Human Myopathy with Giant Abnormal Mitochondria

Abstract. *The study of children with symmetrical nonprogressive muscle weakness and hypotonia has previously led to the discovery of two new disorders of striated muscle. A third myopathy in a child with hypotonia and proximal weakness is reported. Cytochemical and electron-microscopic studies reveal large, bizarre, interfibrillary and subsarcolemmal mitochondria in muscle obtained by biopsy. Smooth muscle of the vascular wall did not show such abnormalities nor did leucocytes or intramuscular nerves with cytochemical techniques. The basal metabolic rate of the patient was within normal limits.*

The utilization of new techniques in the study of the muscle cell in diseases of striated muscle in human beings has led to the separation of two distinct clinicopathologic entities from what has been described previously as muscular dystrophy (*1*). This report describes a third myopathy in which there are abnormal mitochondria in an 8-year-old girl who has been observed for at least 3 years to have weakness of the proximal girdle muscles and the musculature of the neck without marked wasting. In light of the findings it is noteworthy the basal metabolic rate of the child was normal (+ 15) and pyruvate and lactate concentrations in the serum were normal. Laboratory findings of this patient are summarized in Table 1.

Two biopsies of skeletal muscle were strained with the modified Gomori trichrome (*p*H 3.4) for mitochondria and endoplasmic reticulum (*2*). The activity of the oxidative enzymes in the mitochondria was studied by reactions for succinic dehydrogenase (*3*), cytochrome A3 (*4*), and reduced diphosphopyridine nucleotide (DPNH) dehydrogenase (*5*). The enzymatic activity of the myofibril was studied by adenosine triphosphatase (*6*). Sarcoplasmic constituents, other than mitochondria and sarcoplasmic reticulum, were studied by their reactions with periodic acid Schiff (PAS) reagent and amylophosphorylase (*7*). Lysosome activity (acid phosphatase) was determined on formol-calcium fixed sections (*8*) that were incubated in Gomori's medium (*9*).

Table 1. Analyses on blood serum, and urine of patient with giant abnormal mitochondria.

Blood and serum findings	
SGOT*	24 units
SGPT†	26 units
BUN‡	10 mg/100 ml of blood
Creatinine	0.5 mg/100 ml of blood
Calcium	10.4 mg/100 ml of serum
Sodium	139 meq/liter of serum
Potassium	4.1 meq/liter of serum
Phosphorus	5.4 mg/100 ml of serum
Lactic dehydrogenase	180 units
Protein bound iodine	3.8 μg
Chloride	108 meq/liter of serum
Urine	
Creatine (24 hr)	1.0 g
Creatinine (24 hr)	0.05 g
Alpha amino nitrogen (24 hr)	571 mg
Serum electrophoresis	
Albumin	4.22 g/100 ml
α_1-globulin	0.15 g/100 ml
α_2-globulin	0.58 g/100 ml
β-globulin	0.50 g/100 ml
γ-globulin §	0.15 g/100 ml

* Serum glutamic oxalopyruvic transaminase. † Serum glutamic pyruvic transaminase. ‡ Blood urea nitrogen. § Most reduction from normal value in γ_{1A} and γ_{1M} bands.

For electron-microscopic studies, the biopsied muscle was immediately fixed at room temperature with 1 percent osmium tetroxide buffered with Palade's solution (*10*), and the preparations were processed according to standard techniques (*11*).

Examination of the tissue verifies the observations of Engel (*2*) who has reported on the localization of the red-staining material in the modified Gomori trichrome stain to the mitochondria and perhaps to the endoplasmic reticulum. In cross section of normal human muscle this is a delicate network; on longitudinal sections minute red dots appear at the junction of the anisotropic (A) and isotropic (I) bands in pairs where mitochondria are commonly found. In the muscle biopsy herein reported, large amounts of interfibrillary red material could be seen in which the size, on cross section, frequently approached the size of a fibril (Fig. 1). On longitudinal sections, this material was arranged haphazardly and was not oriented with definite banding. Enzymatic oxidative reactions were intensely active at the same sites as seen in the modified trichrome. The most active was succinic dehydrogenase, next cytochrome A3, and least reduced diphosphopyridine nucleotide (DPNH) dehydrogenase (Fig. 2). The fibrillary adenosine triphosphatase stains demonstrated only a separation of the fibrils at the location of the red material seen in sections stained with the modified Gomori stain (Fig. 2). The acid phosphatase stain on the material fixed with formol-calcium showed only an occasional black dot in the cross section of a given muscle cell but there were many such dots in the cells of the vascular walls. Small clear areas appeared also incorporated into areas of excess enzymatic activity.

