ultrastructural analysis

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INTRODUCTION

Breathing is endurance work which starts just after delivery and lasts until the death of the individual. The complex act of breathing involves the coordinated use of many muscles. Among these the diaphragm has for centuries been a source of interest to physiologists as well as to morphologists.

Traditional interpretation of the diaphragm function has been that it behaves as a unit (Boyd & Basmajian, 1963) and that it uses the abdominal contents as a fulcrum to expand the rib cage. However, recent observations in the dog suggest different actions on the rib cage (De Troyer, Sampson, Sigrist & Macklem, 1981). The diaphragm consists of two muscles that act differently on the cage. The action of the costal part increases the dimensions of the lower rib cage, whereas the crural part has an expiratory action, decreasing its dimensions. The mechanical action of the diaphragm is determined by the contractile properties of the muscle fibres as well as by the pattern of its motor neuron activation, the muscle fibre composition of this muscle system being well suited to the task. Approximately 53·9% to 59·8% of fibres in the adult rat diaphragm are of the red oxidative type, which are highly resistant to fatigue, while 15·4% to 18·8% are of fast-twitch, oxidative glycolytic type which, though intermediate, are relatively resistant to fatigue. The remaining 23·1% to 28·4% of fibres are of the fast-twitch glycolytic variety, which are susceptible to fatigue (Gottschal, 1981). The above proportions vary among species. In dogs and mice fast-twitch glycolytic fibres are believed to be absent (Faulkner, Maxwell, Ruff & White, 1979). In the cat diaphragm, however, differences in the proportion of each muscle fibre type have been observed between the abdominal and thoracic surfaces but not between different regions. Approximately 55% of slow-twitch oxidative fibres (Type I) were noted on the abdominal surface and 25% on the thoracic surface. However, fast-twitch oxidative glycolytic (Type II A) and fast-twitch glycolytic (Type II B) fibres were found to be distributed in a reverse proportion in comparison with the slow-twitch oxidative fibres. More fast-twitch oxidative glycolytic (intermediate) and fast-twitch glycolytic fibres were found on the thoracic surface (Sieck *et al.* 1983). No such gradient of fibre distribution was observed, however, in the rat diaphragm (Metzger, Scheid & Fitts, 1985). Therefore, it might be said that in the rat diaphragm no one surface is preferentially recruited whereas, in the cat, the motor units recorded from electrodes in contact with the abdominal surface are consistently recruited during normal quiet breathing. In contrast, motor units that are recorded from electrodes in contact with the thoracic surface show far less consistency in their recruitment. These

results may explain the distributional differences in fibre-type composition across the muscle (thoracic *v.* abdominal surface) diaphragm of the cat (Sieck *et al.* 1983). Nevertheless, it still remains controversial whether there exists a variation in the fibre type distribution between the different regions as well as across the diaphragm (Gunther, 1952 1953; Nishiyama, 1966; George & Susheela, 1961; Riley & Berger, 1979; Yellin, 1972). The aim of the present study was to give a complete morphological description of the rat and rabbit costal diaphragm on the basis of histochemical, ultrastructural and morphometrical estimates.

MATERIALS AND METHODS

Five 5 months old male Wistar rats (300 g) and five 1 year old female Belgian rabbits (4·6 kg) were used. The animals were killed with an overdose of sodium pentobarbitone (rabbits) or by inhalation of O_2/CO_2 mixture. After death the left and right costal hemidiaphragms were excised, placed flat on a cork plate, and then moistened with phosphate-buffered saline. The whole hemidiaphragm was then rolled up (like a Swiss roll), starting from its sternal region, to form a 'roulade' (Fig. 1). The 'roulade' was positioned vertically and glued to a small piece of cardboard by means of Tissue Tek II and frozen in Freon 12 cooled by liquid nitrogen. Serial cross-sections 10 μm thick were cut from the frozen 'roulade' using a cryostat kept at -20 °C. Alternate sections were stained for myofibrillar adenosine triphosphatase (ATPase) activity after acid (pH 4·2–4·5 (rat) and pH 4·3–4·6 (rabbit) and alkaline (pH 10·2 (rat) and pH 9·6 (rabbit)) pre-incubation and for NADH-tetrazolium reductase (Dubowitz & Brooke, 1973). The histochemical profile based on the staining reactions of each fibre in serial sections was established and classified as Type I, corresponding to slow-twitch oxidative, Type IIA, corresponding to fast-twitch oxidative, and Type IIB, corresponding to fast-twitch glycolytic (essentially according to Peter *et al.* 1972).

Morphometry

For the morphometric analysis, a cross-section through the whole 'roulade' was chosen, to cover the entire cross-section profile of the hemidiaphragm. Point and intersection counting was performed on a square lattice test system with a total of 100 points. The test system was superimposed on the glass screen of a Reichert projection microscope. For estimation of the muscle fibre volume density (V_{vf}), muscle fibre surface density (S_{vf}), and muscle fibre numerical density (N_{Af}), a final magnification of $\times 600$ was used. For performing the stereological analysis the cross-section of the 'roulade' was arbitrarily divided into three regions: close to the sternal end of the costal diaphragm, called the ventral region, and the middle portion and crural end of the diaphragm called the medial and dorsal regions respectively. From each region six fascicles per section were chosen at random. Three of them faced the thoracic and three the abdominal surface of the hemidiaphragm. All stereological variables were obtained by the standard procedures described by Weibel (1979). Cross-sectional areas of the three muscle fibre types were measured using a HIPAD Digitizing Pad (Bausch & Lomb Houston Instrument) image analysing system connected with an ABC 800 computer. Diameters of muscle fibres were calculated by the conversion of their cross-sectional areas, assuming a circular shape of their profiles.

Electron microscopy

For electron microscopy, thin (1–2 mm wide) longitudinal strips were cut from the three above-described hemidiaphragm regions (ventral, medial and dorsal), stretched

