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Original

Mitochondria in smooth muscle cells of viscera

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Abstract

The spatial density of mitochondria was studied by thin-section electron microscopy in smooth muscles of bladder, iris and gut in mice, rats, guinea-pigs and sheep. Morphometric data included areas of muscle cell profiles (~6,000 muscle cells were measured) and areas of their mitochondria (more than three times as many). The visual method delivers accurate estimates of the extent of the chondrioma (the ensemble of mitochondria in a cell), measuring all and only the mitochondria in each muscle cell and no other cells. The digital records obtained can be used again for checks and new searches. Spatial density of mitochondria varies between about 2 and 10% in different muscles in different species. In contrast, there is consistency of mitochondrial density within a given muscle in a given species. For each muscle in each species there is a characteristic mitochondrial density with modest variation between experiments. On the basis of data from serial sections in the rat detrusor muscle, mitochondrial density varies very little between the muscle cells, each cell having a value close to that for the whole muscle. Mitochondrial density is different in a given muscle, e.g., ileal circular muscle, from the four mammalian species, with highest values in mouse and lowest in sheep; in mice the mitochondrial density is nearly three time higher that in sheep. In a given species there are characteristic variations between different muscles. For example, the bladder detrusor muscle has markedly fewer mitochondria than the ileum, and the iris has markedly more.

Key words: mooth muscle, muscle cells, mitochondria, morphometry, comparative quantitation

Introduction

Mitochondria are well in evidence in all smooth muscle cells in every organ of the body; their role in aerobic metabolism is as well established as it is essential. Since the smooth muscles of viscera, or visceral muscles, serve a variety of mechanical roles (even more than vascular smooth muscles do) and have specialized functions in each organ, one might ask whether the mitochondrial apparatus (also known as the chondrioma, that is, the ensemble of the mitochondria in a cell) shows differences in various organs and animal species and ages of life, for example. There is no answer to that question, in published work, and smooth muscle physiologists have

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to make do with the assumption that the chondrioma is the same in all muscle cells, except in certain pathologic conditions. Even a basic morphological parameter such as the spatial extent of the chondrioma within smooth muscle cells has not been studied systematically, in spite of the possibility that quantitative data will help to understand structure and function of those muscles. In addition, related problems of morphogenesis, in evolutionary terms and over the life of a single organism are still very unclear and in need of new observations.

The present study is an attempt (a first attempt in the literature) to put on a quantitative basis the extent of the chondrioma (referred to as spatial density or simply density of mitochondria) in a few smooth muscles of the gut, bladder and iris, in some mammalian species. Wider afield, especially in plant studies and in animal cells grown *in vitro*, chemical and structural differences within populations of mitochondria have been found and are well documented (1, 2).

There are many examples of variation in size and distribution of mitochondria in different cell types. For example, well documented are those in fish muscles with different fibre types (3–5) and in pancreatic acinar cells (6). Fernandez-Vizarra and colleagues (7) have observed differences in number, function, protein composition and morphology in mitochondria from whole-organ homogenates of brain, heart, liver, kidney and skeletal muscle of the rat.

What is particularly interesting, however, is that the chondrioma is in a dynamic state and its volume and functional properties change under special circumstances. This has become clear since the classic studies by Gottfried Schatz on yeast (e.g., 8, 9). Among the studies of adaptive changes, those on skeletal muscle under condition of endurance training are quite substantial (e.g., 10, 11). Buser et al. (12) found an increase in mitochondria in skeletal muscles of growing rats after cold adaptation, paralleling the increase in oxygen consumption.

Variations in structure and function of mitochondria clearly exist; however, the field is so vast and the techniques employed so varied that it is hard to draw general conclusions, even when there are thorough investigations and good solutions of the technical problem of quantitation of mitochondria (13, 14). The difficulty of preservation *in vitro* of the *in vivo* conditions is recognized (15). This matter is highlighted by a simple comparison of the complex internal architecture of mitochondria documented by cryo-electron microscopy (16, 17) with the (rarely published) images of mitochondria after homogenization (but see a good documentation in Hendgen-Cotta et al. [18]). In addition, data on cell lines and other cells grown *in vitro* are not fully representative of cells living within tissues and organs. Also some chemical variations may reflect functional states rather than the existence of different populations of mitochondria.

In contrast since the original observations of Veltri et al. (19) the variation in DNA content between mitochondria of different cell types has been repeatedly confirmed.

There is also some evidence suggesting the possibility that even in a single cell there is functional heterogeneity in mitochondria (20). Subpopulations of mitochondrial, structurally and metabolically distinct were described by Kuznetsov and Margreiter (21), by Riva et al. (22) and by Hollander et al. (23). However, Hendgen-Cotta and colleagues (18) provide strong evidence against the existence of discrete subpopulations of mitochondria in cardiac muscle of the mouse. Battersby and Moyes (24) had already shown absence of subpopulations of mitochondria in skeletal muscles. In this field, terms such as types, populations, heterogeneity, subpopulations and similar carry theoretical aspects that are far from simple, to be added to any transient functional variations in any set of organelles.

The method used in this study is based on electron microscopy, transverse sections of a variety of smooth muscles and a morphometric analysis of micrographs. The methodological aspect of the work is important, as will be discussed, and the method is chosen not in preference but as an alternative to several other strong ap-

proaches in use, particularly those discussed in two extensive publications (13, 14).

The numerical data that will be presented, extracted from electron micrographs, are not used as precise quantitative values but as indicators of constancy, or change, or variation or difference between muscles.

Materials and Methods

Materials

Animals used for this study were: adult Sprague-Dawley rats (body weight 170–250 grams); adult female sheep (ewes; body weight about 35 kg); adult mice, Swiss strain (body weight 25–35 grams); and guinea-pig (body weight 200–500 grams).

Tissues were also examined from guinea-pig fetuses, from very young guinea-pigs aged from 1 to 17 days, and from guinea-pig aged 24 to 36 months (aged guinea-pigs). All the procedures involving materials from animals complied fully with the UK Home Office Regulations under a Personal and a Project License.

Microscopy

All the materials were dissected from freshly killed animals and, after a short passage in Krebs solution, were immersed in fixative, at room temperature. Relaxation of the smooth muscles was maintained or obtained by a brief incubation (20–120 seconds) of the tissue in a Ca^{2+}-free version of Krebs solution at room temperature. Strips of muscle (tenia coli of guinea-pigs) and hollow organs (bladder and gut) were immersed in fixative while maintained in a degree of physiologic distension, mainly in order to avoid contracture and excessive shortening or distortion of the natural arrangement of the muscle cells. The fixative was glutaraldehyde, buffered with Na^{+}-cacodylate and used at a concentration ranging between 3% and 5% in different experiments. All the tissues were post-fixed in osmium tetroxide and dehydrated in graded ethanols and epoxy-propane, before embedding in Araldite.

Sections of about 0.1 μm thickness were cut with glass knives, collected on 200-mesh copper grids or on single-hole grids, and stained with uranyl acetate and lead citrate. They were examined and photographed in an electron microscope. Single electron micrographs were taken on film at medium magnification (8,000 to 24,000x) and were followed by photographic montages at low magnification (3,000 to 8,000x). Positive prints enlarged 2.5x were made, the montages assembled, and all areas of interest scanned on an A3 flatbed scanner at 600 dpi resolution. Some montages were assembled electronically by scanning directly the electron microscope plates at 2,400 dpi resolution.

Morphometry

The microscope images, in digital form, were analyzed with the software FreeHand version 10 and version MX (both originally produced by Micromedia, but no longer supported by Adobe). This required the use of Apple desktop computers operating with an obsolete operating system (earlier than OX 10.6).

The structures of interest were ‘traced’, in order to record and measure their outline, perimeter and area. Strictly speaking this is not a ‘tracing’ procedure: rather, a series of points in linear sequence is placed by hand (by clicking the computer mouse) sequentially along the contour of the object of interest, e.g., a muscle cell or a mitochondrion. The points around an individual object, joined to each other by straight segments, form a polygon; the software then replaces each segment with a Bezier curve for each point, and a smooth profile of the object thus emerges. The precision of this outlining procedure can be controlled by the number of points (mouse clicks) that one enters over the profile of an object (a judicious number of points produces a more faith-

ful outline that an excessive number of points). In practice all the membrane-based elements in a tissue were traced, including those of blood vessels, nerves and other non-muscle cells. For the present study only the outlines of muscle cells, their nucleus and their mitochondria were used.