Staining with hematoxylin and eosin, hematoxylin Van Gieson, and Gomori trichrome stains revealed no breakdown of intracellular architecture other than separation of muscle fibrils. However, the average cross-section diameter of the fibers was 27 μ which was small for the patient's age (*12*). No central nuclei were noted. No increase in activity of oxidative enzymes was seen in the walls of the blood vessels or in the intramuscular nerves. Leucocytes seen in intramuscular blood vessels demonstrated no increase of oxidative enzymes.

Observations on the ultrastructure reveal mitochondria in the muscle cells

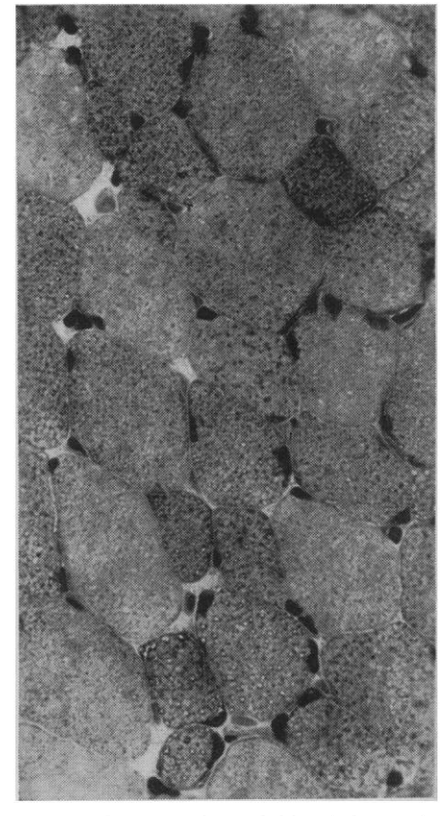

Fig. 1. Cross section of biopsied muscle showing localization of red-staining material in the interfibrillary space. Note clear areas (modified trichrome-frozen section; about × 212).

