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Mitochondrial myopathy in rats fed with a diet containing beta-guanidine propionic acid, an inhibitor of creatine entry in muscle cells

Z. Gori, V. De Tata, M. Pollera and E. Bergamini
Istituto di Patologia Generale, II Cattedra, Via Roma 55, I-56100 Pisa, Italy

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Summary. In rats with phosphoryl-creatine depletion (fed a standard Randoin–Causeret diet containing 1% beta-guanidine propionic acid) abnormal mitochondria were observed in slow skeletal muscles, often containing paracrystalline inclusions very like those induced by ischaemia or mitochondrial poisons and in human mitochondrial myopathy.

Keywords: mitochondrial myopathy

Beta-guanidine propionic acid (GPA) is a synthetic analogue of creatine that is readily absorbed at the intestinal level and then inhibits the entry of creatine into muscle cells and causes depletion of creatine and creatine phosphate from muscle (Fitch *et al.* 1974). The addition of GPA to diet leads to the replacement of creatine and creatine phosphate by GPA and GPA-phosphate and may impair functioning of the creatine phosphate energy-shuttle (Bessman & Carpenter 1985).

It is well known that GPA administration to experimental animals may lead to changes in metabolism (e.g. Fitch *et al.* 1974; 1975), function (Fitch *et al.* 1975; 1978; Petrofsky & Fitch 1980) and structure (Schields *et al.* 1975; Laskowski *et al.* 1981) of the skeletal muscle. In this report, we show that a prolonged administration of the drug causes mitochondrial alteration in rat muscle fibres (but not in capillary endothelia) very like that induced experimentally by ischaemia (Hanzlikova & Schiaffino 1977; Heine & Shaeg 1979) or by exposing animal muscles to certain mitochondrial poisons (Melmed *et al.* 1975; Sahgal *et al.* 1979) and described in human mitochondrial myopathy (see e.g. Scarlato & Cerri 1983).

Materials and methods

Animals

Random groups of male Sprague–Dawley rats (100 g initial body weight) were fed *ad libitum* either with a standard Randoin–Causeret diet in pellets (manufactured by Piccioni, Bergamo, Italy) or the same diet containing 1% GPA. Addition of the drug to diet had no significant effect on food intake or daily body weight gain (Schields & Whitehair 1973) and had a significant effect on muscle weight gain very lately (Table 1). Treated animals appeared to be healthy on casual inspection and exhibited a barely detectable decrease in the running performance (as in Schields & Whitehair 1973; Laskowsky *et al.* 1981).

Correspondence: Professor Ettore Bergamini, Istituto di Patologia Generale, II Cattedra, Via Roma 55, 56100 Pisa, Italy.

Table 1. Effect of the administration of a diet containing 1% GPA for 2, 4 and 8 weeks on body weight gain and on the weight gain of the extensor digitorum longus (EDL) and of the soleus muscles (S) of rat

Treatment	GPA	Body weight (g)	EDL (mg)	S (mg)
2 weeks	–	154±7.8 (9)	73±6.3 (9)	60±4.3 (9)
	+	157±5.9 (10)	69±3.5 (10)	56±3.7 (10)
4 weeks	–	178±7.0 (7)	82±5.7 (7)	66±5.2 (7)
	+	183±10.6 (7)	73±5.6 (7)	62±5.3 (7)
8 weeks	–	360±13.6 (10)	119±6.3 (10)	101±6.7 (10)
	+	258±12.8 (10)	96±5.0 (10)*	76±2.4 (10)**

Means ± SEM are given, in parentheses the number of cases. * and ** denote statistically significant differences ($P<0.05$ and $P<0.01$ respectively, Student's t-test).

Ultrastructural studies

On days 7, 14, 28 and 56 after the beginning of the administration of the drug, fast (the extensor digitorum longus, EDL) and slow (the soleus, S) muscles were rapidly removed under nembutal anaesthesia (5 mg/100 g b.W.) and samples processed for electron microscopy. Small blocks of tissue fixed in cacodylate-buffered 2.5% glutaraldehyde were washed in 0.1 M phosphate buffer (pH 7.3) and post-fixed on 0.1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3), dehydrated and finally embedded in Polybed. Sections were stained with uranyl acetate and lead citrate and observed with a Siemens Elmiskop I microscope.

Biochemical methods

Muscles were quickly frozen with metal clamps precooled in liquid nitrogen and stored at −70°C. The frozen muscles were homogenized in 0.6 M $HClO_4$ (5 volumes) and centrifuged at 1000 g for 10 min. The supernatant was neutralized with $KHCO_3$ 2 M and centrifuged to remove the $KClO_4$ sediment. All the steps were carried out at 0°C. Creatine and phosphoryl-creatine were assayed by the colorimetric procedure described by Ennor & Rosenberg (1952). GPA and phosphorylated GPA were assayed according to Fitch and Chevly (1975) by using the colorimetric reaction described by Bonas *et al.* (1963).

Chemicals

GPA was synthesized and purified according to Rowley *et al.* (1971) and incorporated into the diet. All products used were of analytical grade.