Several thousand muscle cells (strictly speaking, those were not muscle cells but muscle cell profiles) and more than three times as many mitochondria were traced individually by hand. Number of objects, and area and perimeter of each one of them were obtained by means of NIH ImageJ.

The morphometric data presented are all based on micrographs, that is, on two-dimensional views of the muscles rather than on three dimensions. However, since the mitochondria are round or oval or, when elongated, have rounded ends, the values for perimeters and areas on 2-D sections were taken as comparable to those (unknown) of 3-D surface and volume of those structures. Most of the data are given in percentage form, that is, the percentage of the cytoplasm area that consists of mitochondria, and the term cytoplasm in this case refers to the whole cell minus its nucleus. Actual measurements of areas and perimeters, in micrometres, were also obtained. For the present purpose the use of percentage values is sufficient, and it is preferred because it avoids the great difficulties of obtaining exact size values when accounting for specimen shrinkage in fixation, compression in sectioning and microscope magnification across different instruments, over a large amount of material. The number of mitochondria per cell profile is not used, because it is regarded as an unsafe parameter for comparative work, since it is impossible to have all the preparation in exactly the same condition of relaxation (or contraction), and the individuality of mitochondria when in groups is sometimes uncertain.

Results

Mouse

In the ileum of the mouse, 139 muscle cells of the circular muscle layer (from 3 experiments; summation of the sectional areas measured: 3,534 μm^2) contained 2,347 mitochondria (summation of the sectional areas measured: 301 μm^2), which occupied 8.94% of the cytoplasm. It is not uncommon to find cell profiles with 15 or more mitochondria, often arranged in large aggregates near the central axis of the cell, although they could be found in any part of the cell profiles (which were disproportionately large by comparison with other species) (Fig. 1A and B).

Sheep

In the circular muscle of the sheep ileum mitochondria in muscle cells appear uniformly distributed and are present, usually singly, in the large majority of cell profiles (Fig. 2A and B). They are located in any part of the cell, including areas immediately beneath the cell membrane and areas adjacent to the nuclear envelope. They form a relatively uniform population in terms of size (as can be judged from single sections of a muscle), by comparison with other muscles.

In 1,226 muscle cells (from 6 experiments; summation of the sectional areas of muscle cell profiles measured: 7,796 μm^2) contained 2,023 mitochondria (summation of the sectional areas of mitochondria: 225 μm^2), which occupied 2.93% of the cytoplasm (Table 1).

Guinea-pig

Ileum

In the ileum of young adult guinea-pigs, 614 muscle cells of the circular muscle layer (from 3 experiments; summation of the sectional areas measured: 3,100 μm^2) contained 2,037 mitochondria (summation of the sec-

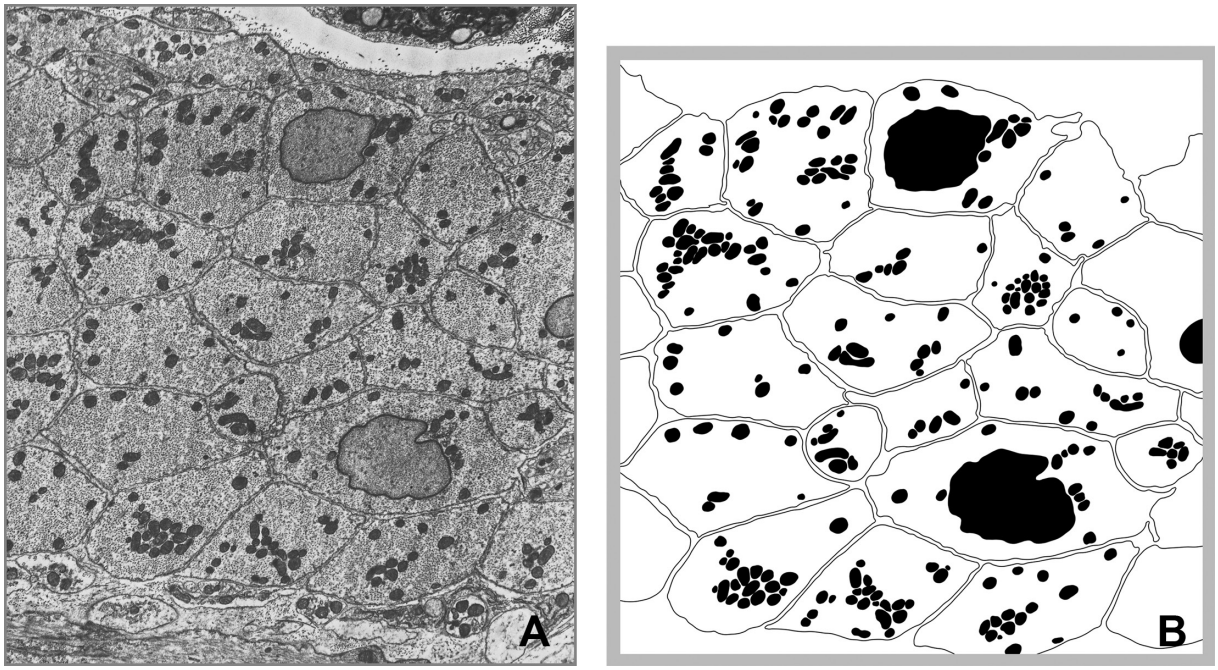

Fig. 1. Ileal muscle of mouse

A. Ileum of mouse, circular muscle layer in transverse section, with submucosa just appearing at top right and longitudinal muscle just appearing at bottom left. Smooth muscle cells of the circular layer (two of them showing the full nucleus profile) are richly endowed with mitochondria, which are highly electron-dense in this preparation and occur singly or clustered in large aggregates; the rest of the cytoplasm appears finely dotted by the relatively uniformly distributed myosin filaments. Width of the microscopic field: ~18 μm.

B. This line drawing is obtained from the micrograph in A, and it covers the same area. The cell membrane of the muscle cells and the envelop of three nuclei are traced in thin lines, and the mitochondria in the full muscle profiles are traced in black-filled small structures. Measurements of mitochondrial density were carried out on images of this type.

tional areas: 126.2 μm^2), which occupied 4.18% of the cytoplasm, calculated from the mean of 3 experiments. No variation was noted across the thickness of the muscle layer (Fig. 3A). In the adjacent longitudinal muscle and in the muscularis mucosae the mitochondrial density (measured on a smaller scale than in the circular layer) was of the same order. In contrast, the accessory muscle cells that form a special layer of circular musculature bordering the submucosa along the entire length of the small intestine (their ultrastructure is described in (25)), consistently had a mitochondrial spatial density at least a third lower than that of the main circular muscle (Fig. 3B), and the same was noted in the ileum of the other species. A large cluster of (apparently) irregularly arranged mitochondria is present beyond both poles of the nucleus. Elsewhere, mitochondria are usually elongated and parallel to the myofilaments; columns of mitochondria along the length of the cell beneath the cell surface are found. Lateral aggregation of mitochondria is rare. Mitochondria are absent in profiles smaller than 1 square micrometer, that is, very close to the tapered ends of the cell. The montages and graphic representation of mitochondrial position do not reveal any evident pattern in distribution (Fig. 3C and D).

Tenia coli

In the tenia coli of young adult guinea-pigs (3 to 5 months of age) mitochondria occupied about 3.9% of the cytoplasm, ranging in 7 experiments from 3.82% to 4.44% (Table 2) (Fig. 4A). During development, spatial density of mitochondria was lower, about 3% around the time of birth (Fig. 4B), then increasing (possibly progressively) (Table 2). At age 19 day the mitochondrial density was within the values of the young adult.