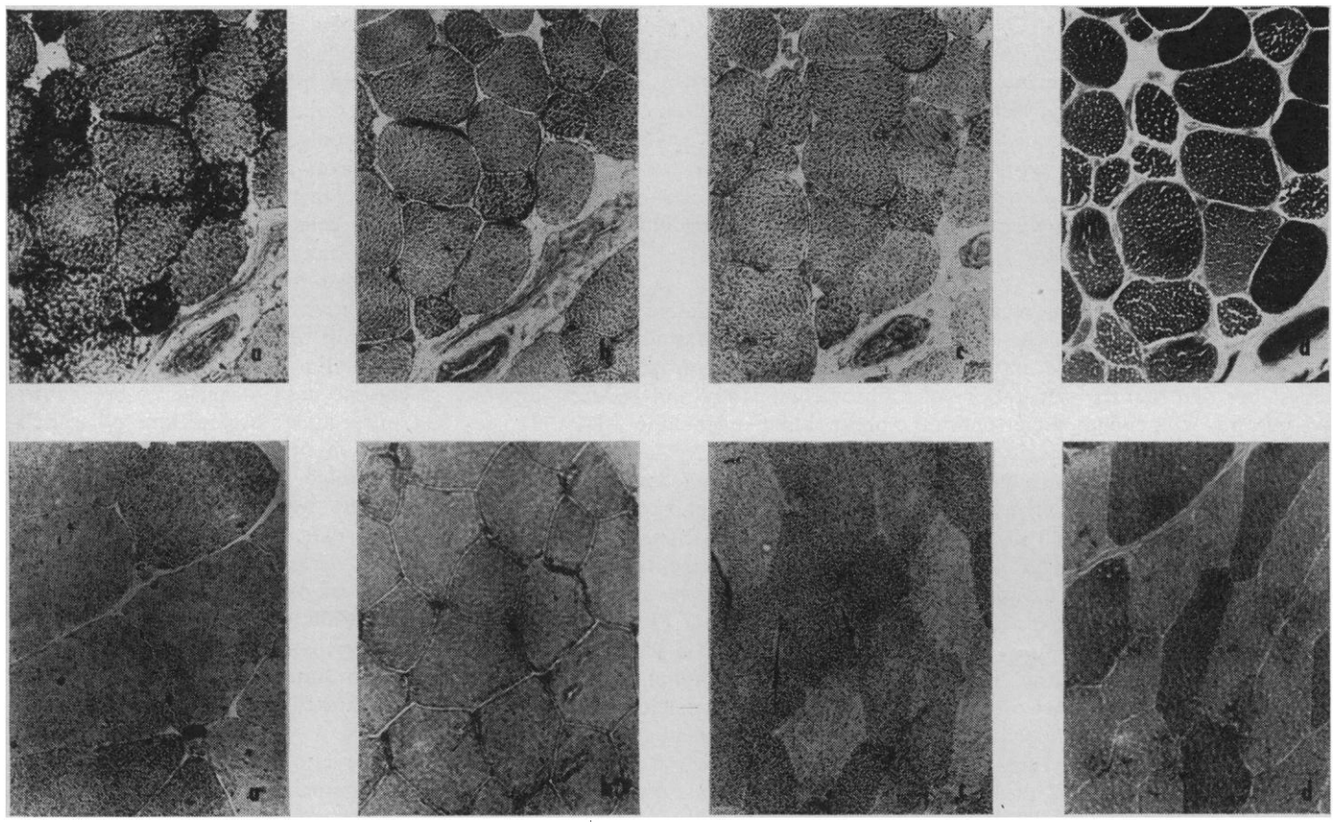

Fig. 2. Cytochemical staining. (Top row) Serial sections showing the same cells in the muscle biopsied from the patient. (Bottom row) Comparative sections from the muscle of a control patient are not serially sectioned. Frozen sections (about × 215). Stains for both rows: *a*, succinic dehydrogenase and phenazine; *b*, cytochrome oxidase; *c*, DPNH; *d*, adenosine triphosphatase.

differing in size and shape from those of normal human and rat muscle. Unusual organelles, probably related to mitochondria, are also frequently seen.

The sarcomeres measure 2.5 μ and show distinct Z, H, A, I, and, occasionally, N bands (Fig. 3*a*). All examined muscle cells contain normal and large mitochondria in varying proportions. The normal mitochondria are located on either side of the Z band; they have an oblong shape and measure, on the average, 1 μ in length. The large mitochondria appear in groups in the zone beneath the sarcolemma and, more frequently, in the interfibrillary space where they are randomly oriented and show considerable variations in