Results

Table 1 shows that GPA administration eventually leads to hypertrophy of both the fast-twitch (EDL) and the slow-twitch (S) muscles. Table 2 shows that this treatment causes a similar depletion in phosphoryl-creatine in the two muscles.

At the electron microscope level, mitochondrial alterations were observed in the subsarcolemmal and perinuclear areas of the S, but not of the EDL muscle; the intermyofibrillar mitochondria retaining a nearly normal appearance. The mitochondrial changes varied with the time of feeding. By week two (Fig. 1*a, b, c*) pleomorphic and enlarged and giant mitochondria (6- to 8-fold larger than normal) were seen, sometimes with a very dense matrix and no visible cristae. Cristae

Table 2. Effect of the administration of a diet containing 1% GPA for 2, 4 and 8 weeks on the content in creatine (C), phosphorylcreatine (PC), GPA and phosphoryl GPA (PGPA) of the extensor digitorum longus (EDL) and of the soleus muscles (S) of rat

Treatment	GPA	C	PC	GPA	PGPA
EDL					
2 weeks	−	37±2.9 (5)	39±1.6 (5)		
	+	17±2.7 (4)**	20±2.6 (5)**	10±0.6(4)	16±1.9 (4)
4 weeks	−	30±2.1 (3)	24±3.8 (3)		
	+	10±0.8 (5)**	6±1.0 (5)	11±0.4(5)	17±1.6 (5)
8 weeks	−	28±1.9(4)	25±1.1 (3)		
	+	10±0.4 (6)**	6±0.9 (6)**	11±0.5(6)	20±0.7 (6)
S					
2 weeks	−	32±4.2 (5)	29±3.2(5)		
	+	19±3.3 (6)*	15±2.7 (6)**	14±1.3(3)	15±2.3 (3)
4 weeks	−	23±1.2(5)	14±3.3 (3)		
	+	7±0.8 (5)**	4±0.4 (5)**	15±0.5(5)	19±2.6 (5)
8 weeks	−	21±1.7(4)	17±2.1 (3)		
	+	8±0.5 (6)**	2±0.4 (6)**	13±0.3 (6)	14±0.7 (6)

Results are given as μmoles/g wet muscle. Means ± SEM are given, in parentheses the number of cases. * and ** denote statistically significant differences ($P<0.05$ and $P<0.01$ respectively, Student's *t*-test).

with an irregular orientation and with a concentric configuration could be detected frequently. By week 4 (Fig. 2*a*, *b*, *c*), in addition to the above-mentioned alterations, abnormal cristae with a tubular instead of the usual plate-like configuration were observed and paracrystalline inclusions could be detected in some small, or normal or giant mitochondria, resembling those described in experimental and human mitochondrial myopathies. On week 8 (Fig. 3*a*, *b*), additional alterations were very elongated forms, and enlargement with a dense matrix and cristae with an irregular often longitudinal arrangement (Fig. 4*a*, *b*). We saw also very thin and elongated worm-like organelles crowded with cristae showing a regular longitudinal orientation (Fig. 5*a*, *b*). Paracrystalline inclusions were seen in many mitochondria in a highly variable number (Fig. 5*b*).

Perhaps we should mention that abnormal mitochondria were embedded in a glycogen-rich sarcoplasm, and that clusters of glycogen granules often were seen included in round-shaped areas, which may not show a limiting membrane and that autophagic vacuoles containing membranous and amorphous material could be observed in these mitochondria-rich areas.

Signs of mitochondrial alterations were not detectable in the EDL muscle, with the exception of an enlargement of subsarcolemmal mitochondria (on weeks 4 and 8 of treatment). On the other hand, heart mitochondria were clearly altered in 8-week treated rats (but never exhibited paracrystalline inclusions) irrespective of their location within the cell (Figs 6*a*, *b*; 7). They were quite pleomorphic, sometimes with a dense matrix and poorly defined cristae or with a very dense matrix and parallel irregularly oriented cristae and well-defined clear intracrystal spaces, or with an increased number of cristae of irregular or concentric orientation. Differences in appearance were

