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Ultrastructural aspects of bilirubin encephalopathy in cochlear nuclei of the Gunn rat

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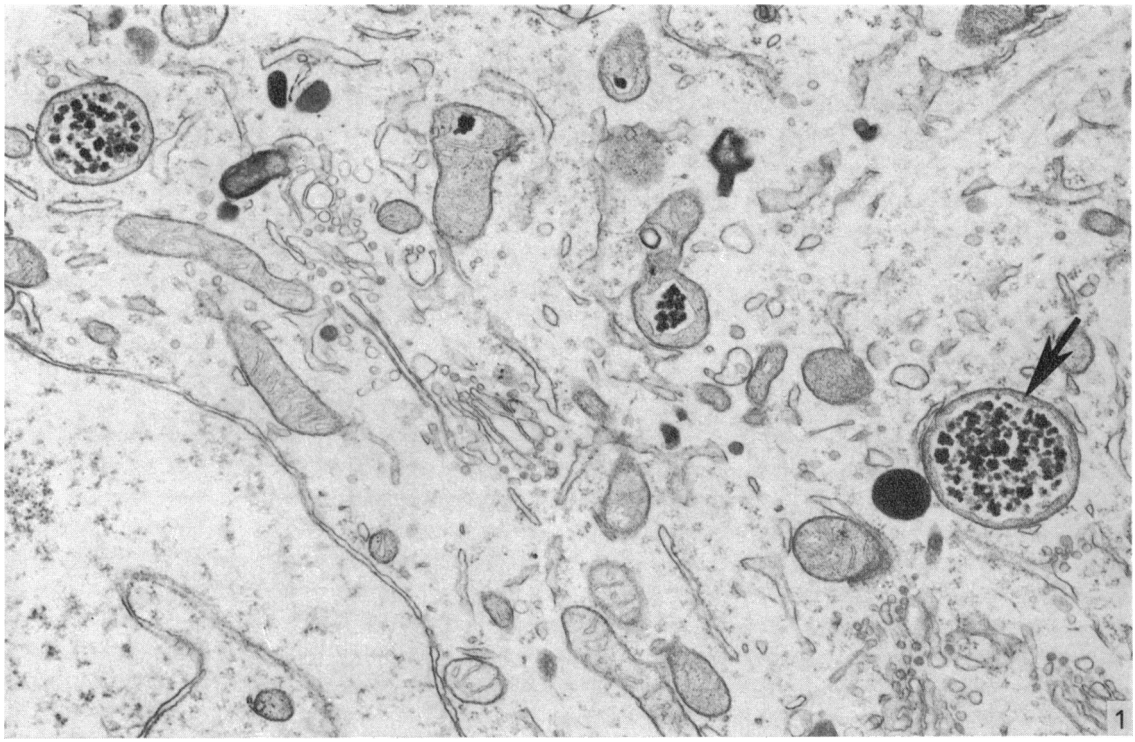
INTRODUCTION

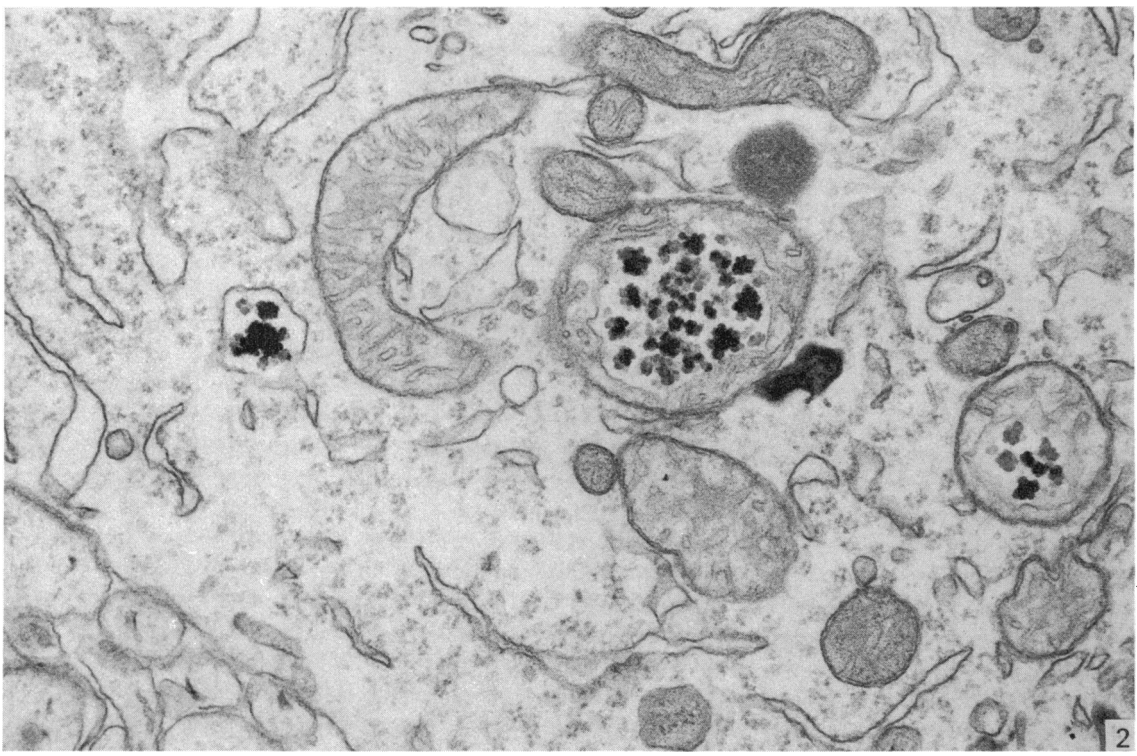
Kernicterus is an affliction of the central nervous system characterized by yellow pigmentation and degeneration of various brain nuclei. There is strong evidence that, in neonatal jaundice, kernicterus results from excessively high levels of unconjugated serum bilirubin, which distributes throughout the brain but has a greater cytotoxic effect on some neuron populations than others, hence the alternative term bilirubin encephalopathy.