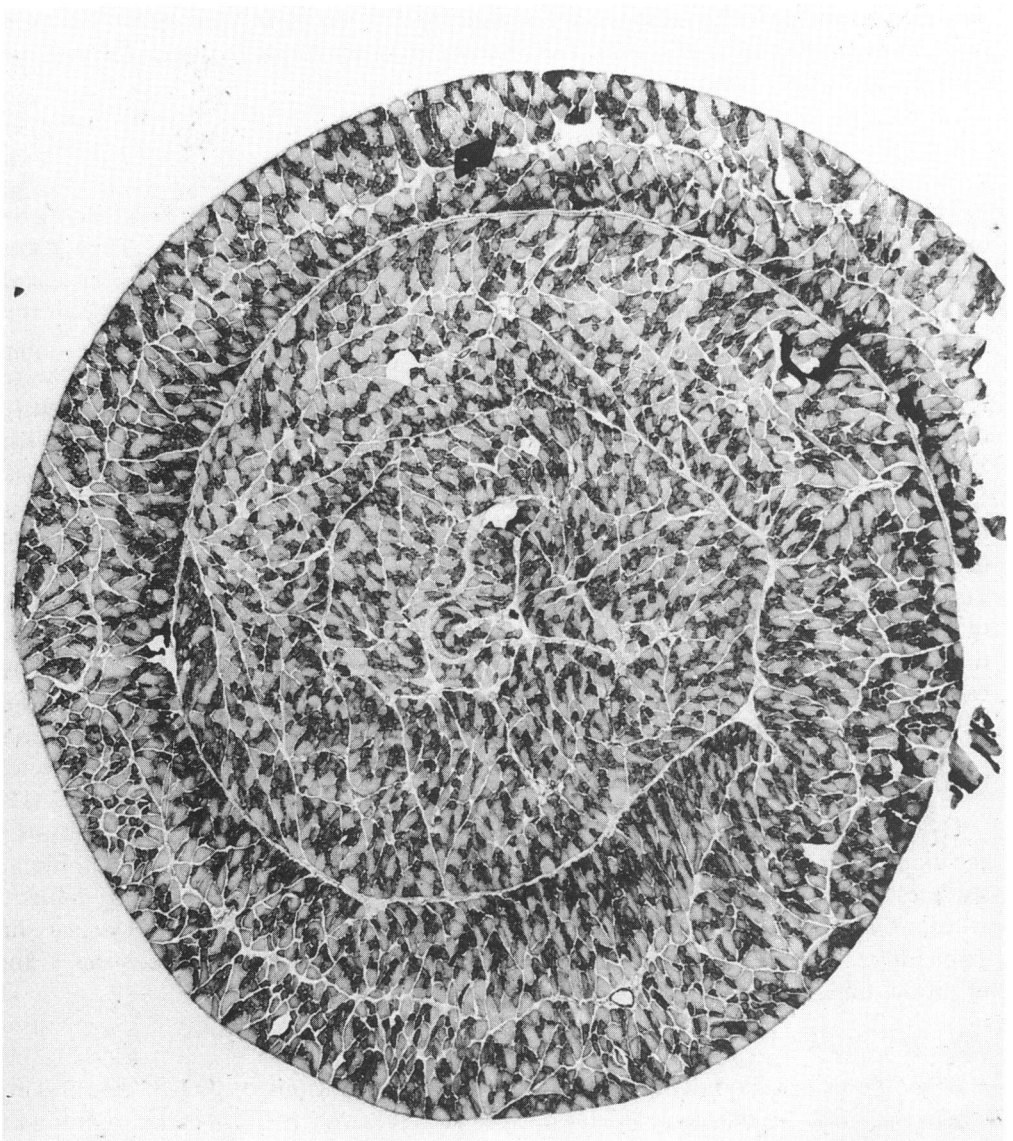

Fig. 1. A frozen cross-section of the whole costal diaphragm of the rat, rolled up into the form of a 'roulade' and stained for NADH-tetrazolium reductase. The ventral (sternal) region is located in the centre of the 'roulade'. The outside surface of the 'roulade' represents the thoracic surface of the diaphragm. ×42.

to more or less physiological length and then fixed in a 2·5% (v/v) solution of glutaraldehyde in Tyrode solution (pH 7·4) overnight at 0 °C. The individual strips were then halved and one part was postfixed in 1% osmium tetroxide and further processed routinely for electron microscopy examination, using vestopal as the embedding material. Thin sections were examined in a Philips 300 electron microscope calibrated, for each 15 pictures taken, with a carbon grating, which had 21 600 lines/cm (E. F. Fullam, Schenectady, N.Y.-No 321). The dimensions of the A-band, M-band, and Z-line were measured from printed micrographs at the final magnification of ×41 220.

The data were analysed by a one-way analysis of variance. Unpaired Student's t test was used to compare the means, when significant differences were detected. The level of significance was set at $P < 0{\cdot}05$. The number (n) of the fibres analysed is shown for each group of data separately.

RESULTS

The costal portion of the diaphragm is thin and flat; its muscle fibres extend more or less radially from the central tendon to attach to the rib cage. They form approximately 8–15 layers of fibres in the rat and 10–30 layers in the rabbit costal diaphragm. The middle region is always thicker than either the ventral (close to the sternal part) or dorsal region (close to the crural part) (Fig. 1).

In each region of the costal diaphragm in general, three types of muscle fibres were found. Their histochemical profiles were essentially similar to those previously described in other skeletal muscles (Burke *et al.* 1971; Burke & Tsairis, 1974; Burke, Levine, Salcman & Tsairis, 1974; Gottschal, 1981; Gunther, 1952; Kugelberg & Edstrom, 1968). On the basis of the ATPase activity, Types I, II A and II B were most frequently present but occasionally a fourth type, II C, was found. The II C fibres were evident after pre-incubation at pH 4·2 in the rat and 4·3 in the rabbit diaphragm (Figs. 2*b*, 3*a*). These fibres are equivalent to an intermediate category according to their mitochondrial content (Figs. 2*d*, 3*c*). However, the II C fibres were rare in the diaphragm of both the rat and rabbit so they have not been included in the calculations.

Fibre type distribution (rat diaphragm)

Type I fibres

Overall, there was a predominance of Type I fibres in each of the three regions of the diaphragm (Table 1), which, however, varied between the thoracic and abdominal surfaces as well as between the ventral, medial and dorsal parts (Tables 1, 3). Similar relations were also encountered between the volume, numerical and surface density of Type I fibres. The latter showed the smallest diameter and lowest cross-sectional areas of all the fibre types measured (Tables 1, 3).

Type II B fibres

These fibres are the next most frequent in all three regions of the rat diaphragm, where they differ significantly (Table 5). The largest numbers of this type were found in the ventral region of the diaphragm. However, there is no evident difference in the number of Type II B fibres between the thoracic and abdominal surfaces in the medial and dorsal regions (Table 5). The Type II B fibres had the highest value of volume density (V_{vf}) and the lowest value of surface density (S_{vf}) (Table 1) – two parameters strongly depending on the size and number of the muscle fibres profiles. The Type II B fibres had the highest value for the cross-sectional area. There were obvious differences between the mean area of the fibres located on the thoracic surface and those found on the abdominal surface in favour of the former (Table 3). The Type II B fibres were most prominent in mass, but not in number, in the rat diaphragm in contrast to the Type I fibres.

Type II A fibres

In the rat costal diaphragm Type II A fibres were in the minority. A significant difference in the distribution of these fibres was clearly seen on the thoracic side of the