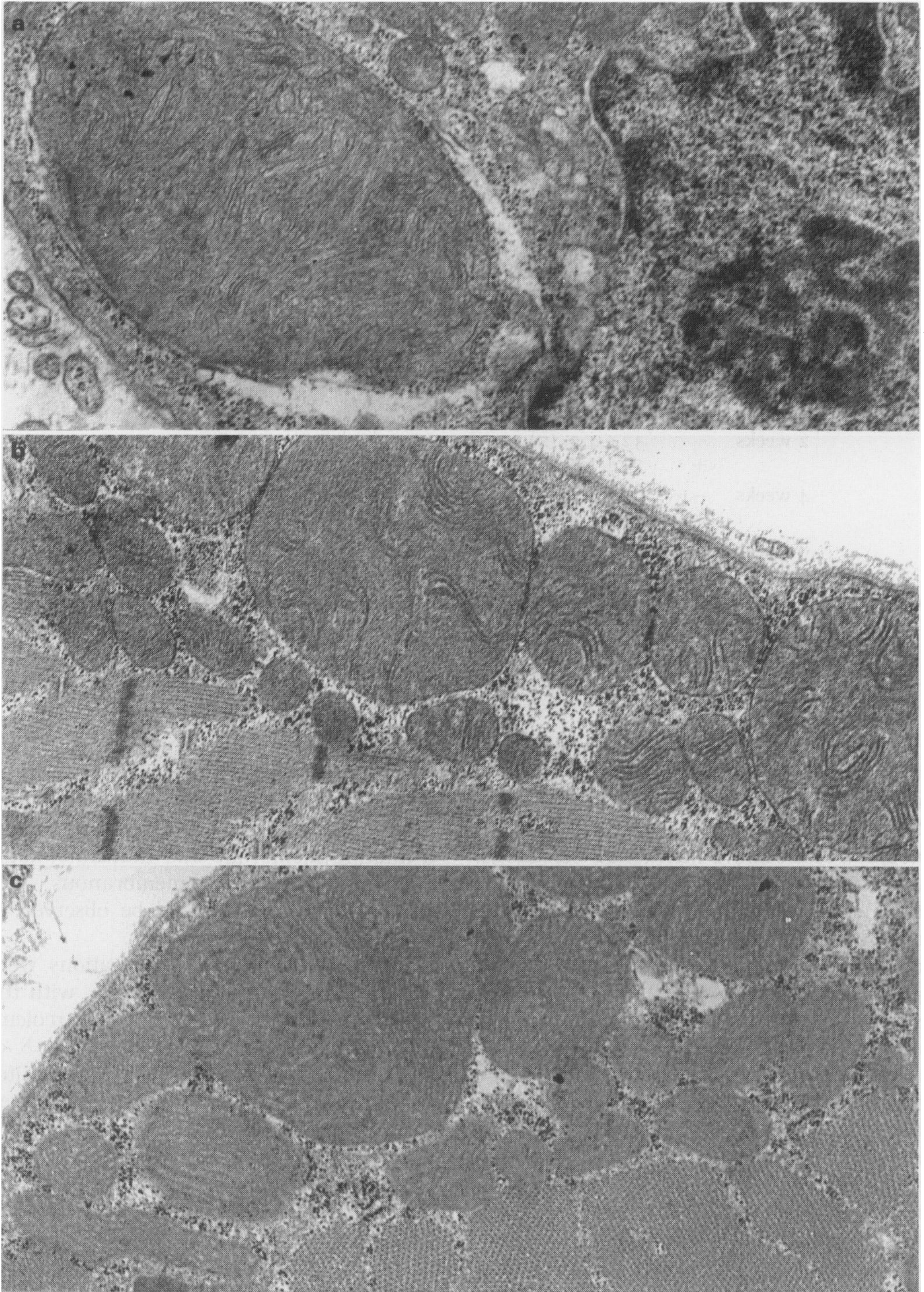

Fig. 1. Mitochondrial alterations in the soleus muscle after two weeks of beta-GPA administration: cristae appear to be increased in number and irregularly oriented; the mitochondrial matrix is electron-dense; giant mitochondria are often seen in the subsarcolemmal area. × 15 700.