There are both clinical and pathological reports, dating from 1907 onwards, indicating that the cochlear nuclei may be damaged in kernicterus (see Dublin, 1951). Clinical reports have associated deafness with kernicterus. Dublin (personal communication) found that in cats, as in human patients who had suffered from kernicterus, the cochlear nuclei were particularly heavily stained with bilirubin. Gerrard (1952), studying kernicterus in two newborn infants, observed that the peripheral auditory machinery appeared normal whereas neurons of the cochlear nuclei had suffered major damage.

While it is clear that bilirubin deposited in the brain of the newborn poses a threat to central auditory neurons that can result in deafness, the precise mode of action is not yet established. Direct ultrastructural observation of the affected neurons can provide information about changes at the organelle level and, by indicating precisely where bilirubin acts inside the neuron, may clarify the molecular mechanisms by which it interferes with vital neuronal functions. In 1938 Gunn reported a mutation which arose in a Wistar rat colony, causing jaundice: the trait seemed to be recessive, with incomplete penetrance. The unconjugated hyperbilirubinaemia in these Gunn rats results from deficient or absent hepatic UDP glucuronyl transferase (Lathe & Walker, 1958; Schmid *et al.* 1958; Arias, 1959). Because (1) this same enzyme deficiency is the operative factor in hyperbilirubinaemia of the human newborn (Lathe & Walker, 1958) and (2) the spontaneously occurring kernicterus syndrome of the Gunn rat is clinicopathologically similar to the syndrome of human kernicterus (Blanc & Johnson, 1959), the Gunn rat is an excellent model for investigations into the mechanisms of kernicterus, or bilirubin encephalopathy.

In vitro experiments have demonstrated that bilirubin inhibits cerebral respiration and uncouples oxidative phosphorylation (Day, 1954; Zetterström & Ernster, 1956), but the sequence of changes whereby bilirubin disrupts such mitochondrial functions *in vivo* is not known. Schenker, McCandless & Zollman (1966) found impaired oxidative phosphorylation in brains of neonatal Gunn rats after repeated administration of sulphonamides, thus focusing attention on mitochondrial function. However, after careful study of the cerebellum, colliculi, basal ganglia and cerebral





cortex, Schutta & Johnson (1971) concluded that the primary site of damage is in the general cytoplasm, and that mitochondrial involvement is secondary. Since bilirubin can impair a number of biological synthetic processes (Odell, 1966), it is clearly important to establish which structural elements are damaged first in particular cell populations. The present report focuses upon ultrastructural changes which occur in the cochlear nuclei of Gunn rats with bilirubin encephalopathy.

MATERIALS AND METHODS

Supplies of breeding animals to start our Gunn rat colony were kindly provided by Dr Antony McDonagh of the University of California College of Medicine, San Francisco, Dr Irwin Arias of Albert Einstein College of Medicine and Dr Carl Hansen of the Veterinary Resources Branch, NIH.

Twenty-one homozygous Gunn rats, aged 2 days to 7 months, with conspicuous jaundice and/or symptoms of CNS disease, were studied: particular attention was paid to 4 weeks old animals, in whom organelle changes have become severe (Schutta & Johnson, 1971). Normal Sprague–Dawley rats and heterozygous Gunn rats of comparable ages served as controls. The brains were fixed by perfusion through a cannula inserted into the ascending aorta. Anaesthesia, ventilation and fixation were carried out according to a programme developed in this laboratory (Williams & Jew, 1975). Blocks containing the ventral cochlear nuclei and the dorsal cochlear nucleus were removed from the fixed brain and processed for electron microscopy. Semithin sections stained with toludine blue facilitated orientation and trimming of the blocks. Thin sections were cut with a diamond knife on a Sorvall MT-2 ultramicrotome, picked up on membrane-coated slotted or 50 mesh copper grids, stained with uranyl acetate and lead citrate and examined with a JEM 100B or a Philips 201 electron microscope.

RESULTS

Gross brain appearance

Yellow staining of Gunn rat brain tissues due to hyperbilirubinaemia was not apparent because of the yellow–orange discoloration produced by glutaraldehyde–hydrogen peroxide fixation. The homozygous Gunn rat cerebellum from 3 weeks on was noticeably reduced in size as compared with controls.

Electron microscopy

Ultrastructural abnormalities were observed in all the age groups of Gunn rats studied, although they were generally least severe in the 2–4 days old animals.