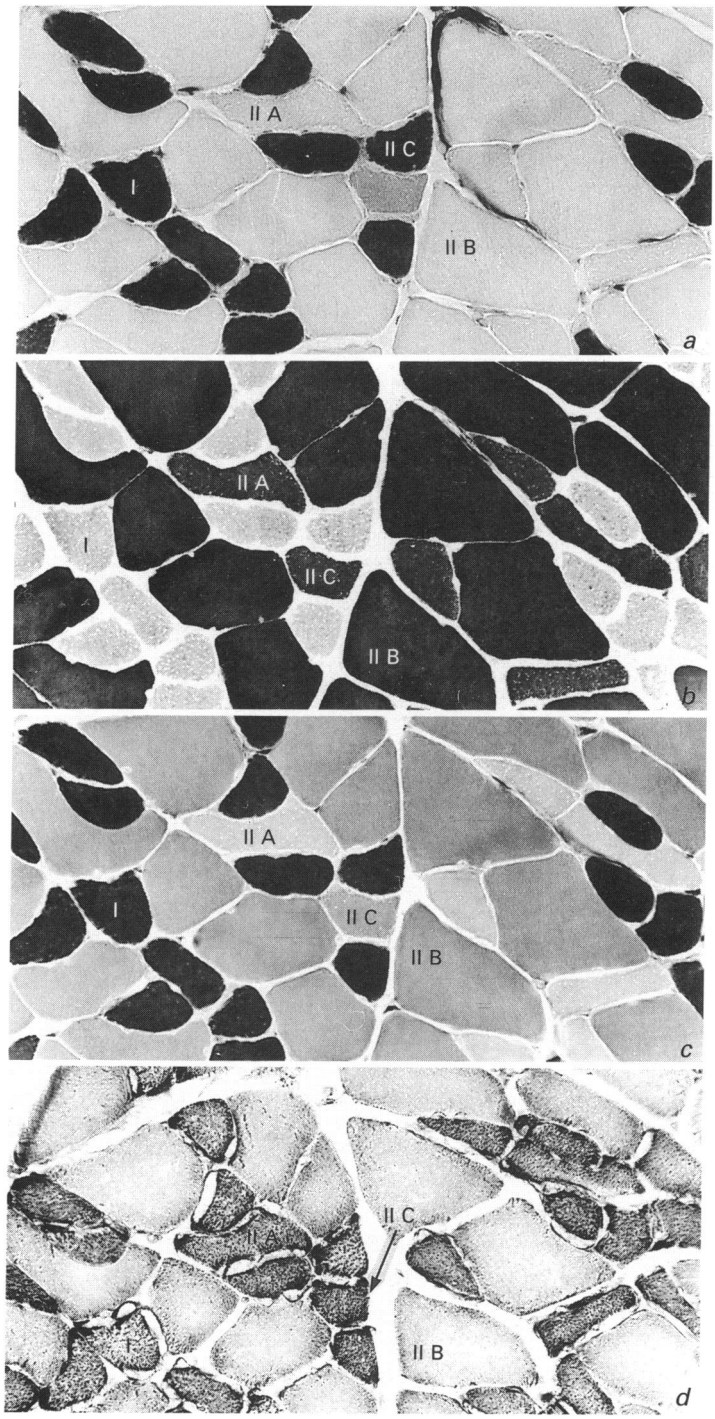

Fig. 2(*a–d*). Comparison of serial sections of the medial region (thoracic surface) of rat costal diaphragm. Fibre typing by staining for ATPase activity after pre-incubation at pH 4·2 (*a*), pH 10·2 (*b*), and pH 4·55 (*c*). (*d*) stained for NADH-tetrazolium reductase. × 120.

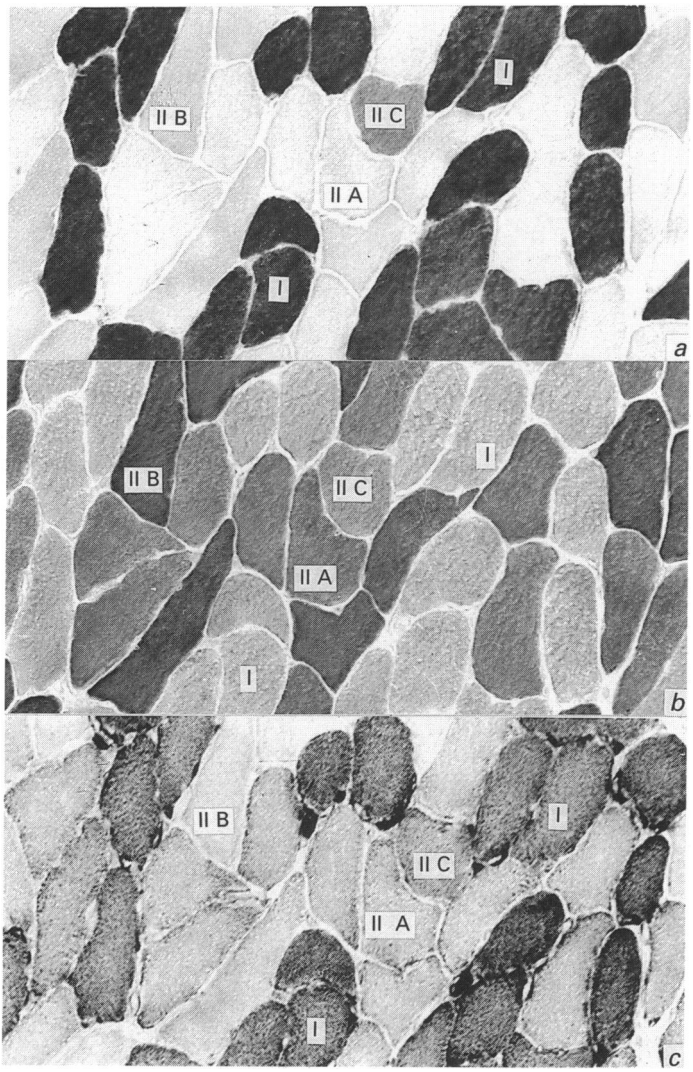

Fig. 3(*a–c*). Comparison of serial sections of the medial region (abdominal surface) of rabbit costal diaphragm. Fibre typing by staining for ATPase activities after pre-incubation at pH 4·3 (*a*), and pH 4·6 (*b*). (*c*) stained for NADH-tetrazolium reductase. × 120.

medial versus dorsal, and ventral versus dorsal, regions (Table 5). The volume density (V_{vf}) values of the Type II A were the lowest in comparison with those of Types I and II B. The surface density (S_{vf}) values of Type II A were similar to those of Type I (Table 1). The similarity of these values for Types II A and I is due to the fact that the diameters of the Type II A fibres are greater (Table 3) than those of Type I, thus compensating for their smaller numbers in the fibre population (Table 5).

Fibre type distribution (rabbit diaphragm)

Type I fibres

The costal diaphragm of the rabbit contained only 18% Type I fibres, which were, however, differently distributed in the three regions investigated (Table 6). The volume

Table 1. *Morphometric estimates of volume density ($V_{vf,A}$) and surface density (S_{vf}) of three types of muscle fibre in rat right diaphragm. The reference space for V_v is the numbers of test points which failed on the muscle fibre profiles only. The numerical density of fibres ($N_{A,f}$) and % occurrence of fibres ($\%_{f,A}$) refer to the area of the test grid (A) of 0·236 cm^2 at magnification ×600. V_v is expressed in %; S_v in $cm^2/cm^3 \times 10^3$.* S.E., *standard error;* C.V. (%), *coefficient of variation;* S.D., *standard deviation of means*