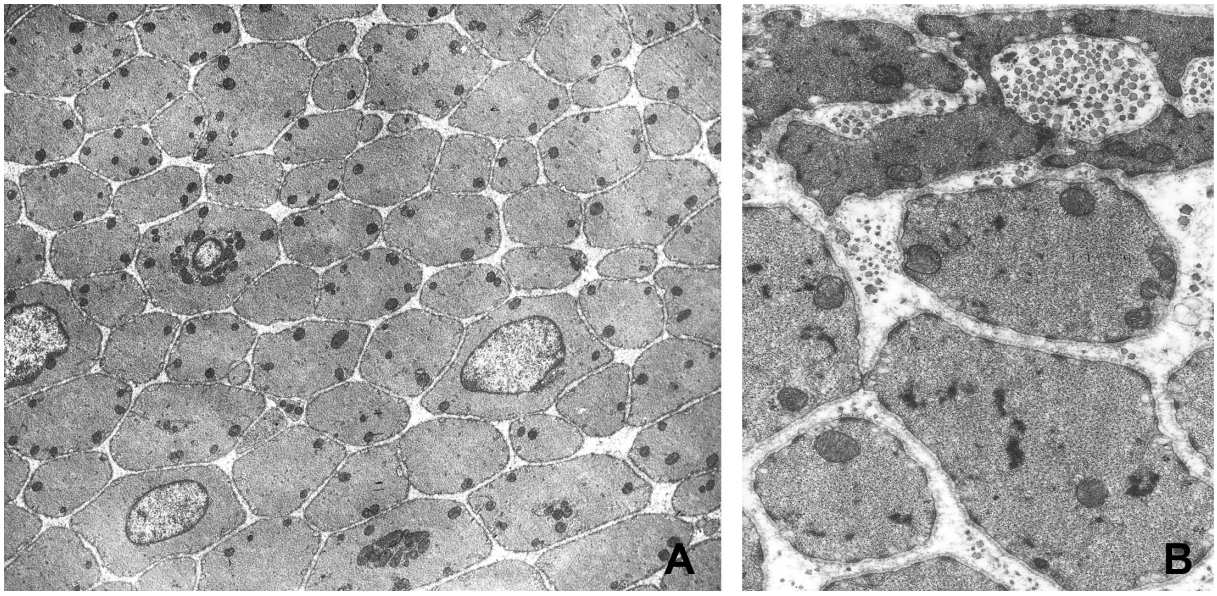

Fig. 2. Ileal muscle of sheep

A. Ileum of sheep, circular muscle layer in transverse section. Smooth muscle cells of the circular layer (four of them showing the full or partial nucleus profile) presenting their mitochondria as electron dense particles in the cytoplasm; most of them occur singly but there is a large cluster of them in the cell profile at bottom middle and another cluster around the pole of a nucleus. Width of field of view: 35 µm.

B. Detail of the circular layer near the border with the submucosa. Some muscle cell profiles display their mitochondria and prominent dense bodies (sites of myofilament insertion); the cell membrane shows numerous dense bands (also for filament insertion) and there is a prominent basal lamina. At the top of the micrograph there are four muscle cells that are smaller, more electron dense, more convoluted in outline, which constitute an accessory thin layer of circular musculature. Collagen fibrils in transverse section (all circular in profile but of non-uniform diameter) are present in the extracellular space. Width of field of view: 8 µm.

Table 1. Mitochondrial density in sheep ileum

Sheep ileum CM	$\Sigma_{1 \text{ to } 6}$	1	2	3	4	5	6
Muscle cells examined, n	1,226	131	854	45	64	72	60
Total muscle cell area, µm^2	7,795.9 µm^2	594.8 µm^2	5,780.9 µm^2	228.2 µm^2	382.8 µm^2	396.7 µm^2	423.1 µm^2
Total area of nuclei	118.68 µm^2	9.57 µm^2	89.49 µm^2	4.84 µm^2	5.37 µm^2	2.75 µm^2	6.66 µm^2
Cytoplasm	7,677.20	585.3	5,691.40	223.3	367.8	394	416.4
Mitochondria examined, n	2,023	217	1,386	77	113	125	105
Mitochondria, total area	224.92 µm^2	16.55 µm^2	165.10 µm^2	5.85 µm^2	12.32 µm^2	12.5 µm^2	12.6 µm^2
Mitochondria, % of area of cytoplasm	2.93%	2.83%	2.91%	2.62%	3.27%	3.17%	3.03%

Circular muscle of sheep ileum; summary of the experiments on 6 animals. In addition to the number of cells (cell profiles) that were measured and their mitochondria (mitochondrial profiles), the Table shows the total area of the cells, the nuclei and the mitochondria measured. The concluding observation (bold column) is the average percentage of mitochondria density (2.93%). The values of the six experiments are also presented individually, to support the conclusion that there was only a modest variability between these animals. All the figures for measurements are given with a decimal point (a numerical artifact and not an indication of the precision of the method) to make it quicker to recognize that they are measurements, here all in micrometres.

In ageing animals, over 2 years old, the values of about 3.6% were at the lower end of the range of the young adults (Fig. 4C and D). In contrast, in animals close to 3 years of age there was a markedly lower value, well below the 3.6% mark (Table 2).