The cochlear nuclei of normal Sprague–Dawley rats and heterozygous Gunn rats were devoid of perceptible abnormalities at all ages studied. In the cytoplasm of the

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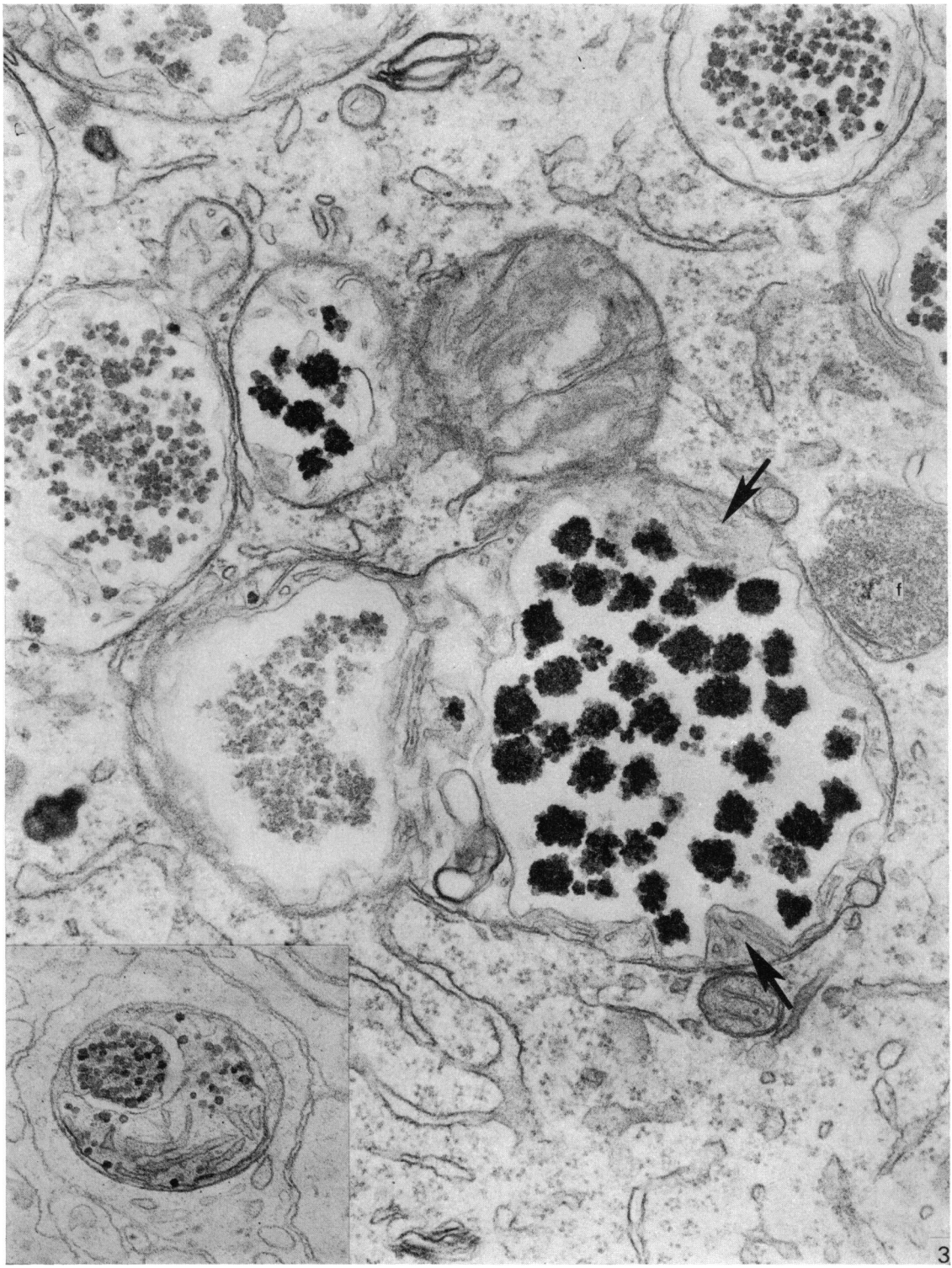
All micrographs are taken from cochlear nuclei of homozygous Gunn rats with bilirubin encephalopathy.

Fig. 1. This electron micrograph illustrates cytoplasmic structures and organelles in an AVCN neuron from a Gunn rat aged 11 days. Several mitochondria are in various stages of forming glycogen-filled 'ex-mitochondrial sacs'. The mitochondrial cristae and matrix are reduced to a thin envelope around the edge of the sac (arrow). ×24300.