Part of diaphragm		Ventral				Medial				Dorsal			
Fibre type	Side of diaphragm	$V_{vf,A}$	S_{vf}	$N_{A,f}$	$\%_{f,A}$	$V_{vf,A}$	S_{vf}	$N_{A,f}$	$\%_{f,A}$	$V_{vf,A}$	S_{vf}	$N_{A,f}$	$\%_{f,A}$
I	Thoracic	26·8	1·66	10·8	45	21·7	1·47	9·6	40	22·4	1·64	9·9	42
		S.D. = 4·8	0·14	S.D. = 2·5	n = 54	S.D. = 3·6	0·15	S.D. = 2·3	n = 96	S.D. = 5·5	0·15	S.D. = 2·6	n = 149
		S.E. = 0·65		S.E. = 0·34		S.E. = 0·36		S.E. = 0·23		S.E. = 0·45		S.E. = 0·21	
		C.V. = 25		C.V. = 23		C.V. = 20		C.V. = 23		C.V. = 19		C.V. = 26	
	Abdominal	21·5	1·61	11·6	40	15·3	1·47	10	40	20·2	1·58	8·6	39
		S.D. = 4·1	0·19	S.D. = 4·7	n = 58	S.D. = 4·2	0·13	S.D. = 3·1	n = 100	S.D. = 3·5	0·08	S.D. = 1·4	n = 121
		S.E. = 0·53		S.E. = 0·61		S.E. = 0·42		S.E. = 0·31		S.E. = 0·31		S.E. = 0·12	
		C.V. = 25		C.V. = 40		C.V. = 38		C.V. = 31		C.V. = 21		C.V. = 16	
IIA	Thoracic	23·1	1·51	5·2	22	16·4	1·31	5·4	22	17·3	1·56	6·6	28
		S.D. = 3·8	0·17	S.D. = 3·3	n = 26	S.D. = 4·9	0·16	S.D. = 2·4	n = 22	S.D. = 5·4	0·15	S.D. = 2·8	n = 99
		S.E. = 0·74		S.E. = 0·64		S.E. = 0·66		S.E. = 0·32		S.E. = 0·54		S.E. = 0·28	
		C.V. = 33		C.V. = 63		C.V. = 37		C.V. = 44		C.V. = 39		C.V. = 42	
	Abdominal	16·3	1·54	7·0	24	20·3	1·50	6·5	26	16·6	1·59	6·0	27
		S.D. = 4·5	0·13	S.D. = 2·8	n = 35	S.D. = 3·7	0·10	S.D. = 1·7	n = 65	S.D. = 4·5	0·18	S.D. = 2·3	n = 85
		S.E. = 0·76		S.E. = 0·47		S.E. = 0·45		S.E. = 0·21		S.E. = 0·48		S.E. = 0·24	
		C.V. = 36		C.V. = 40		C.V. = 25		C.V. = 26		C.V. = 33		C.V. = 38	
IIB	Thoracic	56·7	0·96	7·8	33	61·7	0·80	8·9	37	60·3	0·87	7·0	29
		S.D. = 5·0	0·18	S.D. = 2·8	n = 39	S.D. = 5·4	0·13	S.D. = 3·3	n = 89	S.D. = 60·3		S.D. = 1·2	n = 106
		S.E. = 0·8		S.E. = 0·23		S.E. = 0·57		S.E. = 0·34		S.E. = 0·62		S.E = 0·11	
		C.V. = 12		C.V. = 19		C.V. = 12		C.V. = 37		C.V. = 13		C.V. = 17	
	Abdominal	62·1	1·00	10	35	64·2	1·03	8·5	34	63·0	0·80	7·4	33
		S.D. = 11·5	0·10	S.D. = 1·2	n = 50	S.D. = 6·6	0·19	S.D. = 2·9	n = 85	S.D. = 11·3	0·13	S.D. = 1·3	n = 104
		S.E. = 1·6		S.E. = 0·12		S.E. = 0·71		S.E. = 0·31		S.E. = 1·1		S.E. = 0·12	
		C.V. = 23		C.V. = 12		C.V. = 14		C.V. = 34		C.V. = 22		C.V. = 17	

Table 2. *Morphometric estimates of volume density ($V_{vf,A}$) and surface density (S_{vf}) of three types of muscle fibre in rabbit right diaphragm. The reference space for V_v and S_v is the numbers of test points which failed on the muscle fibre profiles only. The numerical density of fibres ($N_{A,f}$) and occurrence of fibres ($\%_{f,A}$) refer to the area of the test grid (A) of 0·236 cm² at magnification ×600.*

Part of diaphragm		Ventral				Medial				Dorsal			
Fibre type	Side of diaphragm	$V_{vf,A}$	S_{vf}	$N_{A,f}$	$\%_{f,A}$	$V_{vf,A}$	S_{vf}	$N_{A,f}$	$\%_{f,A}$	$V_{vf,A}$	S_{vf}	$N_{A,f}$	$\%_{f,A}$
I	Thoracic	22·8	1·03	4·0	20	24·1	0·97	3·7	21·6	18·3	0·91	3	12·5
		S.D. = 3·4	0·18	S.D. = 0·8	n = 15	S.D. = 2·9	0·05	S.D. = 1·2	n = 15	S.D. = 3·5	0·04	S.D. = 0·1	n = 16
		S.E. = 0·85		S.E. = 0·20		S.E. = 0·74		S.E. = 0·32		S.E. = 1·4		S.E. = 0·04	
		C.V. = 18		C.V. = 20		C.V. = 15		C.V. = 34		C.V. = 22		C.V. = 3	
	Abdominal	41·8	0·75	4·2	31	30·5	0·90	3·5	21·3	27·5	1·39	6·5	27·0
		S.D. = 5·1	0·24	S.D. = 0·5	n = 14	S.D. = 4·9	0·06	S.D. = 1·0	n = 13	S.D. = 7·0	0·26	S.D. = 2·1	n = 16
		S.E. = 1·2		S.E. = 12		S.E. = 1·3		S.E. = 0·26		S.E. = 1·9		S.E. = 0·5	
		C.V. = 15		C.V. = 11		C.V. = 19		C.V. = 28		C.V. = 30		C.V. = 32	
IIA	Thoracic	48·6	0·92	9·0	46	45·7	1·13	7·7	45·0	53·8	0·97	11·5	47·9
		S.D. = 7·1	0·29	S.D. = 2·4	n = 31	S.D. = 10·9	0·29	S.D. = 2·8	n = 23	S.D. = 3·5	0·07	S.D. = 2·1	n = 36
		S.E. = 1·6		S.E. = 0·4		S.E. = 1·9		S.E. = 0·5		S.E. = 0·7		S.E. = 0·4	
		C.V. = 18		C.V. = 26		C.V. = 30		C.V. = 36		C.V. = 7		C.V. = 18	
	Abdominal	24·8	1·13	5·3	39	37·6	1·04	6·2	37·8	45·5	1·07	10	41·6
		S.D. = 3·8	0·07	S.D. = 0·5	n = 25	S.D. = 4·7	0·08	S.D. = 1·5	n = 20	S.D. = 11·3	0·06	S.D. = 0·1	n = 16
		S.E. = 0·95		S.E. = 0·12		S.E. = 0·9		S.E. = 0·3		S.E. = 2·5		S.E. = 0·02	
		C.V. = 19		C.V. = 9		C.V. = 15		C.V. = 24		C.V. = 29		C.V. = 1	
IIB	Thoracic	28·5	1·16	6·2	32	30·1	0·97	5·7	33·3	27·8	1·50	9·5	39·5
		S.D. = 2·5	0·15	S.D. = 2·6	n = 23	S.D. = 8·7	0·16	S.D. = 2·5	n = 19	S.D. = 4·9	0·09	S.D. = 0·7	n = 25
		S.E. = 0·5		S.E. = 0·5		S.E. = 1·8		S.E. = 0·5		S.E. = 1·1		S.E. = 0·16	
		C.V. = 11		C.V. = 41		C.V. = 36		C.V. = 43		C.V. = 20		C.V. = 7	
	Abdominal	33·3	0·98	4·0	29·8	31·7	1·15	6·7	40·8	26·9	1·26	7·5	31·2
		S.D. = 7·1	0·11	S.D. = 1·4	n = 27	S.D. = 7·4	0·16	S.D. = 1·7	n = 15	S.D. = 0·7	0·04	S.D. = 0·7	n = 10
		S.E. = 2·2		S.E. = 0·15		S.E. = 1·4		S.E. = 0·32		S.E. = 0·18		S.E. = 0·18	
		C.V. = 26		C.V. = 15		C.V. = 28		C.V. = 25		C.V. = 3		C.V. = 9	