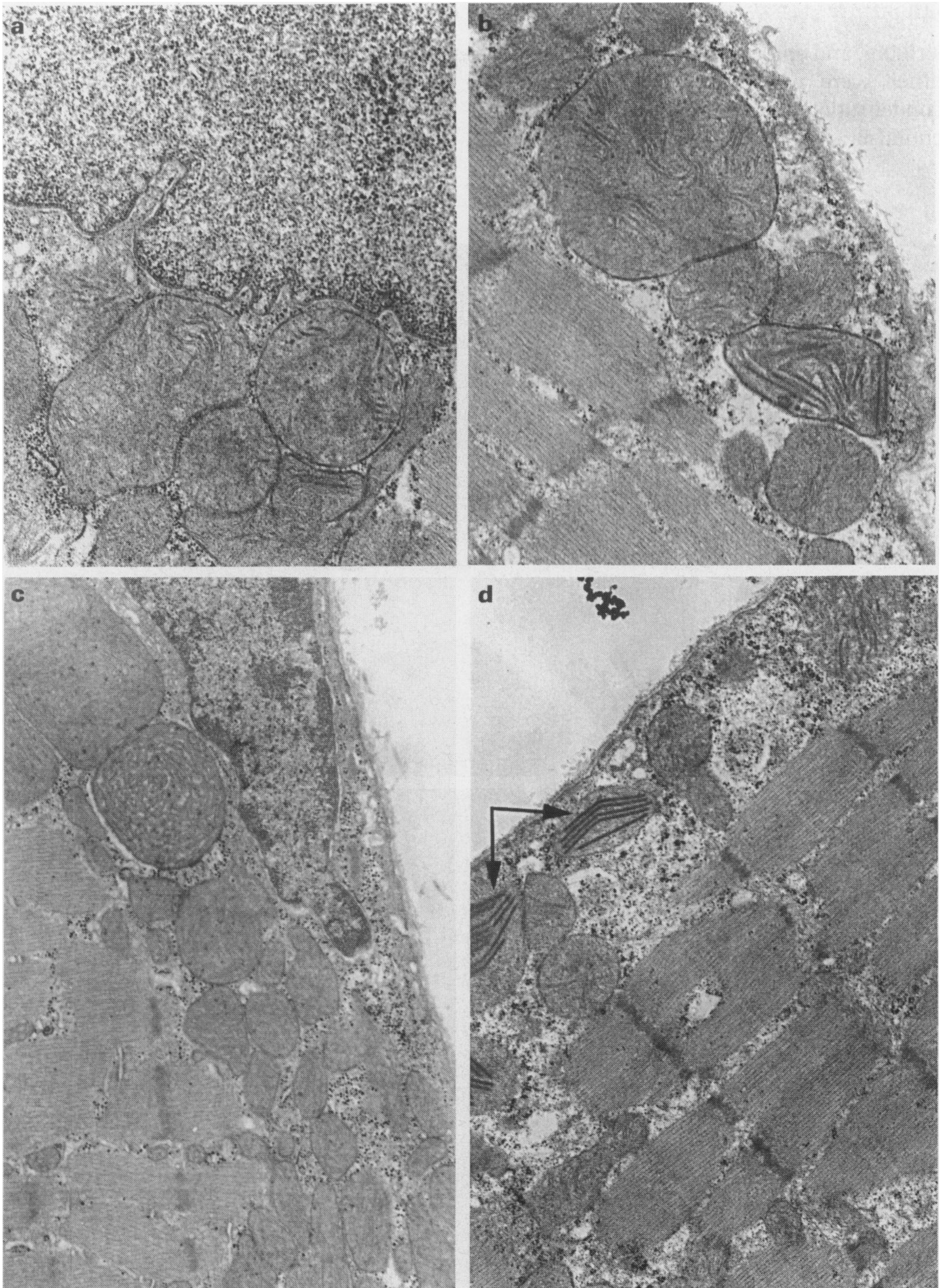

Fig. 2. Soleus muscle. Four weeks of beta-GPA administration. *a*, Enlarged mitochondia are a frequent feature, × 6400. *b*, A giant mitochondrion whose cristae are irregularly oriented, × 9600. *c*, Sometimes the cristae exhibit a tubular-like configuration, × 6400. *d*, Paracrystalline structures (arrows) are present in many mitochondria, × 9600.

striking, and mitochondria very close to each other were in a very different stage of condensation. Round-shaped giant mitochondria (10–15 μm in diameter) were detected (Fig. 7). Mitochondria were seen which contain small clusters of osmiophilic lipid droplets (Fig. 6*a*). However, paracrystalline inclusions were not detectable.