Fig. 2. Glycogen accumulations are seen in mitochondria and in a small dilatation of ER. The 'ex-mitochondrial sacs' are bound by a membrane which appears to be formed of residual cristal membranes. From 11 days old Gunn rat. ×36100.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jaundiced animals, located within large neurons of the cochlear nuclei, and most particularly those of the anteroventral cochlear nucleus (AVCN), were many and various membrane-bound vacuoles. Different kinds of vacuoles were distinguished on the basis of their contents and their associations with particular organelles. One (most prominent) type of vacuole was inside mitochondria. Although, as a general rule, the inner and outer mitochondrial walls remained intact, the residual mitochondrial cristae and matrix were reduced to an inner irregular rim (Figs. 1, 2). The term 'ex-mitochondrial sac' has been coined to describe the large structures within which recognizable mitochondrial material was reduced to a thin envelope around the edge of the vacuole (Fig. 1, arrow). Vacuoles in mitochondria frequently appeared to have irregular limiting membranes which, beyond all reasonable doubt, were residual cristal membranes of the original organelles. A careful study of many cells showed that, most often, the vacuoles contained collections of electron-dense granules, having the ultrastructural characteristics of glycogen. It may be mentioned at this point that other workers endorse this interpretation (Schutta, Johnson & Neville, 1970). The glycogen appeared in both its common forms, i.e. as singly occurring granules or beta particles and as clumps or rosettes of alpha glycogen particles (Fig. 3). Although smaller granule-containing vacuoles were present in neuron cell bodies and neurites of animals as young as 2 days (Fig. 3, inset), large neurons of 4 weeks old animals provided us with the biggest ones and also with the greatest numbers of these vacuoles. The glycogen granules varied widely in electron density, sometimes within the same vacuole and often within the same mitochondrion (Fig. 4). Partitioning of an 'ex-mitochondrial sac' into multiple vacuoles was not uncommon, the compartments being separated by residual cristae and matrix (Fig. 4). The individual vacuoles of one mitochondrion could contain glycogen particles of different kinds (Fig. 4). In addition, we encountered some intramitochondrial vacuoles that contained filamentous material, and others that were devoid of electron-dense inclusions.

In cochlear nuclei of jaundiced Gunn rats we also found aggregations of glycogen particles in dilated profiles of the rough endoplasmic reticulum as well as within some elements of the smooth ER (Fig. 5). Even where these glycogen-containing vacuoles or cisterns were not studded with ribosomes, their association with rough ER cisternae was consistent (Fig. 5). Some portions of the cell appeared to be taken over completely by glycogen-filled rough ER and polysomes lying between the cisternae (Fig. 6), the situation being comparable in appearance to regular Nissl bodies with an addition of abnormal inclusions.

Sometimes one pole of an ex-mitochondrial sac showed signs of breaking down (Fig. 9), with extrusion of the glycogen granules. The presence of packaged glycogen in the vicinity of the extrusion site provided evidence that the ex-mitochondrial glycogen becomes membrane-bound (Fig. 9).

Another development, seen as early as 8 days in icteric animals, was the appearance

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Fig. 3. The centre of the field is dominated by ex-mitochondrial sacs; the mitochondrial cristae (arrows) having been displaced to the edges of the sacs by accumulations of glycogen. Interestingly, glycogen particles take the dispersed β form in some sacs, whereas in others they are in the form of rosettes (α particles). A small membrane-bound organelle containing filamentous elements (*f*) is also seen. From 4 weeks old Gunn rat. × 43700. Inset: mitochondrial vacuolation accompanied by glycogen deposition may be seen in neurons and neurites of Gunn rats as young as 2 days. × 45000.