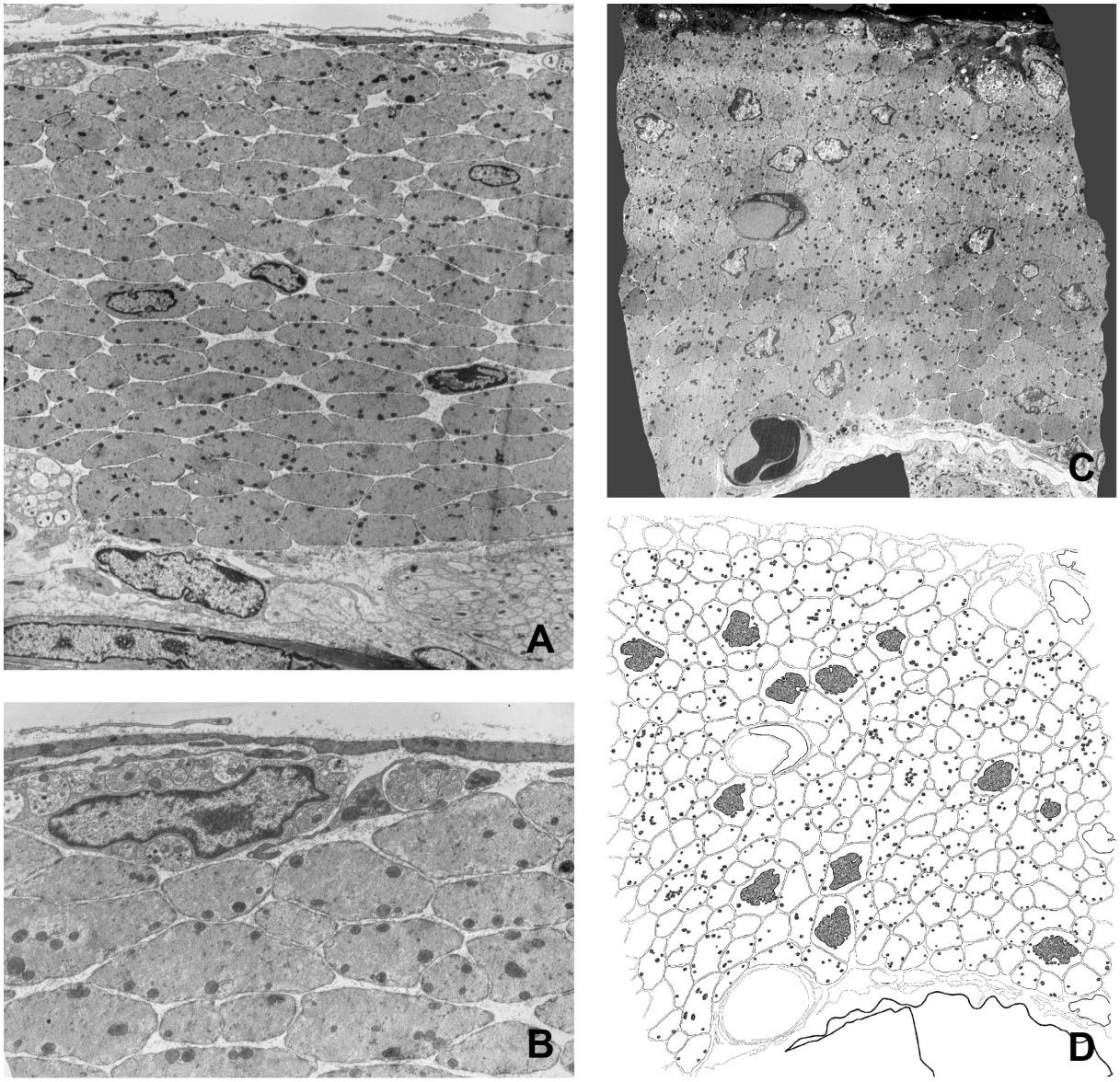

Fig. 3. Ileal muscle of guinea-pig

A. General view of the circular muscle layer of the ileum, in transverse section at low magnification: submucosa at top, a small part of the longitudinal muscle layer at bottom left, part of a myenteric ganglion at bottom right, with a fibroblast between circular and longitudinal muscle layers. The cytoplasm of the muscle cells is studded with particles which are the mitochondria and are highly electron-dense in this preparation. Width of field of view: 50 µm.

B. Higher resolution view of the innermost part of the circular muscle layer. The submucosa is at top and a very thin layer of flattened muscle cells separates it from the circular muscle layer proper. A large nerve bundle is visible, consisting of a glial cell and more than two dozen axons, several of which are laden with axonal vesicles; another, smaller nerve bundle runs adjacent to it. Width of field of view: 19 µm.

C. A montage of the circular muscle layer in its full thickness, as used for the quantitative work. The submucosa is at top, and part of a myenteric ganglion is at the bottom. Mitochondria are highly electron-dense and easy to identify and to trace for counting and area measurement. Width of field of view: 60 µm.

D. This graphic image of the musculature is obtained from the micrographs of Fig. 3C, by tracing the outlines of all the cells (muscle cells, endothelial cells, fibroblasts), the nuclei of the muscle cells (then filling them in dark grey) and the mitochondria of all the full muscle cell profiles (then filling them in black), before counting them and measuring the area of each of them individually.

Table 2. Mitochondrial density in guinea-pig tenia coli

Guinea-pigs	animal code	Number of muscle cells	Number of mitochondria	Spatial density of mitochondria
fetus, term	BMT	83	170	2.87%
newborn	BQE	31	96	2.84%
newborn	BRY	64	138	3.08%
newborn	BQI	74	254	3.05%
4 day old	BQQ	111	243	3.08%
4.5 day old	BQH	54	172	2.99%
9 day old	BQK	32	114	3.12%
17 day old	BRQ	50	162	3.46%
adult	BPK	121	350	4.02%
adult	BPJ	209	617	3.82%
adult	BQP	75	307	3.84%
adult	BPW	84	244	3.91%
adult	AJG	148	451	3.99%
adult	AJH	214	705	4.44%
adult	BIJ	252	881	4.08%
aged (~2 year old)	BSJ	55	246	3.67%
aged (~2 year old)	BSK	267	778	3.52%
aged (~3 year old	BQT	117	451	2.80%
aged (~3 year old)	BQZ	46	212	2.75%

Spatial density of 4,591, mitochondria in 2,087 muscle cell profiles from the tenia coli of guinea-pigs, from birth to old age. The three numerical columns give the number of muscle cell profiles measured, the number of mitochondria in those cell profiles, and the percentage area of the muscle cell cytoplasm occupied by those mitochondria.

Bladder

In the guinea-pig bladder detrusor muscle, 139 muscle cells had 345 mitochondria, which occupied 2.63% of the cytoplasmic area (weighted average; the percentages from 3 experiments were 2.90%, 2.55% and 2.51%).

Iris

In the sphincter pupillae - the ring of usually entirely smooth musculature around the pupil - mitochondria were particularly abundant: in 172 muscle cell profiles (from 3 experiments) there were 1,045 mitochondria occupying 9.07% of the cytoplasm (the percentages from the three experiments, each with about 60 cells, were 9.60%, 9.26% and 8.41%). In this muscle, mitochondria are often clustered or stacked sideways, and have an extensive association with cisternae of sarcoplasmic reticulum. The average size of the profiles is smaller than in other muscles and their distribution quite irregular and matching the irregular shape of the muscle cell profiles (Fig. 5A and B).

Rat

Ileum

In the ileum of the rat, in 749 muscle cell profiles of the circular muscle layer from 4 experiments 3,421 mitochondria occupied 6.61% of the cytoplasm (the percentages from the 4 experiments were 7.32%, 6.06%, 6.43% and 6.14%) (Fig. 6A–D). The spatial density of mitochondria was not affected by the muscle being shortened in contraction or being elongated in relaxation (data not shown); the average size of the mitochondrial profiles, however, was much greater in the contracted muscle, roughly in proportion to the expanded sectional

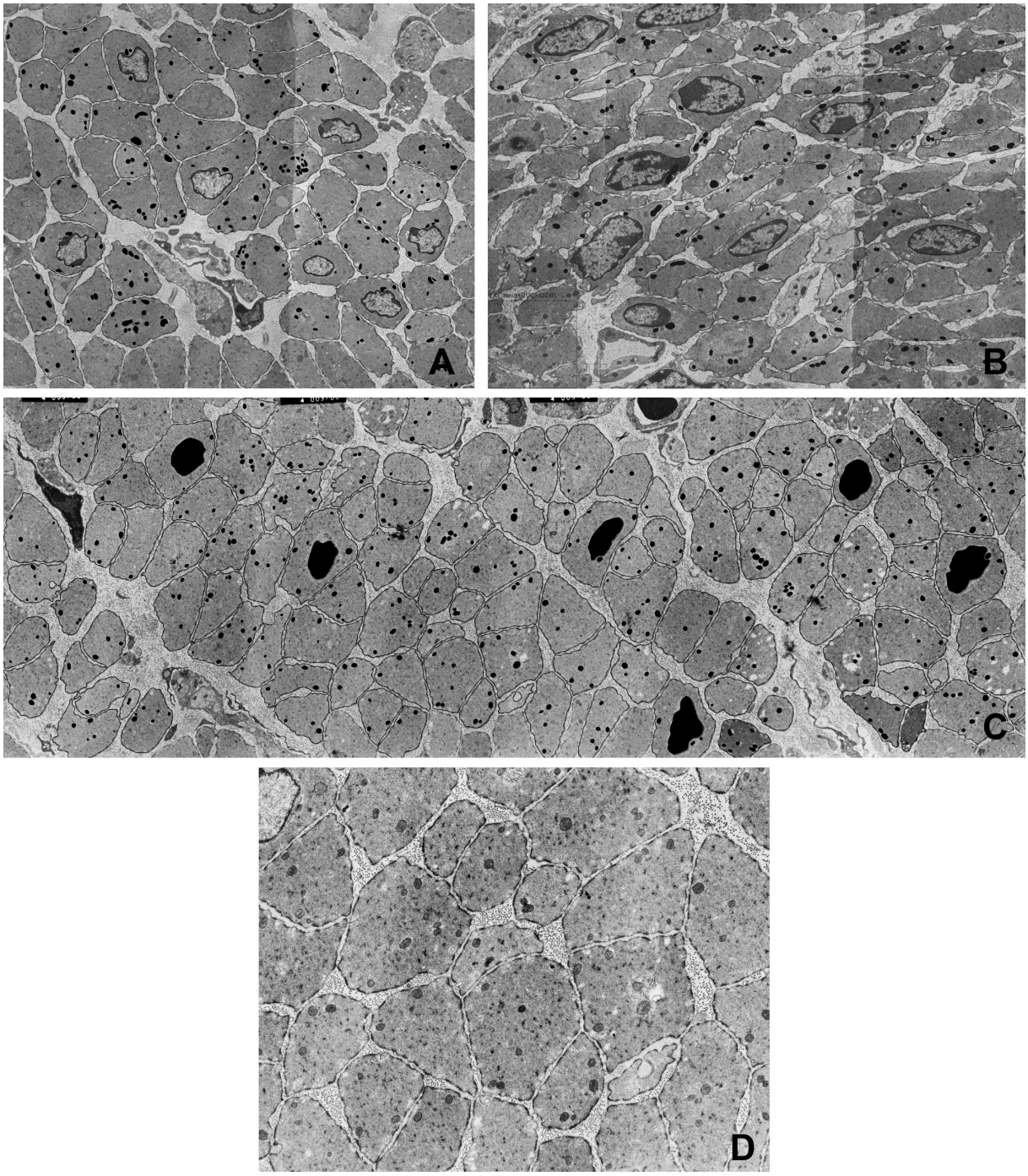

Fig. 4. Tenia coli of guinea-pig

A. Part of a montage from an adult guinea-pig tenia coli sectioned transversely. For the morphometric work in this montage the muscle cell profiles were traced and a black line was superimposed on the cell membrane of the muscle cells (the cell outlines thus look particularly sharp). In the muscle cell profiles that are fully included in this field of view the mitochondria have also been traced and filled in black, in preparation for counts and measurements. Width of field of view: 50 µm.