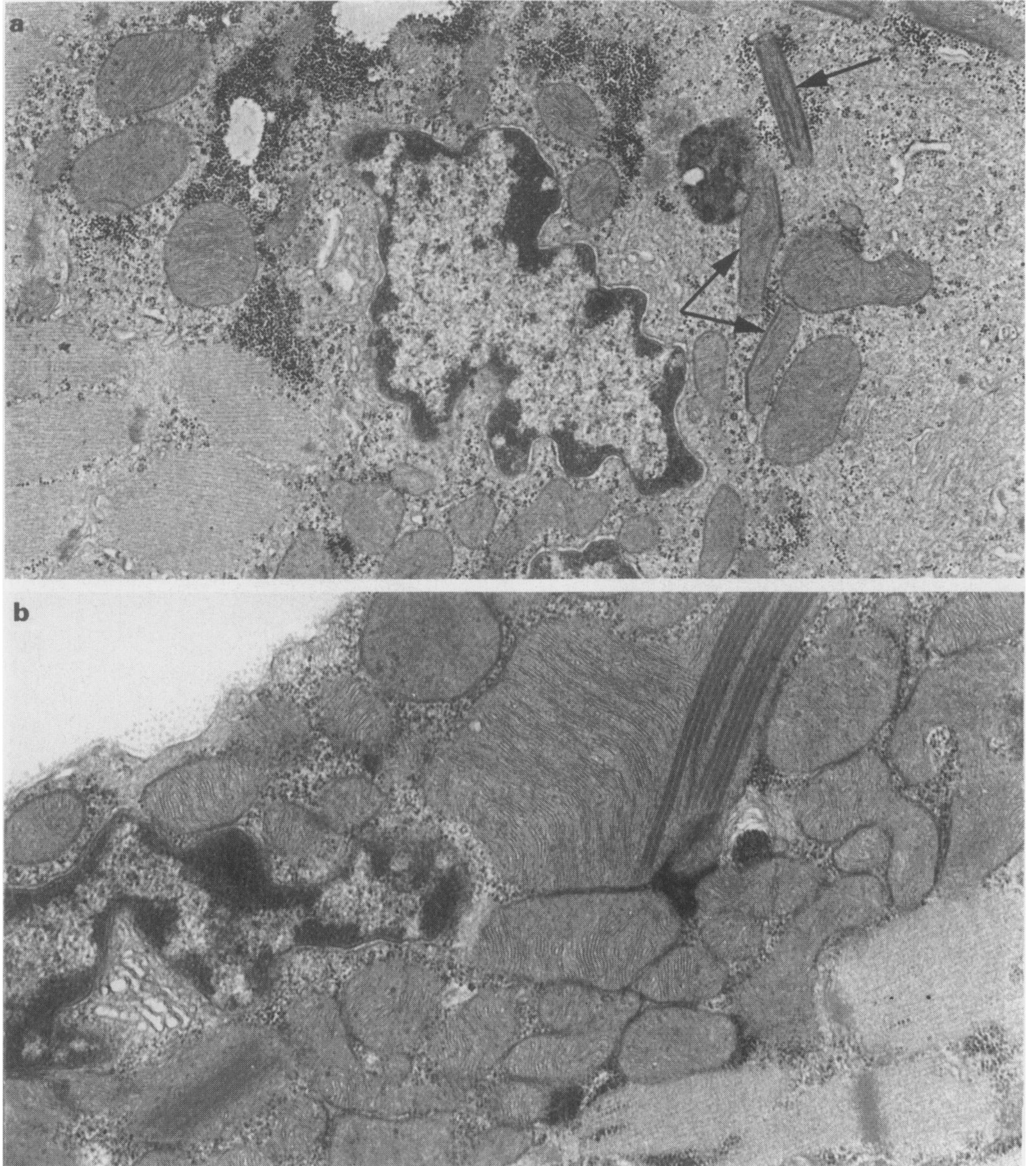

Fig. 3. Soleus muscle. Eight weeks of beta-GPA administration. *a*, Paracrystalline structures are a frequent feature (arrows). Cristae are very large and parallely oriented, ×9600. *b*, Cluster of mitochondria. Paracrystalline inclusions are frequently seen in giant mitochondria, ×12 800.

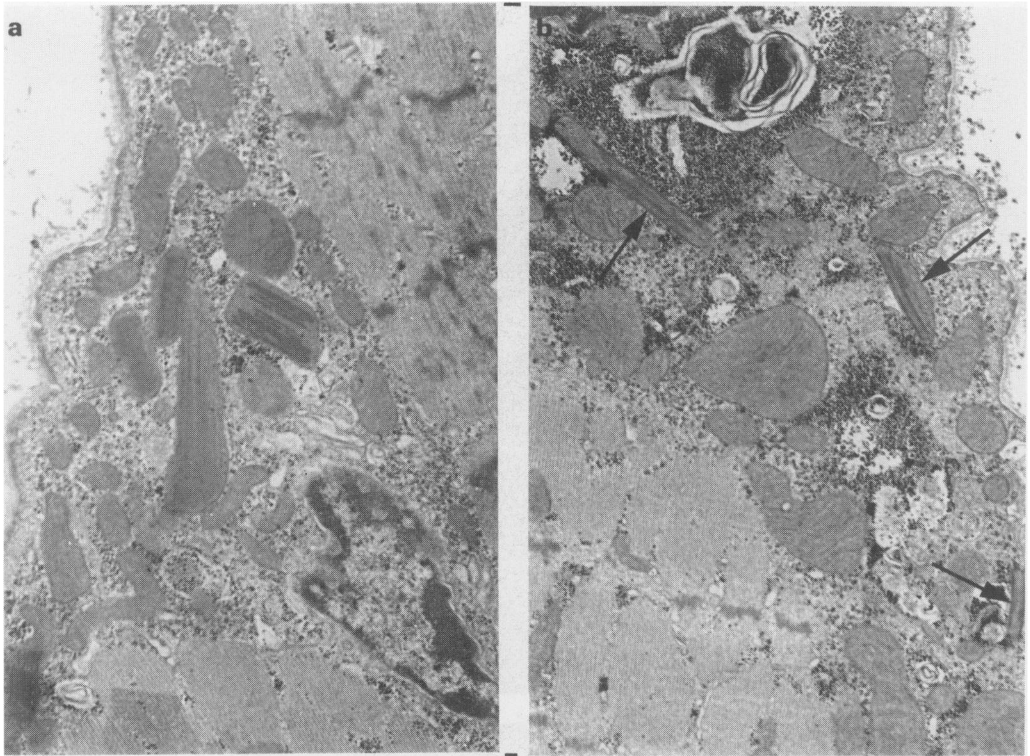

Fig. 4. Soleus muscle. Eight weeks of beta-GPA administration. *a* and *b*, Many mitochondria show paracrystalline structures (arrows), ×9600.