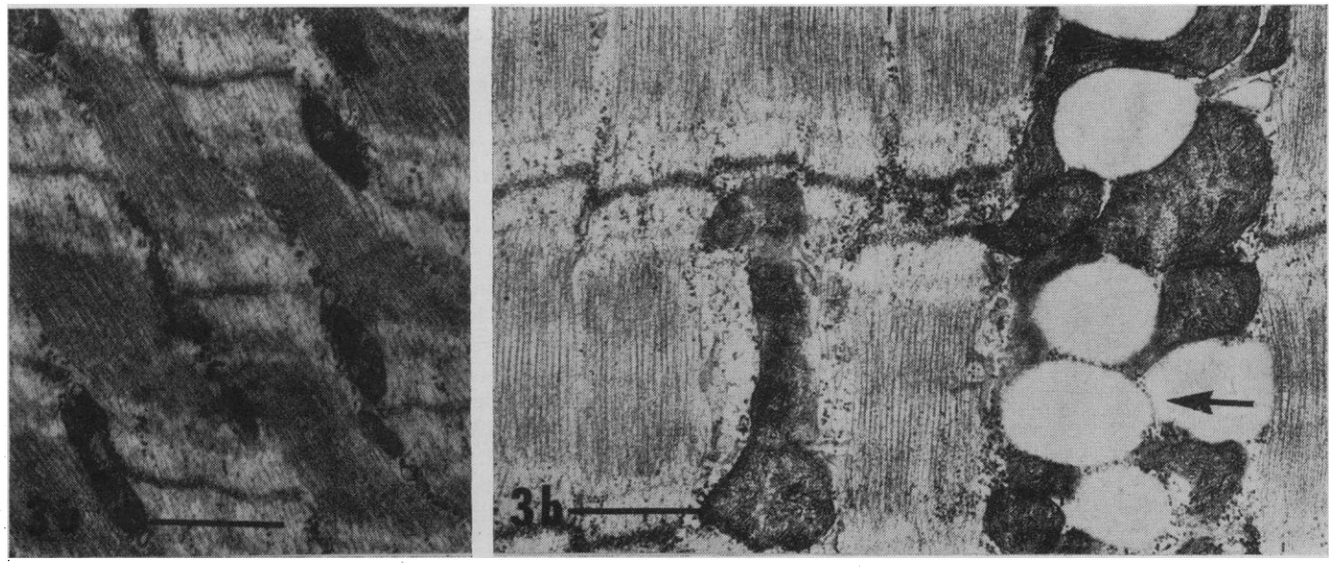

Fig. 3. *a*, Normal field. Mitochondria measure 0.5 to 1 μ (about × 20,000). Line indicates 1 μ unless otherwise stated. *b*, Rows of large mitochondria. Arrow: membrane around empty zone (about × 20,000).

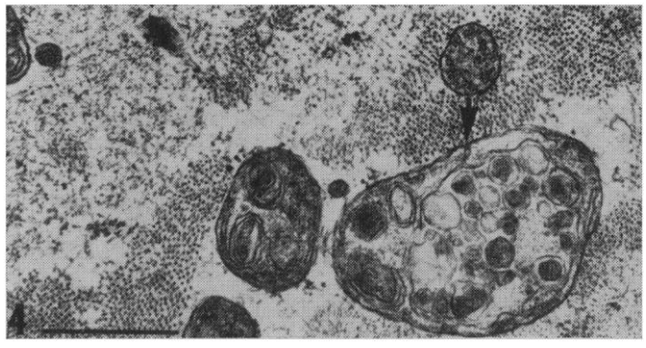

Fig. 4 (above). Organelles containing membranous and rounded dense structures. Arrow: continuity of outer limiting membrane with inner membranes (about × 19,000).

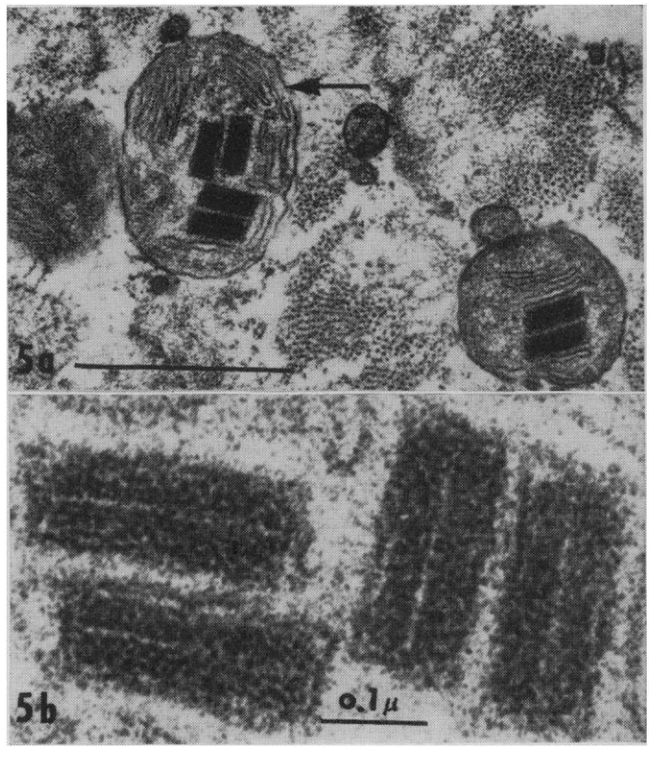

Fig. 5 (right). *a*, Two unusual organelles containing rectangular bodies. Arrow: finger-like profiles reminiscent of cristae mitochondriales (about × 34,000). *b*, Enlargement of rectangular bodies. Granules, 50 to 100 Å in diameter, are discernible (about × 150,000).

size and shape (Fig. 3*b*). The average size of a mitochondrion on longitudinal sectioning is 4 μ. These groups of mitochondria extend over the length of several sarcomeres. Interspersed between the mitochondria are rounded zones of low density upon which the adjacent mitochondria abut; at these points the contour of the mitochondria is evenly curved. The clear zones appear to be bound by a membrane (Fig. 3*b*) and resemble similar structures described by Andersson-Cedergren (*13*) in the mouse intercostal muscle and referred to as spherical bodies.