B. Part of a montage from the tenia coli of a newborn guinea-pig, in transverse section. All the muscle cell profiles are traced and black line are superimposed on the cell membranes. In the muscle cell profiles the mitochondria have also been traced and filled in black, in preparation for counts and measurements. Width of field of view: 35 µm.

C. Part of a montage from the tenia coli of an aged guinea-pig, in transverse section. All the muscle cell profiles are traced and black lines are superimposed on the cell membranes. In the muscle cell profiles the mitochondria have also been traced and filled in black, in preparation for counts and measurements. Width of field of view: 90 µm.

D. Plain electron micrograph with a detail from the montage and tracing in C showing the cytological features of the tenia muscle cells in an aged animal. Width of field of view: 35 µm.

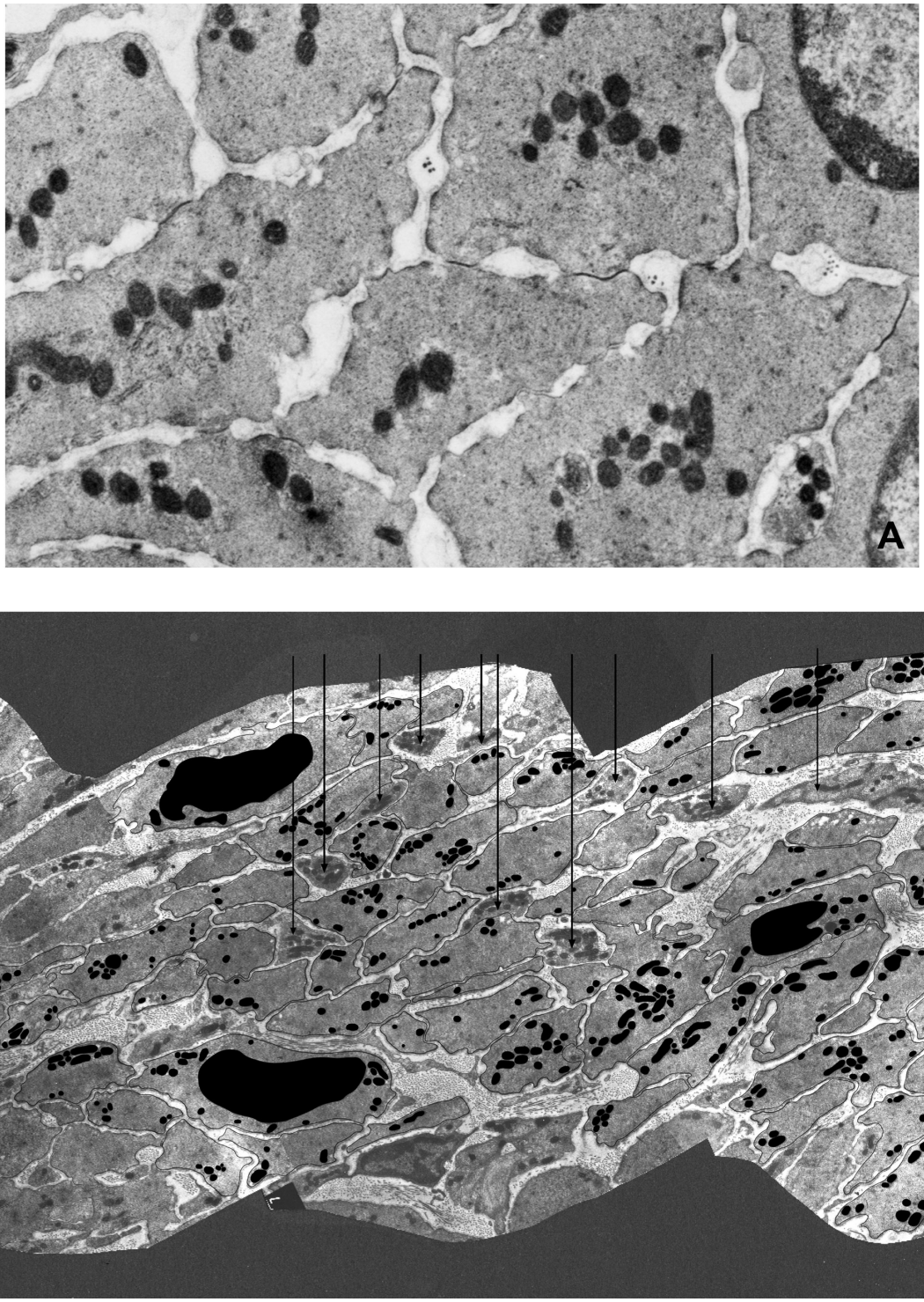

Fig. 5. Sphincter pupillae of guinea-pig

A. Iris of guinea-pig, sphincter pupillae muscle in transverse section (radial to the pupil). Smooth muscle cells present highly irregular outlines and large numbers of mitochondria. Width of field of view: 24 µm.

B. Iris of guinea, sphincter pupillae in transverse section (montage), with muscle cells traced (thin black lines) and muscle cell mitochondria traced and filled in black, superimposed on the electron micrographs. The high spatial density of mitochondria in this muscle compared with other muscles is well in evidence. Small, untraced profiles (arrowed) are axons, and they too are particularly rich in mitochondria. Width of field of view: 32 µm.