Discussion

Our results show that beta-GPA administration has a remarkable effect on the rat, since it induces selectively a typical mitochondrial myopathy in the slow muscles and causes relatively minor ultrastructural abnormalities in the fast muscle and in myocardial tissue. Laskowsky *et al.* (1981) could observe only minor signs of mitochondrial alteration (dilation) in the pectoralis and gastrocnemius muscles of chicks fed a similar diet. Since creatine depletion was similar in the two types of muscle, differences in the reaction to poison perhaps should be attributed to the properties of type 1 fibres themselves, that use larger amounts of oxidative energy (e.g. Lehninger 1975). Incidentally, Hanzlikova & Schiaffino (1977) observed a mitochondrial myopathy in the soleus muscle of rat after sublethal ischaemia. GPA administration (Fitch *et al.* 1974; 1975; and unpublished) and ischaemia (Gaja *et al.* 1973) decrease the levels of adenine nucleotides in cells, due to leakage and/or degradation by the AMP-metabolizing enzymes. This might be the right perspective to understand why GPA causes mitochondrial myopathy in type 1 fibres only, which contract at a higher frequency and use a larger amount of energy per unit of time and have a shorter percentage time for recovery in comparison with type 2 and heart muscle fibres. Mitochondrial myopathy has been observed also in human muscle fibres (Shellens & Ossentjouk 1969; Antoci & Pizzolitto 1984) and appears to be quite rare in heart muscle cells (Huebner & Grantzow 1983).

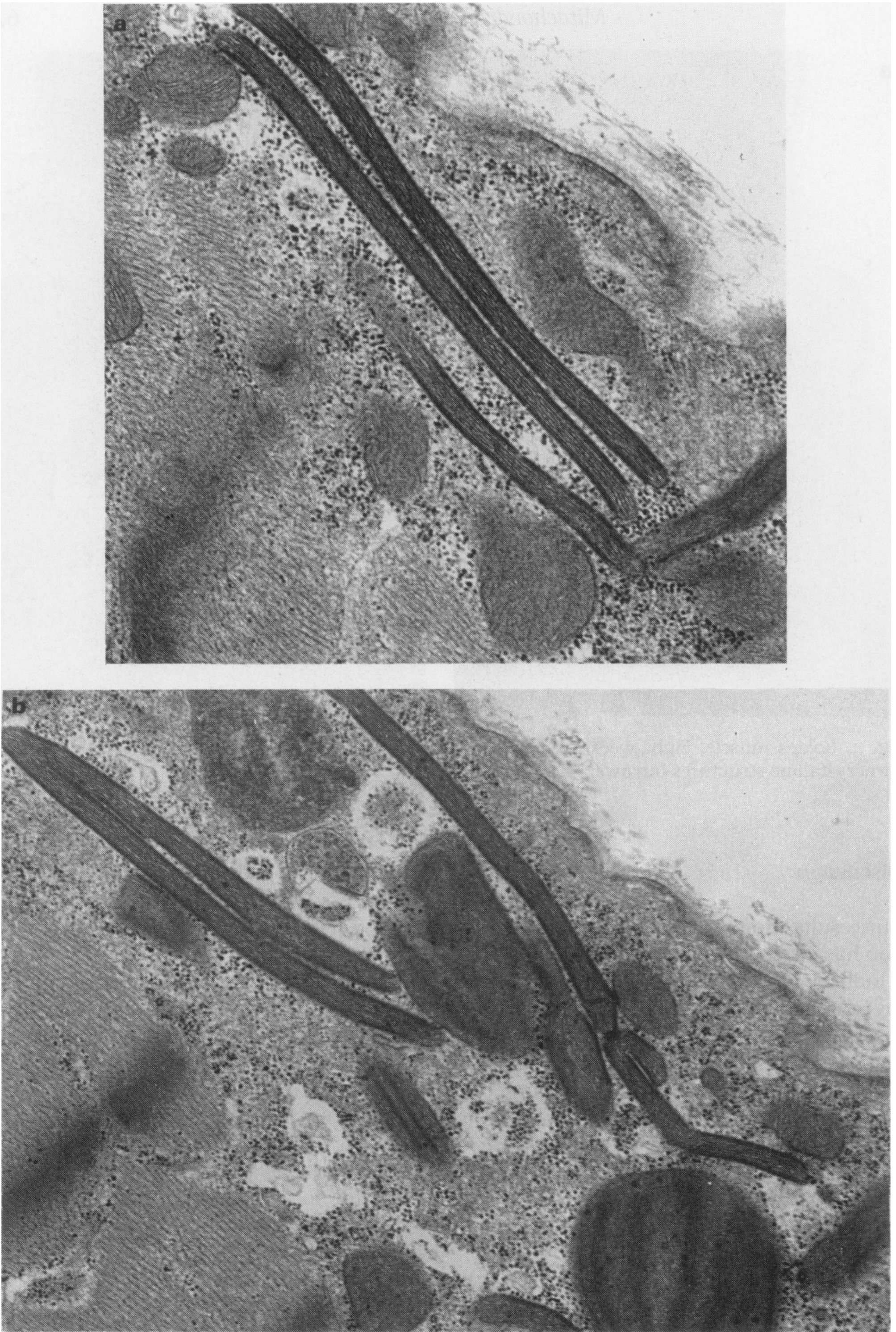

Fig. 5. Soleus muscle. Eight weeks of beta-GPA administration. *a* and *b*, Peculiar worm-like paracrystalline structures are frequently seen, either free in the cytoplasm or inside the mitochondria, × 18 400.