In addition to the many usual and large mitochondria, a significant number of unusual organelles is noted. These appear as oval bodies defined by a single limiting membrane about 70 Å thick. They measure 1 to 3 μ. The interior of these organelles is filled with circular membranous or rounded profiles, 0.1 to 0.3 μ in diameter, and of varying density. An occasional internal membrane appears to be continuous with the limiting membrane (Fig. 4.).

Several oval bodies contain finger-like membranous profiles which are reminiscent of mitochondrial cristae. The thickness of each crista-like membrane is about 100 Å and the space between the two membranes measures 75 to 250 Å in width (Fig. 5*a*). In addition, rectangular densities 0.25 by 0.50 μ are seen. This unique material on higher magnification seems to be made of confluent spherical densities about 50 to 100 Å in diameter which are arrayed to form linear densities. Distinct lateral lines are noted about 100 Å thick which confine the rectangle (Fig. 5*b*).

Several significant differences exist between the findings in this case and those in the case of hypermetabolism and mitochondrial abnormalities reported by Luft *et al.* (*14*). In this case, the abnormalities in the structure of the mitochondria were noted in the organelles in the interfibrillar space as well as in the peripheral subsarcolemmal and perinuclear zones in contrast to Luft's case where only the mitochondria in the perinuclear zones were involved. Furthermore, the structural abnormalities noted here were not seen in Luft's case. The rod-like particles, the rounded and cylinder-shape inclusions seen by Luft *et al.* were not observed in the case reported here.

The unusual oval organelles (Figs. 4 and 5*a*) probably are altered mitochondria, as suggested by the results of cytochemical techniques. The virtual absence of acid phosphatase activity from the striated muscle fibers in the biopsy seems to exclude the possibility that these organelles are lysosomal. The nature of the rectangular bodies is unknown. It is tempting to suggest that the 50 to 100 Å spherical structures may represent elementary bodies as seen by Fernandez-Moran (*15*), Parsons (*16*), and Stoeckinius (*17*). However, elementary particles are not seen with the usual preparatory techniques applied in this case.

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18. We thank Carol Hoff for her assistance in preparation of the cytochemical studies and Arthur Siegel for photographic aid. These studies were made possible by grant 45630 from the Muscular Dystrophy Association of America and PHS grant No. NB-04613-01.

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Lobuloalveolar Differentiation in Mouse Mammary Tissues in vitro

Abstract. *Administration of lobuloalveolar mammogenic hormones (for a period insufficient to induce alveolar differentiation) to immature mice prior to cultivation of their mammary glands in chemically defined, hormone-supplemented media resulted in lobuloalveolar growth in vitro comparable to that of late pregnancy. No such growth occurred in cultures of mammary tissues from mice not treated with hormones. The use of tissues from hormone-treated donors might serve as a valuable tool for studies concerned with differentiation in vitro.*

The nature of the hormonal influence on the growth and differentiation of mammary glands in mice (*1*) and rats (*2*) has been established by studies of animals deprived of various endocrine glands. Such studies, however, do not permit precise delineation of the actions of hormones on mammogenesis, since the possible influence of the remaining endocrine glands or of other organismal effects cannot be eliminated. Methods in which organs are cultured in vitro offer an advantage in analyzing the role of hormones in growth and differentiation, provided successful cultivation of postnatal tissues can be attained in chemically defined media. Stimulation of secretion in cultures of mouse mammary lobules has been reported (*3*). Rivera (*4*) and Prop (*5*) induced some lobuloalveolar differentiation by cultivating mammary tissues of young mice in media containing serum and hormones, but a suitable method is still lacking for inducing such growth in a medium that is entirely chemically defined. Although Lasfargues (*6*) reported that mammary tissues of young adult mice showed slight lobular development when cultured in a defined medium containing only ovarian steroids as hormones, Rivera failed to obtain such growth in cultures of young adult mammary tissues (*4*) in media containing ovarian and adrenocortical steroids along with insulin. Thus, the induction in vitro, in defined media, of full lobuloalveolar development comparable to that seen in pregnancy has yet to be reported. The fulfillment of this condition, with which this report is concerned, may permit organ culture techniques to be applied to this and similar morphogenetic phenomena.