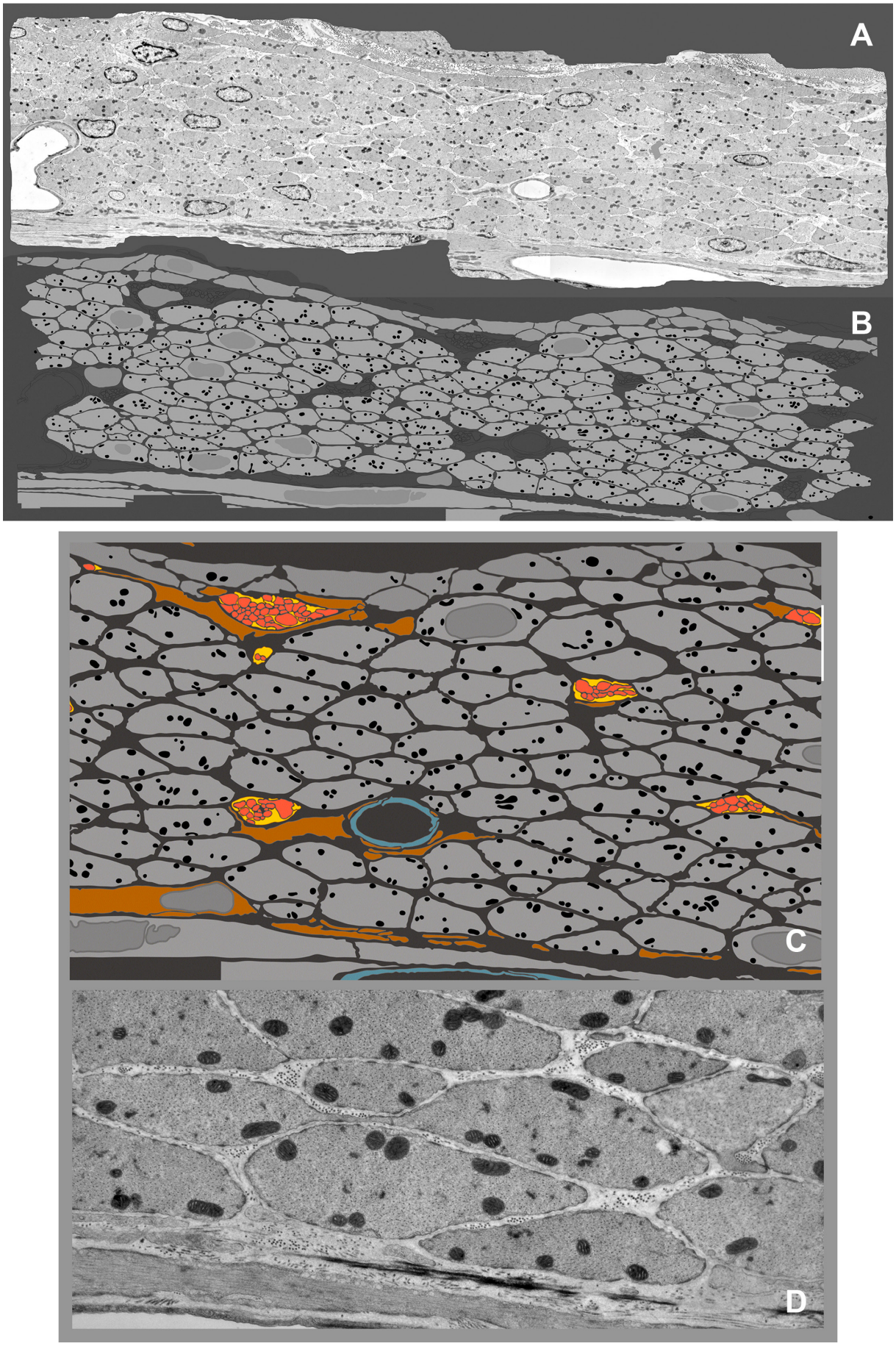

Fig. 6.

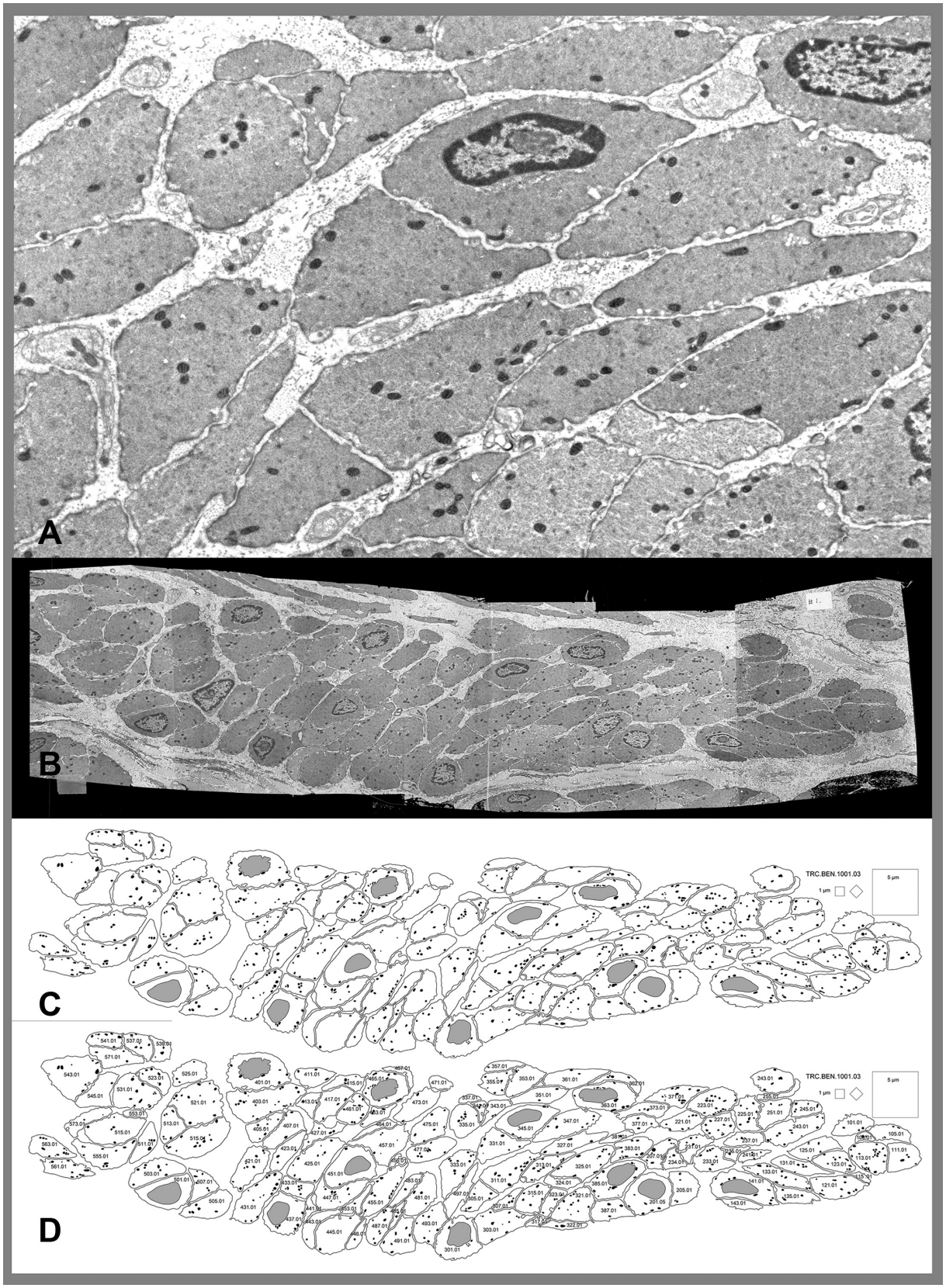

Fig. 7.

area of the cells, a difference accounted for by some change in shape of the mitochondria and by some change in their orientation.

Bladder

In the urinary bladder detrusor muscle 419 muscle cell profiles (from 3 experiments) contained 2,155 mitochondria, which occupy about 3.13% of the cytoplasm sectional area (the averages in the 3 experiments were 3.24%, 3.04% and 3.16%). Distribution and size are similar to those of the small intestine (Fig. 7A). In an experiment on the rat detrusor a muscle bundle of 127–135 muscle cells was examined in serial sections over a depth of about 100 μm (Fig. 7B–D). The mitochondria density in each section of the series varied from 3.06% to 3.24%. On the basis of serial sections, in this bundle of some 130 muscle cells, in exact transverse section (the number of cells included varies slightly along the series, due to mid-bundle terminations and origins of muscle cells) the spatial density of mitochondria was uniform, as one would expect even if there were variations in the single cells. The consistent value of the spatial density works more as a validation of the measurement method than an original observation. In this preparation, as in all smooth muscles, individual muscle

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Fig. 6. Ileal muscle of rat in four views

A. Ileum of rat, circular muscle in transverse section. Four views from the same preparation. Montage of electron micrographs of the full thickness of the circular muscle layer, with cells of the accessory muscle layer just appearing at top right and some longitudinal muscle cells at bottom; the two clear structures are blood vessels. At this magnification only the muscle cells, their nuclei and their mitochondria can be recognized. Width of field of view: 160 μm.

B. In this line picture, covering approximately the area of the photographic montage and obtained with the tracing procedure, the muscle cells are in grey and their mitochondria in black. All the other elements of the tissue, including the extracellular space and non-muscle cells (with the exception of the layer of accessory circular muscle cells at the top have been darkened.

C. A detail from the traced montage as shown in B, with added tracings of the nerve bundles (axons in red and glia in yellow), of a blood vessel (endothelium in blue) and of the other non-muscle cells (in brown). Width of field of view: 62 μm.