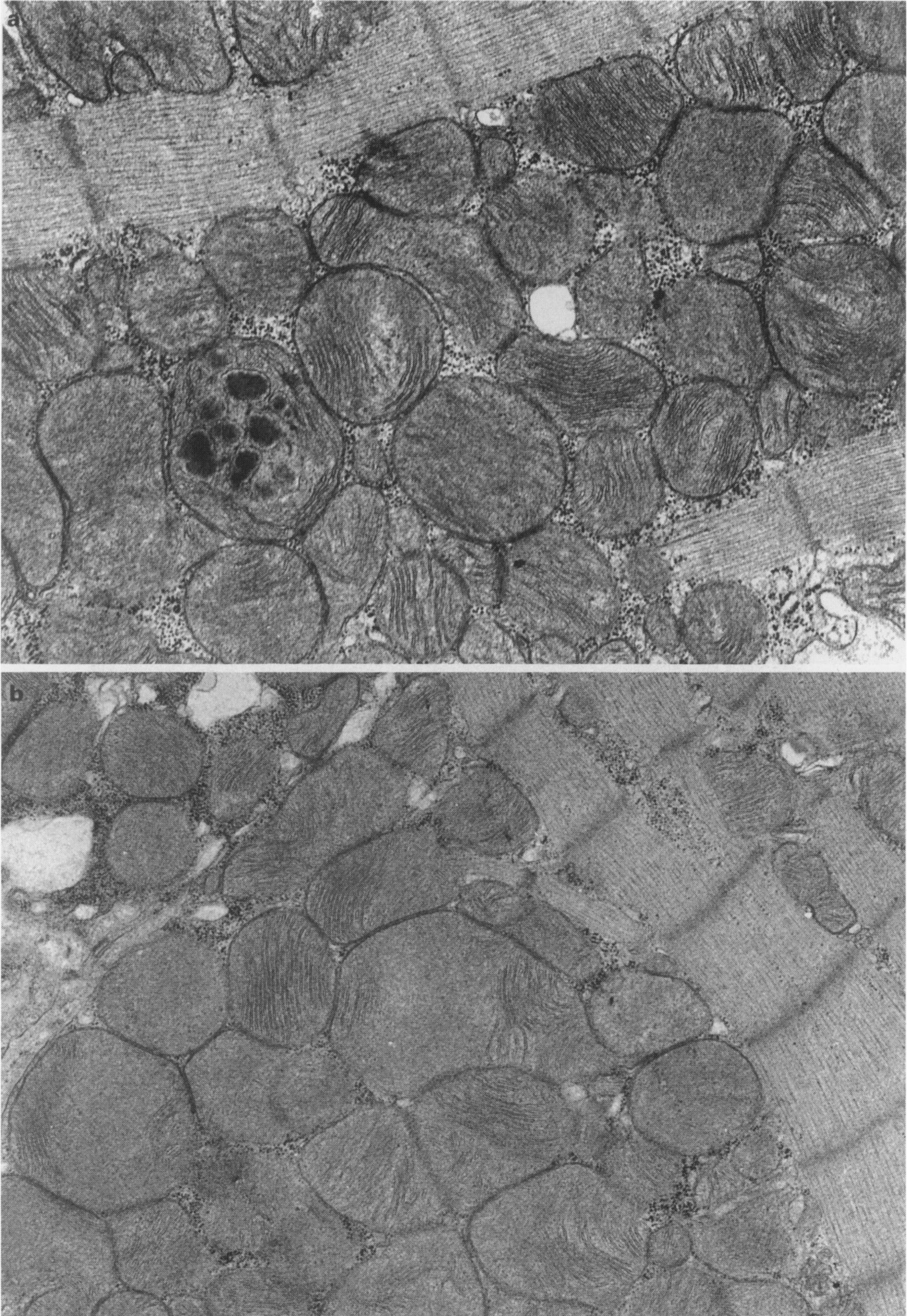

Fig. 6. Heart muscle. Eight weeks of beta-GPA administration. Masses of pleomorphic mitochondria showing parallel irregularly oriented cristae and a dense matrix. *a*, One mitochondrion contains a small cluster of osmiophilic lipid droplets. *a*, ×13 800; *b*, ×9200.