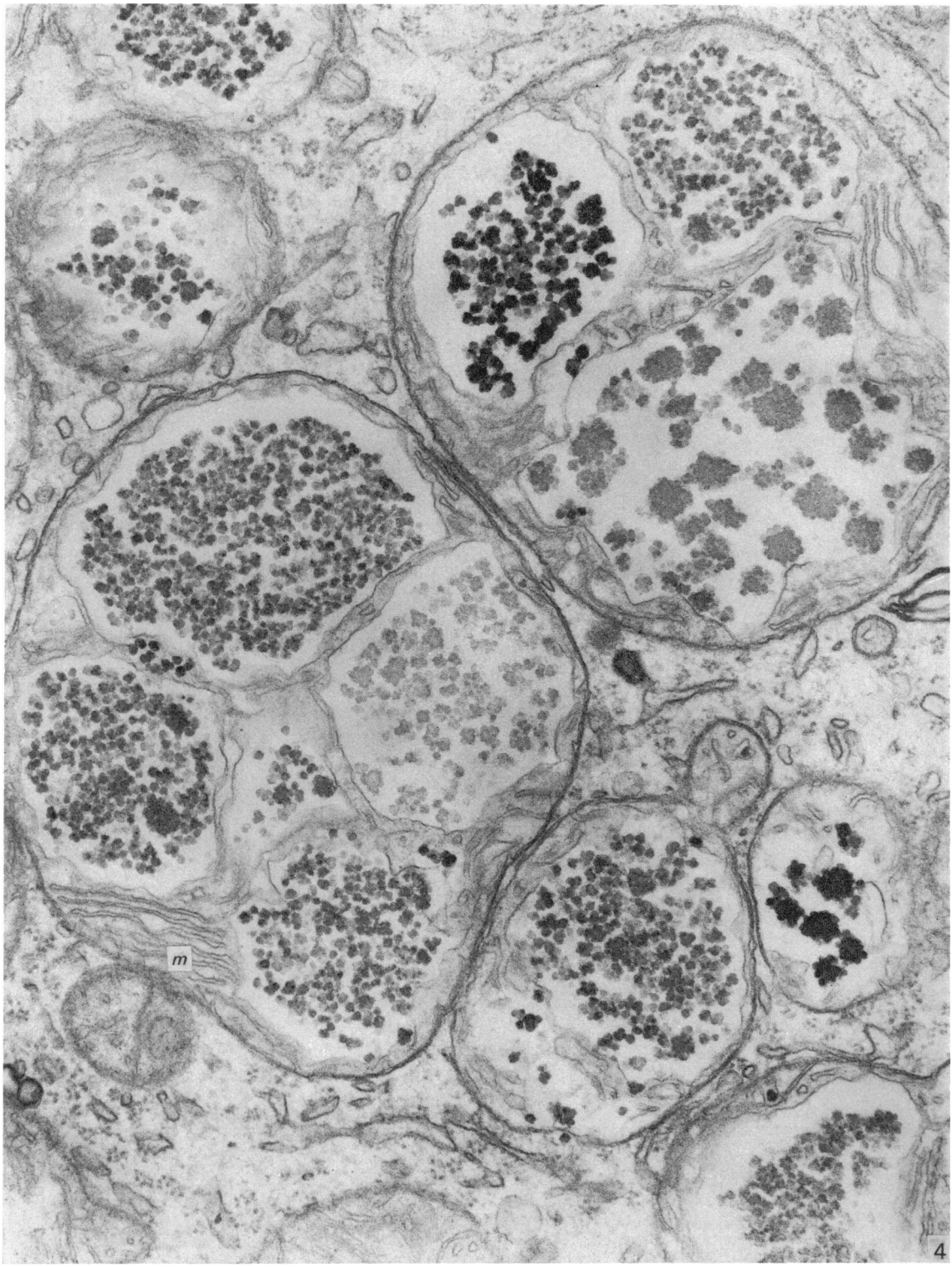


Fig. 4. Large amounts of glycogen are embedded in these ex-mitochondrial sacs which are subdivided by partitions fashioned out of mitochondrial cristae and matrix. Remnants of these elements (*m*) provide evidence that these sacs are of mitochondrial origin. ×44600.

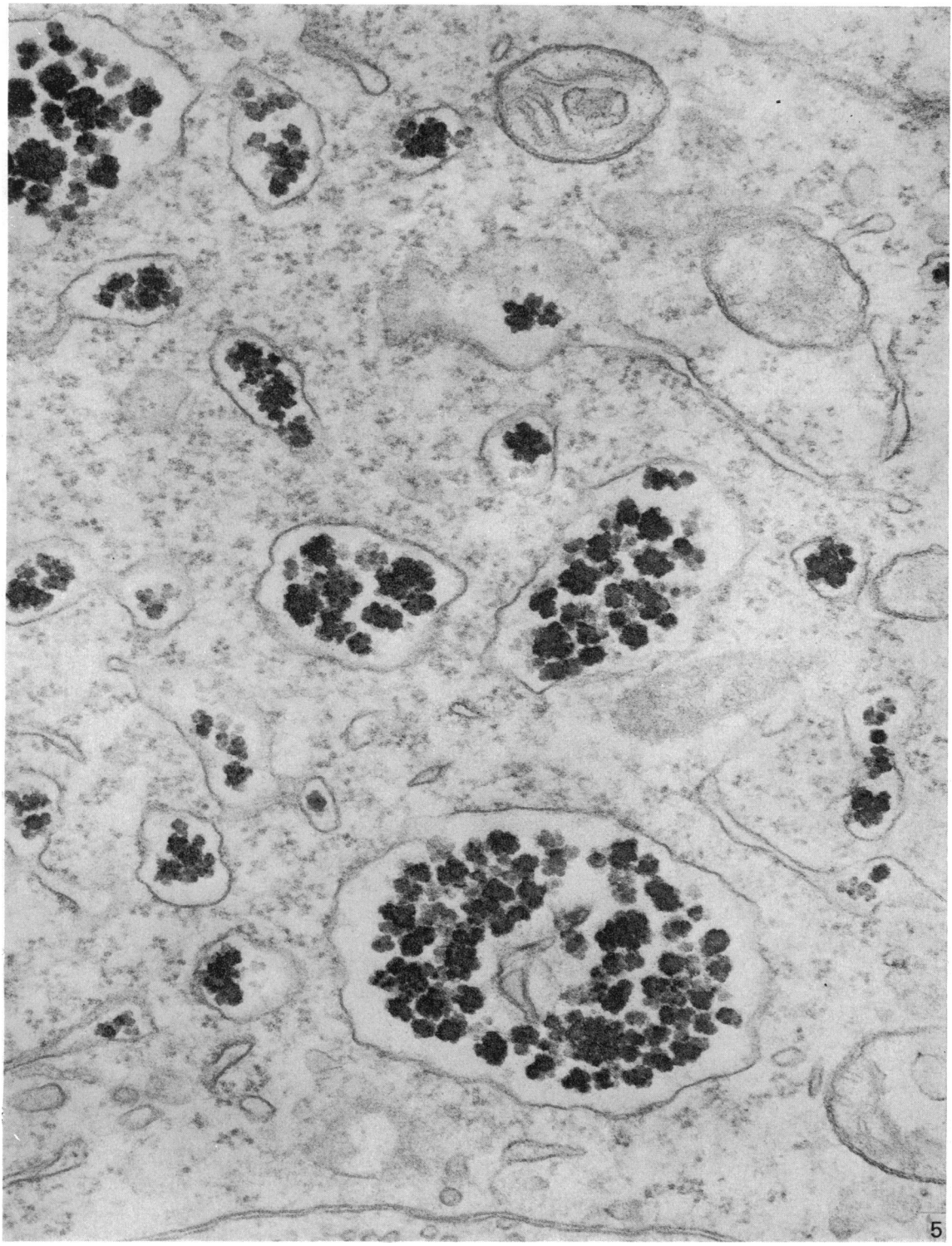
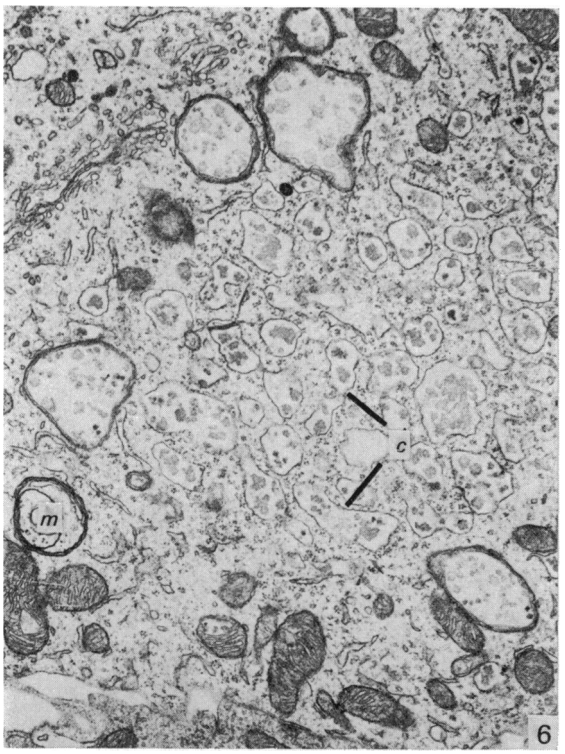