D. A micrograph with further detail of the muscle layer, as in B, showing muscle cells, their mitochondria, as they appeared under the microscope. Even before tracing, the mitochondria are optically dense and easy to recognize and measure. Width of field of view: 18 μm.

Fig. 7. Detrusor muscle of bladder of rat

A. Smooth muscle cells in transverse section, surrounded by extracellular space with collagen fibrils and by small nerve fibres. In all the muscle cell profiles there are mitochondria, round or oval dark structures; the nucleus is visible in three cell profiles. The same group of cells is also visible right of centre of the micrographs below. Width of the microscopic field: 21 μm.

B. Low-magnification of a muscle bundle in transverse section (the same as in A.) that was used for serial sections. A few muscle cells in oblique section lie around the bundle. The enlargement in B–D. is shown through the calibration squares on 1 and 5 μm in the tracings, near the right border. Width of field of view: 100 μm.

C. Tracing of the muscle bundle in B for measuring mitochondrial spatial density. The cell membrane of all the muscle cells of the bundle, the mitochondria and the nuclei are traced; the nuclei are filled in grey and the mitochondria are filled in black. There are 136 muscle cell profiles in the bundle at this level, 11 nuclei and 765 mitochondria. The sectional area of each muscle cell profile (minus the nucleus) and of each mitochondrion was measured, in order to obtain the mitochondrial spatial density.

D. The same image as in C, to which numbering has been added. Each muscle cell profiles bears a double set of digits, the first indicating the assigned number to that cell and the second indicating the number of the section in the series. For example the nucleated cell profile at the top of A, which also appears in all the other images, is labelled 341.01, being cell n. 341 in the first section of the series.

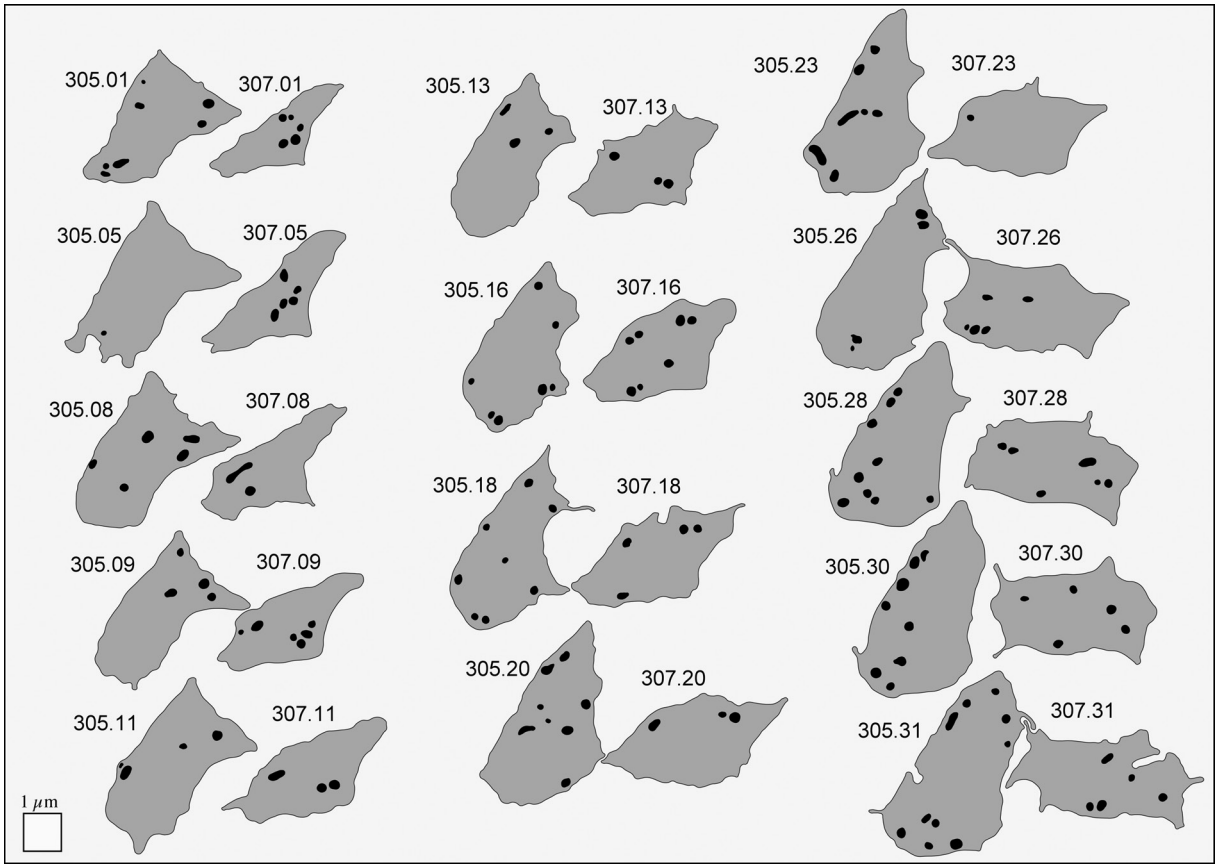

Fig. 8. Muscle cells of the detrusor muscle of rat bladder.
From the same preparation illustrated in Fig. 4, two muscle cells (number 305 and 307), shaded in grey, are followed in some serial sections (from level 01 to level 31); they are non-nucleated at these levels. Their mitochondria are filled-in in black. Over the sequence there is a remarkable change in shape of the profiles of the two cells, whose space relationship also varies rapidly; it should also be noted how un-smooth these cell profiles are. The number of mitochondria varies considerable between the individual profiles; however, their average spatial density over the series is close to the one calculated for the whole bundle. A calibration square (1 μm) is at the bottom left.

cell profiles have a large variation in the number and spatial density of mitochondria, some small profiles, and occasionally even a large profile showing no mitochondria. Mitochondria are again absent in cell profiles of less than 1 square micrometres. However, each muscle cell followed in serial sections, collecting the data from up to 30 profiles at different serial levels, shows a mitochondrial density very close to the average density of the entire bundle (Fig. 8). The variation is of the order of 2–4%.

Other animal species

Data on other animal species were collected but were not sufficiently extensive for proper quantitation.

Rabbit

In a large preparation of the ileum, 311 muscle cell profiles of the circular layer contained 1,277 mitochondria, which occupied 4.76% of the cytoplasmic sectional area.

Shrew

In the shrew (a small mammal with a body weight below 10 g), in a large preparation of the ileum 232

muscle cell profiles of the circular layer contained 1,311 mitochondria, which occupied 5.91% of the cytoplasmic sectional area.

Amphibians, fishes, reptiles and birds

Mitochondrial density in smooth muscles of amphibian was assessed in margin of the present study. Typically, in the small intestine of the Axolotl the mitochondrial density in muscle cells was 1.8%. In intestinal muscles of frog, Xenopus and bullfrog the mitochondrial density was consistently well below the 2% mark. Similar values were obtained from intestinal muscles of fish (ray fish and angler fish). In a few hundred muscle cells of the small intestine of turtles and chickens the mitochondrial density was consistently within the 2.0–2.5% range.

Discussion

The first concern to discuss is about the reliability of the procedures used, and their limitations. Many methods have been used for quantitative studies of mitochondria, the majority based on biochemical assays (13, 14, 26). Some of them have great attraction, but all have considerable and well-acknowledged limitations; in addition, the very question of what is meant with quantitative evaluation of the chondrioma has complex theoretical aspects.

On the one hand, the morphometric method used in this study, based on electron micrographs and a graphics software, can be very accurate in outlining the objects to be measured, in obtaining their dimensions and in producing a permanent and accessible digital record of the entire procedure – images, measurements, calculations. All the materials can be revisited for checks and further analysis. The mitochondria (all of them and only them) can be readily identified microscopically, and there is no risk of contamination from other cellular elements or from mitochondria of other cell types within the tissue. A further attraction of the method is that the data are partly figurative, rather than entirely numerical, a possible advantage in the eye of morphologists.