Table 3. *Morphometric estimates of rat right hemidiaphragm, from ventral, medial and dorsal parts respectively, ±, standard deviation of means;* n, *number of fibres*; S.E., *standard error;* C.V., *coefficient of variation in %*

		Fibre Type I		Fibre Type II A		Fibre Type II B	
		Thoracic surface	Abdominal surface	Thoracic surface	Abdominal surface	Thoracic surface	Abdominal surface
Mean fibre area (μm^2)	Ventral	898·4 ± 225	782·2 ± 254	1096 ± 303	887·0 ± 321	3436 ± 1230	2821 ± 1046
		n = 127	n = 100	n = 128	n = 125	n = 125	n = 125
		S.E. = 20	S.E. = 25	S.E. = 26	S.E. = 28	S.E. = 110	S.E. = 93
		C.V. = 25	C.V. = 32	C..V = 27	C.V. = 36	C.V. = 35	C.V. = 37
	Medial	901·2 ± 195	789·1 ± 159	1171 ± 376	775·4 ± 176	3020 ± 1127	2111 ± 812
		n = 126	n = 125	n = 127	n = 125	n = 125	n = 125
		S.E. = 17	S.E. = 14	S.E. = 33	S.E. = 15	S.E. = 100	S.E. = 72
		C.V. = 21	C.V. = 20	C.V. = 32	C.V. = 22	C.V. = 37	C.V. = 38
	Dorsal	792·4 ± 162	742·3 ± 165	983·5 ± 276	771·1 ± 196	2276 ± 716	1654 ± 704
		n = 125	n = 125	n = 126	n = 125	n = 125	n = 125
		S.E. = 14	S.E. = 14	S.E. = 24	S.E. = 17	S.E. = 64	S.E. = 63
Mean fibre diameter (μm)	Ventral	33·49 ± 4·7	31·15 ± 5·1	36·99 ± 5·2	33·08 ± 5·9	64·90 ± 12·8	58·82 ± 11·5
		S.E. = 0·4	S.E. = 0·5	S.E. = 0·4	S.E. = 0·5	S.E. = 1·1	S.E. = 1·0
		C.V. = 14	C.V. = 16	C.V. = 14	C.V. = 17	C.V. = 20	C.V. = 20
	Medial	33·68 ± 3·6	31·72 ± 3·1	38·22 ± 5·4	31·23 ± 3·5	60·91 ± 11·5	50·09 ± 10
		S.E. = 0·4	S.E. = 0·3	S.E. = 0·5	S.E. = 0·3	S.E. = 1·0	S.E. = 0·9
		C.V. = 10	C.V. = 10	C.V. = 14	C.V. = 11	C.V. = 18	C.V. = 20
	Dorsal	31·60 ± 3·2	30·56 ± 3·4	34·98 ± 5·3	31·10 ± 3·8	34·98 ± 5·3	44·64 ± 10·7
		S.E. = 0·3	S.E. = 0·3	S.E. = 0·5	S.E. = 0·3	S.E. = 0·5	S.E. = 0·9
		C.V. = 10	C.V. = 11	C.V. = 15	C.V. = 12	C.V. = 15	C.V. = 24

Table 4. *Morphometric estimates of rabbit right hemidiaphragm, from ventral, medial and dorsal parts respectively.* ±, *standard deviation of means;* n, *number of fibres*; S.E., *standard error;* C.V., *coefficient of variation in %*

		Fibre Type I		Fibre Type II A		Fibre Type II B	
		Thoracic s. n = 60	Abdominal s. n = 60	Thoracic s. n = 60	Abdominal s. n = 60	Thoracic s. n = 60	Abdominal s. n = 60
Mean fibre area (μm^2)	Ventral	1757 ± 366	1847 ± 490	1367 ± 253	1391 ± 293	1681 ± 478	1592 ± 414
		S.E. = 47	S.E. = 63	S.E. = 32	S.E. = 30	S.E. = 61	S.E. = 53
		C.V. = 20	C.V. = 26	C.V. = 18	C.V. = 21	C.V. = 28	C.V. = 26
	Medial	2692 ± 997	3284 ± 717	2127 ± 442	1692 ± 425	1930 ± 526	2141 ± 657
		S.E. = 128	S.E. = 92	S.E. = 57	S.E. = 54	S.E. = 67	S.E. = 84
		C.V. = 37	C.V. = 21	C.V. = 20	C.V. = 25	C.V. = 27	C.V. = 30
	Dorsal	1972 ± 699	2195 ± 786	1692 ± 375	1846 ± 375	1811 ± 386	1660 ± 340
		S.E. = 90	S.E. = 101	S.E. = 48	S.E. = 48	S.E. = 49	S.E. = 43
		C.V. = 35	C.V. = 35	C.V. = 22	C.V. = 20	C.V. = 21	C.V. = 20
Mean fibre diameter (μm)	Ventral	47·06 ± 4·8	48·08 ± 6·3	41·84 ± 4·5	41·53 ± 3·9	45·85 ± 6·2	44·63 ± 5·9
		S.E. = 0·81	S.E. = 0·81	S.E. = 0·58	S.E. = 0·50	S.E. = 0·80	S.E. = 0·76
		C.V. = 10	C.V. = 13	C.V. = 10	C.V. = 9	C.V. = 13	C.V. = 13
	Medial	57·53 ± 10·9	64·28 ± 7·0	51·77 ± 5·4	46·13 ± 5·8	49·18 ± 6·3	51·59 ± 8·1
		S.E. = 1·4	S.E. = 0·90	S.E. = 0·69	S.E. = 0·74	S.E. = 0·81	S.E. = 1·0
		C.V. = 18	C.V. = 10	C.V. = 10	C.V. = 12	C.V. = 12	C.V. = 15
	Dorsal	49·37 ± 8·6	52·10 ± 9·0	46·18 ± 4·7	48·22 ± 5·1	47·76 ± 5·0	45·72 ± 4·8
		S.E. = 1·1	S.E. = 1·1	S.E. = 0·60	S.E. = 0·65	S.E. = 0·64	S.E. = 0·61
		C.V. = 17	C.V. = 17	C.V. = 10	C.V. = 10	C.V. = 10	C.V. = 10

Table 5. *Test on differences between the occurrence of fibre types in three parts of the rat and rabbit diaphragm (ventral, medial, dorsal). For each part differences are given t tests and relevant degrees of freedom* (D.F.) *and P values (P). The results of analysed means* ($N_{A,f}$; $\%_{f,A}$) *are given separately (see Tables* 3 *and* 4)