Fig. 5. Accumulations of glycogen particles are frequently seen in dilated profiles of rough and smooth ER. × 52600.



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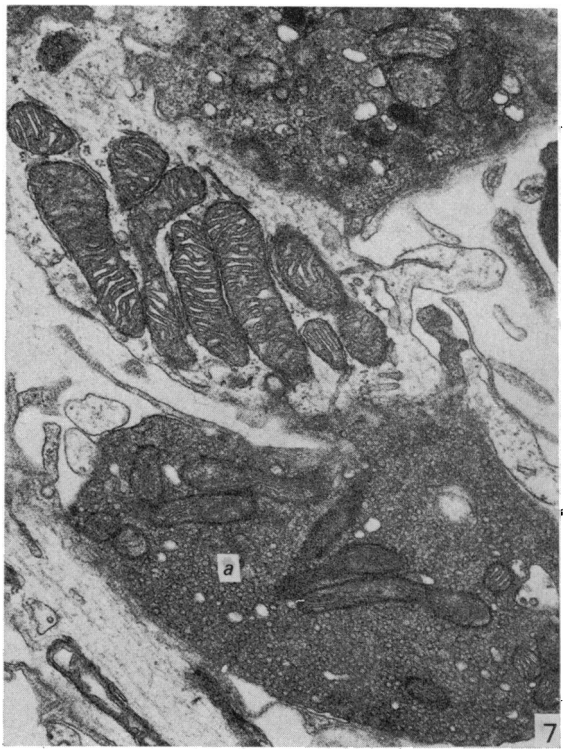

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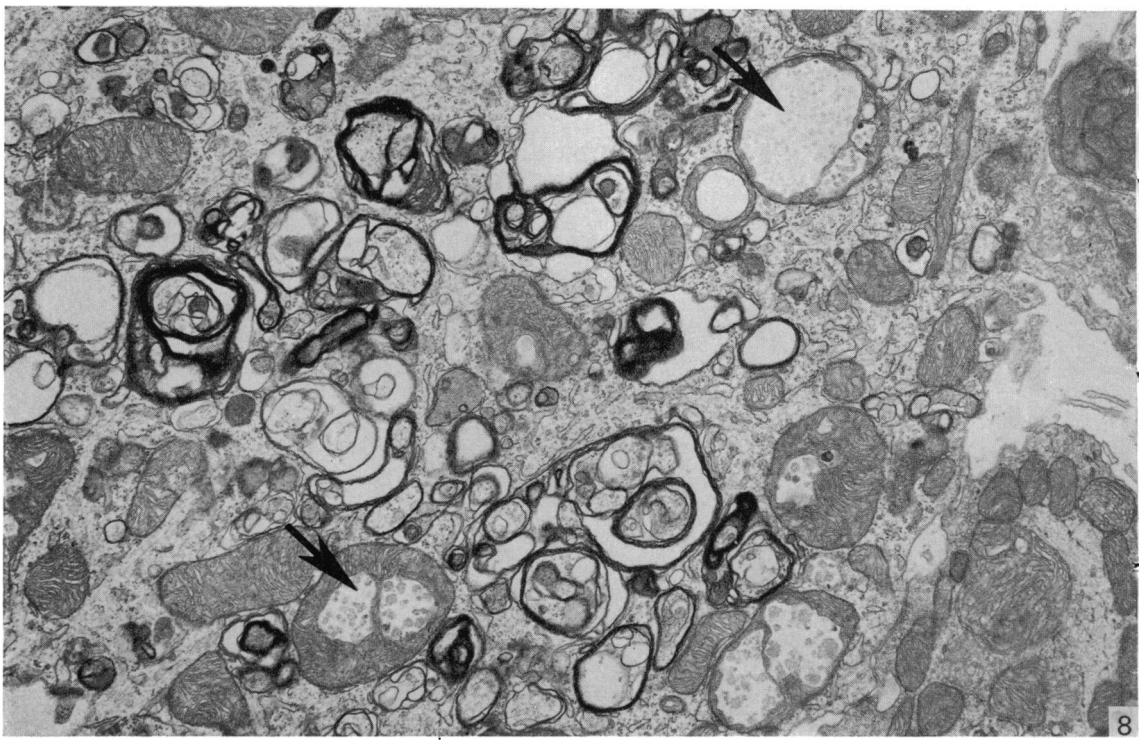