On the other hand, a difficulty of the method is its reliance on adequate preservation of the tissues for microscopy. Preparation artifacts affecting the mitochondria (such a shrinkage, swelling, cutting compression) are hard to avoid entirely, and more difficult still it is to recognize whether an artifact is present or not. A further important weakness of this work is that all mitochondria were regarded as equal, varying only in size and number, which is surely an oversimplification, even if it is currently the basic assumption in the literature on smooth muscle. In any case, data on the extent of the chondrioma only show the maximum “mitochondrial capacity” of a cell or a tissue, not what activity the organelle plays at any functional moment. In other fields, chemical and structural differences among mitochondria have been observed, as was mentioned in the Introduction.

The first conclusion to draw from the data is that the mitochondrial density, that is, the percentage of the cytoplasm volume (or of the cell volume minus the nucleus) that is occupied by mitochondria, varies markedly in different smooth muscles, roughly from less than 2 to almost 10 percent. Even the highest values observed are small by comparison with those of skeletal muscles and very small by comparison with those of cardiac muscle. For example, Park, *et al.* (6), who used citrate synthase activity as an index of mitochondrial density in human muscles, obtained relative values of 222, 115 and 48 for cardiac, skeletal and smooth muscles, respectively; however, since respiration activity varied in the same proportion, the authors concluded that the respiratory activity per mitochondrion is similar in the three types of muscle. In smooth muscles of mammals it seems that the 10% spatial density is the upper limit for mitochondrial density, and in fact it is not a very com-

mon value, and the lower limit is around 2%, over a range of species and muscles. Looking at other Vertebrates, including fishes, reptiles and amphibians (preliminary observations not illustrated here) the mitochondrial density was lower than in mammals but it never fell below about 1.8%. In smooth muscles of mammals it seems that 10% spatial density is the upper limit for mitochondrial density, and in fact it is not even a very common value, and the lower limit is around 2%, over a range of species and muscles. Looking at other Vertebrates, including cold-blooded species of fishes, reptiles and amphibians (preliminary observations, not illustrated here) the mitochondrial density was lower than in mammals but it never fell below about 1.8%.

The large differences in mitochondrial spatial density in different muscles that are observed by microscopy are regarded as genuine differences between the tissues (and not as artifacts), and variations of this magnitude raise many questions. The variation may have biological significance in two respects. First, the variation in the spatial density of mitochondria may impart (but not necessarily) some different functional property to each muscle. Second, the structural features of the chondrioma are the outcome of life-long morphogenetic processes, and should not be interpreted solely as an expression of the functional 'needs' of the tissue.

The second conclusion is that the ample variation of mitochondrial density is in contrast with its relative uniformity within a given muscle in a given animal species. It is true that different experiments on the same material show some variability, and, even if some variability is introduced and is accounted for by the method used, some of it is surely a property of the tissue itself. So, for example, in the case of the tenia coli variability between preparations is quite evident. Nevertheless, there is no overlap in the range of the values for the tenia with those for the ileum of the same species, allowing the conclusion to be made that there is a range of spatial density values that is characteristic of each muscle type or animal species. Similarly, while the results from the guinea-pig ileum are not identical in different experiments, nevertheless there is no overlap in the data from the experiments on the ileum of mice, rats, guinea-pigs and sheep; each one of the four species seems to have a characteristic non-overlapping range of values for their chondrioma.

A third point concerns the variability of mitochondrial density among the cells of a given muscle, suggesting the presence of some species-specific mechanisms. In an individual muscle cell profile the value of mitochondrial spatial density has no interest: it amounts to a measurement on a small fraction of a one hundredth of the volume of that cell (a section of 0.1 μm thickness out of an elongated cell 600–800 μm long). Serial sections, in contrast, are interesting because they allow measurements to be made on several profiles from the same muscle cell. In the work with serial sections the larger the number of sections of a single cell that are examined, the closer the value of mitochondrial density for that cell gets to the average value for the whole muscle. How much variation would remain if one had access to all the sections of a single cell through its entire length, cannot be established. However, the true variation between cells would be quite small, certainly well below +/–5%. This is the conclusion offered by the rat detrusor muscle; its validity also for other smooth muscles seems probable, but remains to be proved.

The mitochondrial density, therefore, appears to be somehow regulated, around small oscillations and with small differences between the cells of the same muscle. What are the possible regulation mechanisms, and how they work, is unknown. It should also be noted that a given percentage value of spatial density depends on at least two factors, the mitochondria themselves on the one hand, and the rest of the cell on the other: for example, mitochondria might be entirely static but their density could fall because of an increase in the volume of the contractile material in the cell. Processes bringing in new mitochondria or eliminating old ones are known in many cell types (27–29), including bladder smooth muscle (30); however, nothing can be said yet as to the role of mitogenesis and mitophagy in the comparative difference described here.

As a fourth conclusion, in the circular musculature of the ileum there is intra-species consistence in mi-

tochondrial density, as discussed, but also a characteristic variation between the four species studied. There is some correlation between mitochondrial density of visceral muscle cells and the body size of the species examined. However, the correlation has only limited merit, because it is rather vague and there are many exceptions (the shrew has values closer to those of the rat than to those of the mouse, for example, and the rabbit has values close to those of the guinea-pig). A link between body size and cell size is an intriguing feature, and one that is observed only in a very few cell types in the body. While more observations will cast light on the nature of these links, the current data simply suggest that among the many factors influencing the muscle structure there is also the body size of the species and its relationship with the basic metabolic rate. Recent studies have cast much doubt on the value of these relationships, centred on the notion of scaling, in all tissues in general (31).

A fifth conclusion is drawn by comparing mitochondria in different muscles from the same species. While along the gut of the guinea-pig variations in the chondrioma are small and probably of little significance (and possibly, as usual, partly accounted for by technical variations), in contrast ileum and urinary bladder show a clear-cut difference. Ileal circular muscle has 30% more mitochondria per unit volume than the detrusor muscle of the bladder. In the rat the difference is even greater, with the bladder having only half the mitochondrial density than the ileum. The differential between gut and bladder is found both in guinea-pigs and in rats, while the differential between guinea-pigs and rats remains the same in the two organs. Such a large difference suggests the occurrence of a substantial difference in the energy access of the two muscles, by mitochondrial respiration and by anaerobic energy production via glycolysis. In a parallel study (32) in the same segment of ileum used for the present work, the mitochondrial spatial density in the myenteric neurons was found to be about twice as large as that in the adjacent smooth muscle, and there was a similar differential between different mammalian species; in contrast, the corresponding enteric glial cells gave values similar to those of muscle cells, that is, much lower than those of the neurons.

The iris has the highest mitochondrial endowment of all smooth muscles, twice as large a chondrioma in the guinea-pig iris than in the bladder (a very large difference was observed also in rats and rabbits, even without quantitative evaluations). No explanation can be offered for this richness in mitochondria in the sphincter pupillae of the iris; in principle, it may be related to some of the unique features of that muscle, such as the high speed of contraction, the minimal external resistance to shortening or the intense exposure to light. Visible light has marked effects on mitochondrial functions (33, 34); whether this has any physiological significance for the musculature of the iris remains to be investigated.

Age-related changes in the chondrioma are well known and rather extensive in some cell types, including skeletal muscle fibres (35–37). Here only preliminary data are available, on the tenia coli of guinea-pigs, and they suggest only a minor reduction in aged animals (except in case of extreme old age). These data are difficult to interpret in view of the continuous changes in muscle cell size and shape, organelles, density of innervation, extracellular materials occurring in the tenia throughout life (38).

Mitochondria are highly dynamic organelles (39–41) and growth, fission, budding, change in shape are linked with the data presented here, yet not understood. The relationship of mitochondria with the endoplasmic reticulum, well explored in preparations in vitro, is not clear in cells *in situ*, even in the case of smooth muscle cells where the movement of calcium between cytoplasmic compartments is essential in the control of contraction. The links of mitochondria with microtubules and the cytoskeleton, crucial in a tissue capable of extensive isotonic contraction, should be explored, especially if detected in a whole tissue in conditions close to natural life.

Declaration

The Author declares that there is no conflict of interest with this work and the preparation of the paper.

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