Part of diaphragm	Fibre type	Ventral		Medial		Dorsal		Ventral/Medial			Ventral/Dorsal			Medial/Dorsal		
		n	%	n	%	n	%	*t*	D.F.	*P*	*t*	D.F.	*P*	*t*	D.F.	*P*
								Rat								
Thoracic side	I	54	45	96	40	149	42	3·2	148	< 0·005	—	—	n.s.	2·3	200	< 0·025
	IIA	26	22	22	22	99	28	—	—	n.s.	2·7	151	< 0·005	1·9	123	< 0·005
	IIB	39	33	89	37	121	29	6·2	126	< 0·001	5·1	193	< 0·001	3·1	143	< 0·005
Abdominal side	I	58	40	100	40	106	39	2·3	152	< 0·025	4·7	200	< 0·001	4·7	160	< 0·001
	IIA	35	24	65	26	85	27	—	—	n.s.	—	—	n.s.	—	—	n.s.
	IIB	50	35	85	34	104	33	4·2	133	< 0·001	3·2	187	< 0·005	12·1	152	< 0·001
								Rabbit								
Thoracic side	I	60	20	60	22	60	13	—	—	n.s.	3·9	28	< 0·001	2·1	28	< 0·01
	IIA	60	46	60	45	60	48	1·7	52	< 0·05	3·9	44	< 0·001	5·6	44	< 0·001
	IIB	60	32	60	33	60	40	—	—	n.s.	5·7	40	< 0·001	6·2	36	< 0·001
Abdominal side	I	60	31	60	21	60	27	2·1	25	< 0·05	3·6	25	< 0·001	4·4	24	< 0·001
	IIA	60	39	60	38	60	42	2·5	43	< 0·025	4·4	43	< 0·001	3·2	43	< 0·001
	IIB	60	30	60	41	60	31	3·9	40	< 0·001	5·6	40	< 0·001	—	—	n.s.

Table 6. *Test of differences of fibre type occurrence between the two surfaces of rat and rabbit diaphragm (thoracic v. abdominal) in the three parts of the muscle (ventral, medial, dorsal). For each surface differences are given t tests, relevant degrees of freedom* (D.F.), *and P values (P). The results of analysed means* ($N_{A,f}$; $\%_{f,A}$) *are given separately (see Tables* 1 *and* 2)

Fibre type	Diaphragm	Ventral			Medial			Dorsal		
		t	D.F.	*P*	*t*	D.F.	*P*	*t*	D.F.	*P*
				Rat						
I	Thorac/Abdom.	—	—	n.s.	—	—	n.s.	5·2	270	< 0·001
IIA	Thorac/Abdom.	—	—	n.s.	2·8	119	< 0·005	—	—	n.s.
IIB	Thorac/Abdom.	7·7	87	< 0·001	—	—	n.s.	—	—	n.s.
				Rabbit						
I	Thorac/Abdom.	—	—	n.s.	—	—	n.s.	5·7	17	< 0·001
IIA	Thorac./Abdom.	22	55	< 0·001	2·5	54	< 0·025	7·4	41	< 0·001
IIB	Thorac/Abdom.	3·3	39	< 0·001	—	—	n.s.	8	32	< 0·001

Table 7. *Thickness (d) of sarcomere bands of three muscle fibre types of rat and rabbit costal diaphragm. Test on the differences between sarcomere bands of rat and rabbit muscle fibre types from the diaphragm. For each sarcomere band differences are given t tests and relevant degrees of freedom* (D.F.) *and P values*

	I			IIA			IIB		
	A	M	Z	A	M	Z	A	M	Z
				Rat					
n	20	—	20	18	9	20	15	14	20
d	1·41 μm	—	65·8 nm	1·38 μm	82·8 nm	68·0 nm	1·29 μm	50 nm	45·4 nm
S.D.	0·02	—	12	0·03	5·1	22	0·02	7·5	6·1
C.V.	1·42%	—	18%	2·2%	6·2%	32%	1·5%	15%	13%
S.E.	0·0004	—	2·6	0·007	1·7	5·0	0·0005	2	1·4
				Rabbit					
n	25	28	24	45	42	50	30	30	29
d	1·46 μm	85·8 nm	108 nm	1·40 μm	77 nm	78·1 nm	1·37 μm	48 nm	53 nm
S.D.	0·02	7·2	11·4	0·02	12	18·1	0·02	0·24	10
C.V.	1·4%	8·4%	10%	1·4%	15%	23%	1·4%	0·5%	18%
S.E.	0·004	1·3	2·3	0·003	1·8	2·5	0·003	0·04	1·8

	I/IIA			I/IIB			IIA/IIB		
				Rat					
t	3·4			17		6·6	9·9	11·9	4·3
D.F.	36		n.s.	33		38	31	21	38
P	< 0·005			< 0·001		< 0·001	< 0·001	< 0·001	< 0·001
				Rabbit					
t	11	3·7	8·5	16	20	18	10	15	7·8
D.F.	68	68	72	53	56	51	53	70	75
P	< 0·001	< 0·001	< 0·001	< 0·001	< 0·001	< 0·001	< 0·001	< 0·001	< 0·001