Three observations seemed pertinent to this problem. (i) Postnatal differentiation of mammary tissues of mice requires hormonal stimulation in vivo (*1*). (ii) Full lobuloalveolar development in mouse mammary glands is achieved in late pregnancy (*1*). (iii) Preliminary experiments indicated that hormone treatment for 15 to 20 days is required for lobuloalveolar differentiation to be induced experimentally in the mammary glands of immature mice. These findings suggest that mammary tissues of immature mice might require extended periods of exposure to hormones in vitro in order to undergo lobuloalveolar differentiation comparable to that seen in pregnancy. However, the period of survival of tissues in organ culture is often limited. As an alternative to merely increasing the period of culture, we therefore decided to "prime" the donor animals. To this end, immature mice received mammogenic hormones for a brief period of time, itself insufficient to initiate lobuloalveolar development. It seemed probable that mammary tissues from such mice would be more likely to undergo lobuloalveolar formation in vitro than tissues from mice not treated with hormones.

The watch-glass method of organ culture, as adapted by Chen (*7*) for liquid media, was used. The basal culture medium was synthetic medium MB 752/1 (*8*), routinely supplemented with penicillin G (35 μg/ml) and L-glutamine (350 μg/ml). The petri-dish culture chambers were incubated at 37°C in a humidified atmosphere of 95 percent O_2 and 5 percent CO_2.

One of the pair of second thoracic mammary glands from the following two types of donor animals was cultured. Intact 4-week-old BALB/cCrgl female mice which had received daily for 7 days subcutaneous injections of 1 μg of estradiol-17β (E-17β), 1 mg of progesterone, 50 μg of ovine mammotropin (MH), and 50 μg of bovine somatotropin (STH), prepared in distilled water [a combination of lobuloalveolar mammogenic hormones in vivo (*1*)]. Intact 3- and 4-week-old BALB/cCrgl female mice served as controls, having been either injected with distilled water for 7 days or subjected to no preliminary treatment. Whole mammary glands were cultured in any one of five types of media. Medium 1 contained no hormone supplements. Medium 2 was supplemented with insulin; medium 3, with insulin, E-17β, progesterone, and aldosterone. Medium 4 was supplemented with insulin, MH, and STH; and medium 5, with insulin, E-17β, progesterone, aldosterone, MH, and STH. The contents of media 2 and 3 will be referred to hereafter as "incomplete hormone supplements"; the contents of medium 4, as "complete hormone supplement." The method used for the addition of hormones to the synthetic medium was described previously (*4*). The concentration of hormones per milliliter of medium was: insulin, 5 μg; E-17β, 0.001 μg; progesterone, 1 μg; aldosterone, 1 μg; MH, 5 μg; and STH, 5 μg.

The medium was usually changed on the 3rd day of culture, and cultures were terminated at the end of 5 days. There were two exceptions to the basic experimental design. In one series of experiments, mammary tissues of 3-week-old control mice were cultured for 12 days. In another series, tissues from mice previously treated with hormones were cultured for 5 days with lobuloalveolar mammogens and then for 5 additional days with secretion-inducing hormones [either aldosterone, MH, STH, and insulin; or cortisol (8 μg/ml), MH, STH, and insulin (*9*)]. The assessment of cell viability, increased growth, and secretory stimulation was based on comparisons of stained whole-mount preparations and histologic sections of cultured glands with their uncultured contralateral controls.

The parenchyma of the second mammary glands of normal 3- to 4-week-old BALB/cCrgl females is organized into branching ducts with end buds of various sizes (Fig. 1*A*). Alveoli are absent. In similar mice injected with E-17β, progesterone, MH, and STH for 7 days (hormone-treated mice), the glands contain more numerous end buds, and possibly a few small alveoli (Fig. 1*C*).

After 5 days of culture, the mammary tissues appeared as follows. (i) In media unsupplemented with hormones, glands from both hormone-treated and control donors degenerated. (ii) In media containing incomplete hormone supplement, glands from both hormone-treated and control donors were only partially maintained. Degen-