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of myelin figures, often containing tongues or remnants of cytoplasm, as well as extremely irregular spaces or vacuoles (Fig. 8). Some vacuole profiles were surrounded by electron-dense condensations of multiple membranes. These membranous bodies varied greatly in size and were often intermingled with full-blown and part-formed glycogen-containing vesicles in mitochondrial envelopes (Fig. 8, arrows).

In addition to the mitochondrial abnormalities described above, hypertrophied and often distorted mitochondria (Fig. 10) were found in most neurons and in some of their dendritic processes as well. Microcavitation, or abnormally spacious extracellular space, was a feature of the neuropil in all cochlear nuclei of jaundiced Gunn rats (Fig. 11). Axonal terminals in many areas showed 'dark degeneration' (presumably condensed cytoplasm) with synaptic vesicle clumping (Fig. 7). Even where axosomatic synapses appeared otherwise healthy, microcavitation (arrows in Fig. 11) was often present in close relationship to the specialized contact sites.

DISCUSSION

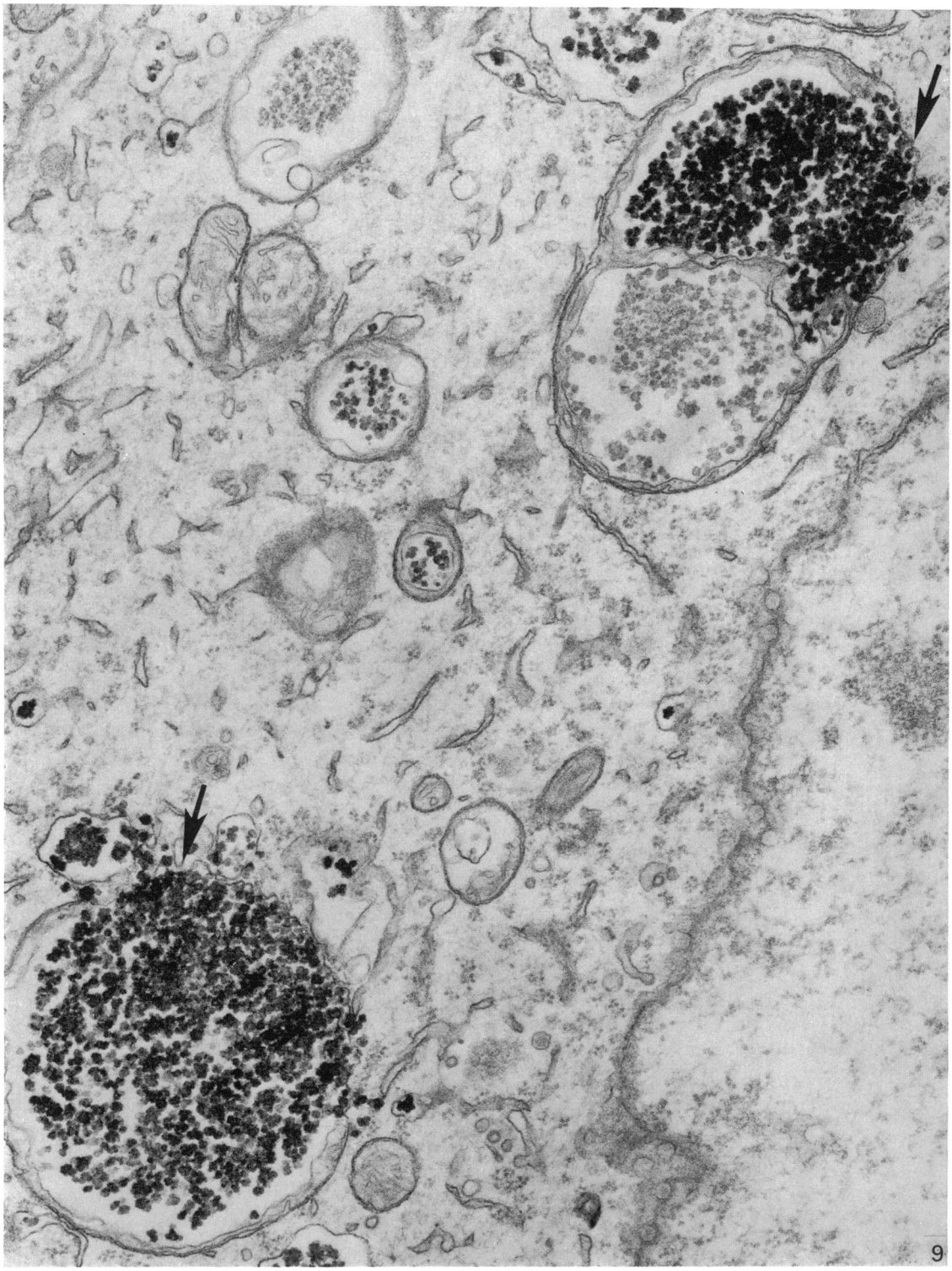
Bilirubin and mitochondrial function

In vitro and *in vivo* biochemical studies have corroborated the claim that bilirubin affects mitochondrial enzyme systems by inhibiting brain respiration (Day, 1954) and uncoupling oxidative phosphorylation (Zetterström & Ernster, 1956; Schenker *et al.* 1966). However, it has been questioned whether or not the bilirubin is delivered to the neurons in the concentration necessary to initiate mitochondrial damage. For example, Diamond & Schmid (1967) were of the opinion that the bilirubin concentrations found in animals with bilirubin encephalopathy did not reach the level necessary to uncouple oxidative phosphorylation *in vitro*. Their evaluation, along with the ultrastructural findings of Schutta *et al.* (1970), did not support the view that inhibition of cerebral respiration and uncoupling of oxidative phosphorylation are the primary neurocytotoxic effects of bilirubin. Histochemical studies on 10–14 day old Gunn rats (Hanefeld & Natzschka, 1971) treated with sulphadimethoxine showed that bilirubin-containing cells exhibited greatly diminished or no oxidative enzyme activity.

Fig. 6. This field of neuronal cytoplasm from a hyperbilirubinaemic Gunn rat (aged 4 weeks) shows the basic organization of a Nissl body, except that RER cisternae (*c*) are filled with glycogen. Some of the mitochondria (those around the modified Nissl body) are distended with glycogen also, while those further away are less affected. This raises the question of the sites of synthesis, possible transfer between organelles, and ultimate disposal of glycogen in this pathological process. A cytoplasmic myelin body (*m*) is also present. × 14 400.

Fig. 7. In jaundiced Gunn rats, at 4 weeks, a significant number of axon terminals show degenerative changes. In this example, degeneration is characterized by darkening of the axonal profile (*a*) with clumping of synaptic vesicles. In the degenerating terminal, there is also an indication of shrinkage and darkening of mitochondria, as compared with those in the adjacent cell process. × 19 700.