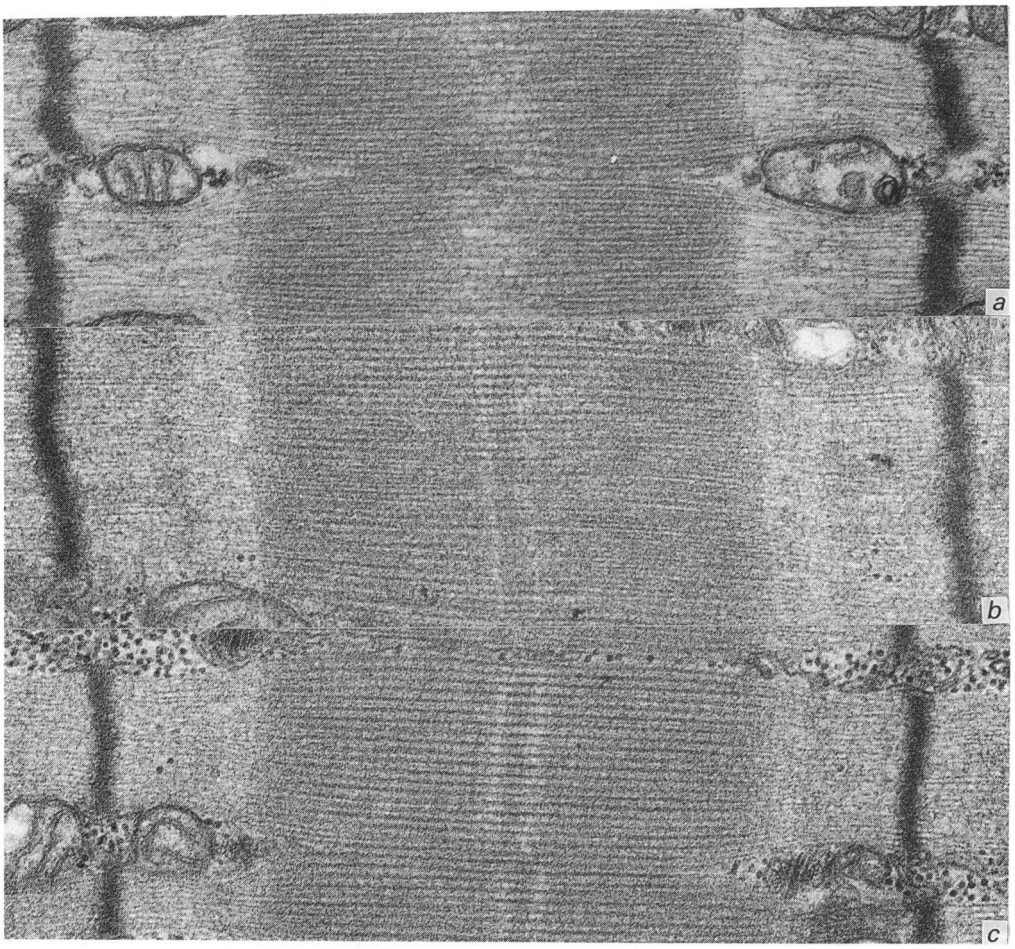

Fig. 4(*a–c*). Longitudinal sections through a single sarcomere of three muscle fibre types of rat costal diaphragm chosen at random. (*a*) Type I fibre, (*b*) Type II A fibre, (*c*) Type II B fibre. × 63 529.

density (V_{vf}) of these fibres was relatively low in all three regions investigated, but showed differences between the thoracic and abdominal surfaces, favouring the latter. The surface density values (S_{vf}) were relatively low (Table 2). The Type I fibres were the largest in diameter and cross-sectional area. (Table 4).

Type II A fibres

In contrast to Type I, Type II A fibres were the smallest in diameter (Table 4) and had relatively low values for their cross-sectional areas. They constituted, however, 42·5% of the whole population of muscle fibres, this being the highest value of the fibre types so far discerned. Their distribution differed markedly between the regions as well as between the surfaces in the same region (Table 6). The volume density (V_{vf}) of Type II A fibres was high and was always found to be highest in the thoracic surface of all three regions of the rabbit diaphragm; they constituted the main mass volume in the rabbit (Table 2).

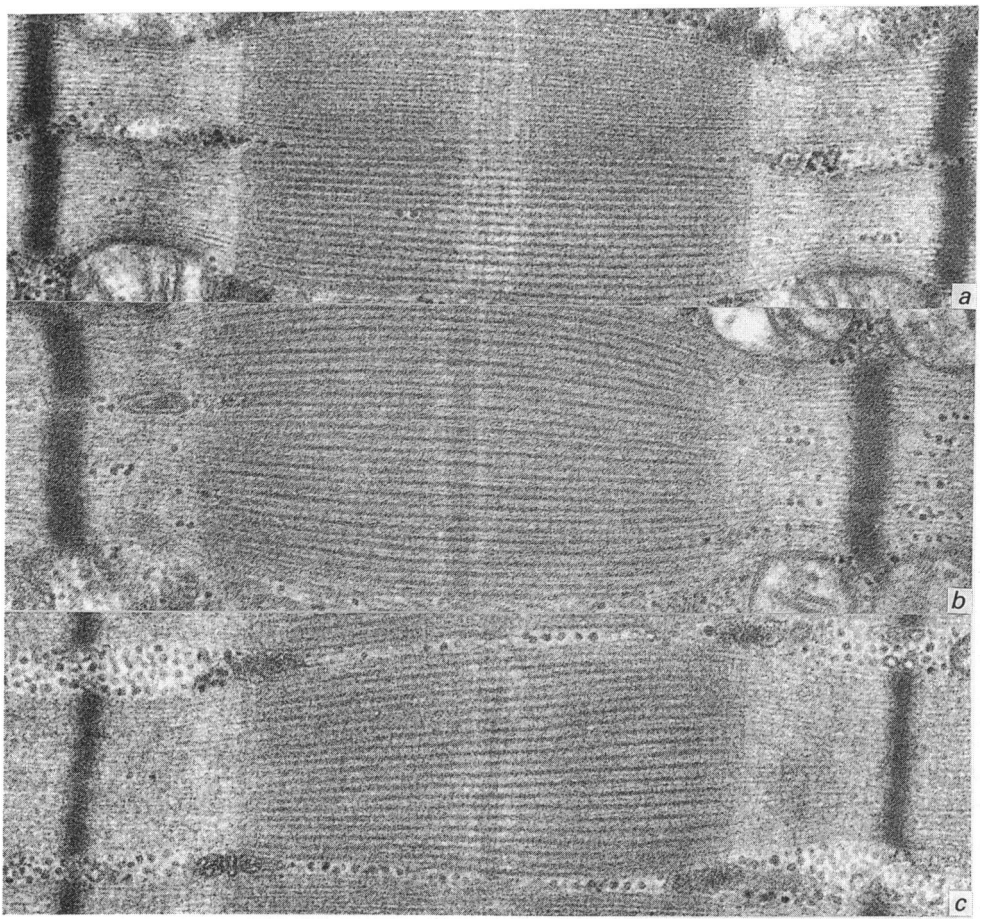

Fig. 5(*a–c*). Longitudinal sections through a single sarcomere of three muscle fibre types of rabbit costal diaphragm chosen at random. (*a*) Type I fibre, (*b*) Type II A fibre, (*c*) Type II B fibre. × 63 529.

Type II B fibres

These fibres followed the preceding type as far as diameter and occurrence were concerned (Table 4). Their distribution showed significant variation between the regions as well as between the sides observed (Table 6). The cross-sectional area of Type II B was similar to that of Type II A fibres, which was due to the fact that they had a very similar diameter (Table 4). Differences between the three regions and their surfaces were found, however, when the volume density (V_{vf}) of Type II B fibres was calculated. Differences in the surface density (S_{vf}) of Type II B were observed only in the dorsal region (Table 2). These differences may have been connected with the different sizes which occurred among the Type II B fibres themselves in these particular regions of the diaphragm.

Electron microscopical observations

On the basis of ultrastructural variability it was possible to identify at least three categories of muscle fibres in the rat and rabbit costal diaphragm. The number and distribution of mitochondria were the most useful characteristics in distinguishing the red, intermediate and white muscle fibres. The fibres which contained large numbers

of mitochondria aggregated close to the sarcolemma and in longitudinal rows between myofibrils were considered as the first type of red fibres. These fibres also had wide Z-lines and M-bands and the widest A-bands (Table 7). In the second type of red fibres, the subsarcolemmal mitochondria were rather less abundant and limited to one or two rows only, but interfibrillar mitochondria were present. The second type of red fibres, however, had very wide Z-lines, wide A-bands and no visible M-bands. After analysis of all the distinguishing characteristics, it may be claimed that the first type of red fibres represent the Type II A fast-twitch red, while the second type may be considered as Type I slow-twitch red, owing to the absence of the M-bands in the sarcomeres of these fibre types.

Those fibres in which the subsarcolemmal mitochondria were sporadically distributed and no interfibrillar mitochondria were present were considered to be white fibres. There were only small elongated mitochondria surrounding the myofibrils at the level of I-bands close to the Z-lines level, forming a three-dimensional network. The Z-lines in these fibres were thin and straight. Their thickness was only about half that in the Type I fibres. The M-bands were also narrow and the A-bands had the smallest dimensions in comparison with the other two types of fibres described. These fibres were considered as Type II B fast-twitch white (Table 7; Fig. 4).