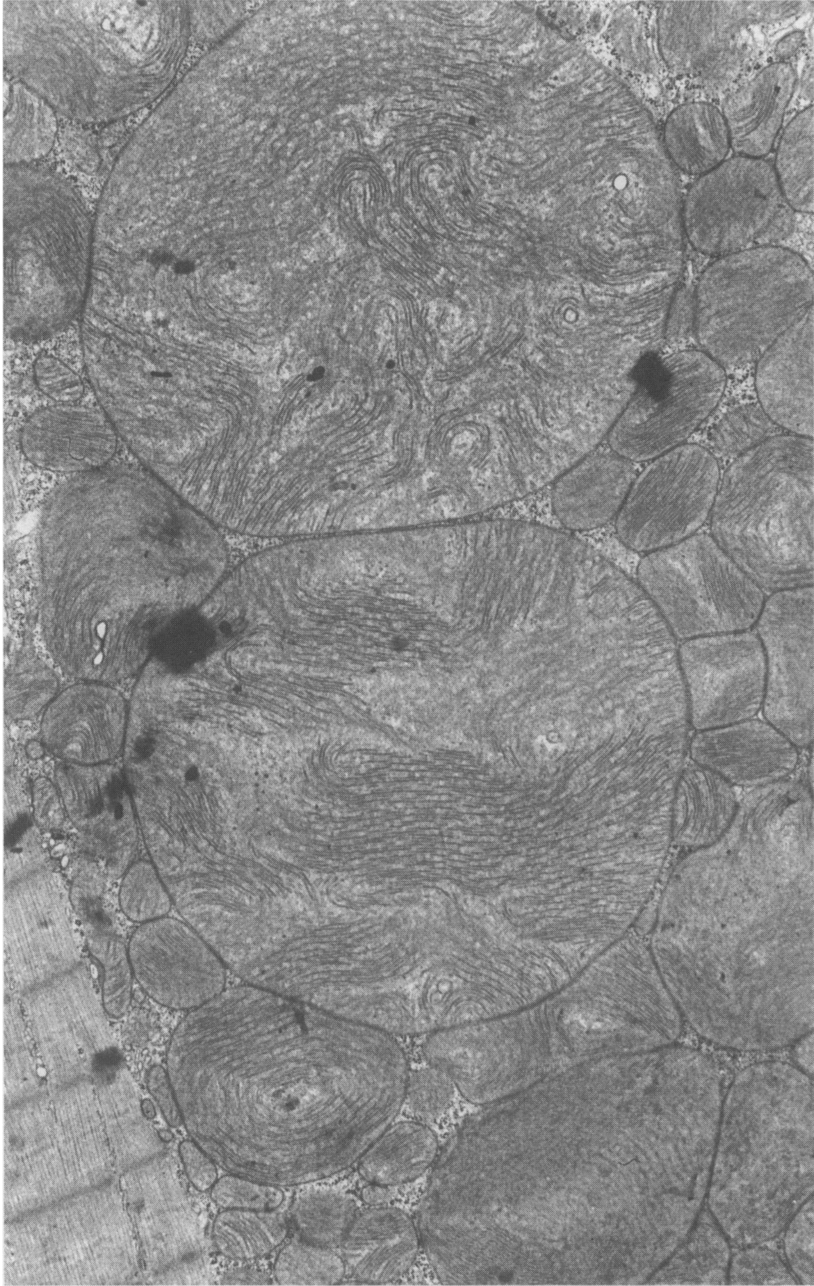

Fig. 7. Heart muscle. Eight weeks of beta-GPA administration. Giant and pleomorphic mitochondria with an increased number of cristae of irregular or concentric orientation, ×4600.

Our observations invite comment with regard to the pathogenesis of mitochondrial myopathy. It has been shown that this myopathy can be induced by many different experimental procedures, all of which affect the production of energy by mitochondria: ischemia (Hanzlikova & Schiaffino 1977); anoxia (Heine & Shaeg 1979); and the acute administration of uncouplers of the oxidative phosphorylation (Melmed *et al.* 1975). In this respect, our model appears to be different in that there is an extramitochondrial biochemical lesion: the alteration of the creatine phosphate-dependent ATP buffering capacity of muscle fibres, and of the transport of energy from mitochondria – the sites of energy production – to myofibrils (Bessman & Carpenter 1985).

Therefore our data rule out the previous hypothesis that the appearance of rod-like intramitochondrial structures is an expression of cellular hypoxia (as suggested by Heine & Schaeg 1979) and may rather indicate that changes in energy metabolism and transfer are the true pathogenetic factors of this myopathy. Because these mitochondrial changes are similar to those seen in human mitochondrial myopathies, perhaps it should be stressed that defects in creatine phosphate might have a role in the pathogenesis of the human disease.

As an additional comment, there is evidence that treatments may not unbalance the energy metabolism of the muscle fibres significantly: muscle glycogen levels are increased in GPA given rats (Baruffi *et al.* 1984; Shoubridge *et al.* 1985; Shoubridge & Radda 1987) very like the cases of post-ischaemic experimental mitochondrial myopathy (Bergamini *et al.* 1977; Hanzlikova & Schiaffino 1977) and of the human disease (Schmalbruch 1984). Perhaps the decrease in the ATP buffering capacity may lead to transient depletions of energy during contraction and stimulate an adaptive response in mitochondria, secondary to an increased protein turnover; inclusions may be of proteinous nature (Hanzlikova & Schiaffino 1977).

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