On the basis of the structural differences described above, the three muscle fibre types distinguished in the rabbit costal diaphragm were considered to be Type I, Type II A, and Type II B fibres. Type I had the widest A-bands, diffuse and wide M-bands and very wide and distinct Z-lines. Type II A had smaller A-bands, narrower M-bands and Z-lines intermediate in width. Type II B fibres, which are fast-twitch white, had the shortest A-bands, narrow M-bands and straight and thin Z-lines. These two elements of the sarcomeres were only about half as thick as in Type I fibres and only about one third as thick as in Type II A. These two characteristics were therefore very useful in the identification of Type II B fibres (Table 7; Fig. 5).

DISCUSSION

Developmentally, anatomically and functionally the diaphragm comprises two muscles (Decramer *et al.* 1984; De Troyer *et al.* 1981, 1982), the costal portion, which is thin and flat, and the crural part, which is thick and has a more complex criss-cross arrangement of fibres. To understand the action of the diaphragm on the respiratory system, it is necessary to evaluate the arrangement of its muscle fibres in its different regions and also across the muscle. In the present work only the costal part of the diaphragm was investigated. The diaphragm of small animal species, such as the mouse, bat, and shrew, is believed to be composed of homogeneous red fibres which are relatively fast and resistant to fatigue, while that of animals intermediate in size, such as the rat and rabbit, is a mixture of all three types of muscle fibres (Gauthier & Padykula, 1966). In general, smaller animals require, per unit mass, a higher metabolic rate than do larger ones. They consume a greater amount of oxygen and breathe extremely rapidly, so that their expiratory as well as their inspiratory muscle fibres use predominantly aerobic metabolism. The medium sized animals, however, have a lower metabolism than small ones; this is believed to be related to the body surface area and thus directly to heat loss (Weibel, 1984). The contraction times determined for the diaphragm of the rat, rabbit, cat and dog suggest that these, too, are related to the body size (rat 18 ms, rabbit 32 ms, cat 39 ms and dog 65 ms) (Sant'Ambrogio & Saibene, 1970). These two groups of animals have a different ratio of oxidative to glycolytic fibres. The present results showed little variation in the proportion of glycolytic (Type

II B fibres) and oxidative (both Type I and II A) fibres between these two species, which should reflect the differences in contraction times found in these two animals. In the rat diaphragm the Type II B fibres (glycolytic) comprise 33·5% as opposed to 64·5% of both oxidative types (I and II A). In the rabbit diaphragm the proportion is very similar, i.e. 34·5% and 65% respectively. But a significant variation was found in the proportion of the oxidative fibres themselves between the rat and rabbit. In the rat diaphragm 40% of Type I fibre was found and 24·5% of Type II A, while in the rabbit diaphragm the proportion was the reverse, i.e. 21·5% and 43% respectively. This relation probably reflects the breathing activity per gram of body weight, which is higher in the rat. Hence, in the rabbit, whose weight is almost ten times that of the rat, the proportion of Type I to Type II A fibres is reversed.

In both animals, rats and rabbits, the muscle fibre type composition of the thoracic surface of the costal diaphragm varied from that of the abdominal surface of the muscle (Tables 5, 6). A similar difference in fibre type composition across the thickness of the diaphragm has also been found in the cat (Riley & Berger, 1979; Sieck *et al.* 1983). The results of Metzger *et al.* (1985) regarding abdominal–thoracic differences in fibre-type composition are the reverse of those found in the present study. These authors' data suggest that in the rat "no one region or side of the diaphragm is preferentially recruited". The present results also showed differences in the proportion of fibre types between diaphragmatic regions. This finding is in contrast to the data presented by Sieck *et al.* (1983), though it is in good agreement with the marked regional differences in fibre-type composition reported by Riley & Berger, also in the cat (1979). The variation in fibre-type composition between the abdominal and thoracic surfaces as well as between the ventral, medial and dorsal regions of the costal diaphragm of both the rat and rabbit (Tables 5, 6) suggest possible functional differences, such as the order of activation of motor units on each surface and region and the relative contribution of fibres on each surface to the overall force developed by the diaphragm under varying conditions. The greater number of fast-twitch fibres (both Types II A and II B) on the thoracic surface in the rabbit may suggest that this surface of the diaphragm might be recruited only during high inspiratory activity. The abdominal surface, which contains a larger number of slow-twitch fibres in the rat diaphragm may, on the other hand, be responsible for slower inspiratory efforts, highly resistant to fatigue in the fast breathing animals.

The qualitative ultrastructural organisation of the rat and rabbit skeletal muscle is entirely similar to that of other vertebrates. The differences observed at the ultrastructural level among the various muscle fibre types are related rather to their metabolic character and are common to all skeletal muscle fibres of vertebrates. It is possible now to discriminate fibre types at the ultrastructural level, since all the differences between the A- and M-bands as well as Z-lines measured for three different muscle fibres of both animals are statistically significant (Table 7). The studies of Payne, Stern, Curless & Hannapel (1975) emphasised the importance of the Z-line width, which was found to be consistently greater in slow or Type I than in fast or Type II fibres. However, the M-band width and structure can also be used to distinguish between subpopulations of Type II fibres (Sjöström, Kidman, Henriksson-Larsen & Ängquist, 1982). We, too, were able to show the correlation between the ultrastructural organisation of sarcomeres and particular muscle fibre types of both the rat and rabbit diaphragm (Table 7).

Large differences for fibre type specific Z-line width exist between Types I, II A and II B in the rat and rabbit. This is, of course, true for our calculations, since the detailed architecture of the Z-line has not yet been completely explained and the definition of

the edges of the Z-line have therefore not been settled. However, the present results show that the structure of the Z-line is fibre-type specific and it should therefore be possible to classify muscle fibre types on the basis of its structure and width (Sjöström *et al.* 1982). The structure and presumed function of the M-band has been recently reviewed in detail by Wallimann & Eppenberger (1985). Sjöström *et al.* (1982) showed that the M-band structure is a characteristic of particular muscle fibre types and therefore fibre typing can be carried out with an acceptable degree of success (Table 7; Figs 4, 5).

SUMMARY

The histochemical and ultrastructural characteristics of the adult rat and rabbit costal diaphragm were investigated. On the basis of enzyme histochemistry, the rat diaphragm was found to contain 42% and 39% Type I, 24% and 25% Type IIA and 33% and 34% Type IIB fibres on the thoracic and abdominal surfaces respectively. The rabbit costal diaphragm contained 18% and 26% Type I, 46% and 39% Type IIA and 35% and 34% Type IIB fibres on the thoracic and abdominal surfaces respectively. Differences in the proportion of each muscle fibre type were also observed between diaphragmatic regions (ventral, medial and dorsal) in the rat as well as in the rabbit. Differences in muscle architecture were also noted on the basis of stereological analysis in estimation of volume density, surface density, numerical density and cross-sectional areas of each muscle fibre type. The fine structural analysis of all three fibre types also showed significant differences in the width of the A-bands and Z-lines between the muscle fibre types of the rat and rabbit costal diaphragm.

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