Fig. 8. Some of the mitochondria are voluminous and show varying degrees of vacuolation and glycogen accumulation (arrows). There are numerous myelin figures surrounding vacuoles, bits of cytoplasm, and other intracytoplasmic debris. From AVCN neuron of 4 weeks old Gunn rat. × 13 600.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Bilirubin and protein synthesis

Bilirubin can also inhibit amino acid incorporation into protein (Cowger, Igo & Labbe, 1965; Greenfield & Majumdar, 1974). But this may be a secondary effect since there is no direct correlation between bilirubin levels and inhibition of amino acid incorporation. Thus, when Gunn rat neuronal cell damage is evident and plasma bilirubin levels are highest at 10–14 days of age, according to Schutta & Johnson (1969), there are no significant differences in amino acid incorporation into neuronal, glial and myelin proteins as compared with normal controls (Greenfield & Majumdar, 1974). These authors have suggested that defective protein synthesis as found in the adult Gunn rat brain results from a time-dependent degenerative disorder or interference in a cellular developmental process. We can therefore almost certainly infer that mitochondrial changes precede major disruption of the protein manufacturing machinery of the neuron.

Bilirubin and lipid metabolism

The toxic effects of bilirubin are not limited to mitochondrial function and amino acid incorporation. Spectral absorption studies by Weil & Menkes (1975) have demonstrated interaction between bilirubin and neonatal brain ganglioside. They suggest that bilirubin may be toxic (*in vivo*) to cell elements rich in ganglioside; and this effect is achieved by interference with plasma membrane function. Earlier, Cowger (1971) had suggested that the electron transport lesion induced by bilirubin may not directly cause cell death, but that the lethal lesion, to which the cell is unable to adjust, may involve multiple cell membrane systems.

Electron microscope correlates of bilirubin intoxication

We can now say that large neurons of the cochlear nuclei of homozygous Gunn rats suffer severe damage. Ultrastructural evidence of mitochondrial changes is presented, and it is clear that these elements are among the earliest structures to undergo changes in bilirubin encephalopathy. In these nuclei we encountered convincing signs of mitochondrial involvement as early as 2 days post-partum, although the numbers of neurons and mitochondria affected were very much fewer than in older animals.

In their studies of cerebellar Purkinje cells, Schutta *et al.* (1970) saw enlarged mitochondria at 3–8 days of age, followed by granule accumulations in mitochondria from 8 days onward. Similar changes took longer to appear in neurons of the cerebellar nuclei, colliculi, cerebral cortex, hippocampus and basal ganglia; enlarged mitochondria being manifest at 8–12 days of age whereas collections of glycogen were seen in these nuclei only in animals aged 2 months and more. Sulphadimethoxine which, according to Lee & Cowger (1974), causes an acute influx of bilirubin into tissues, including the brain, did not accelerate the incorporation of glycogen into mitochondria, even though it did appear to precipitate early and severe

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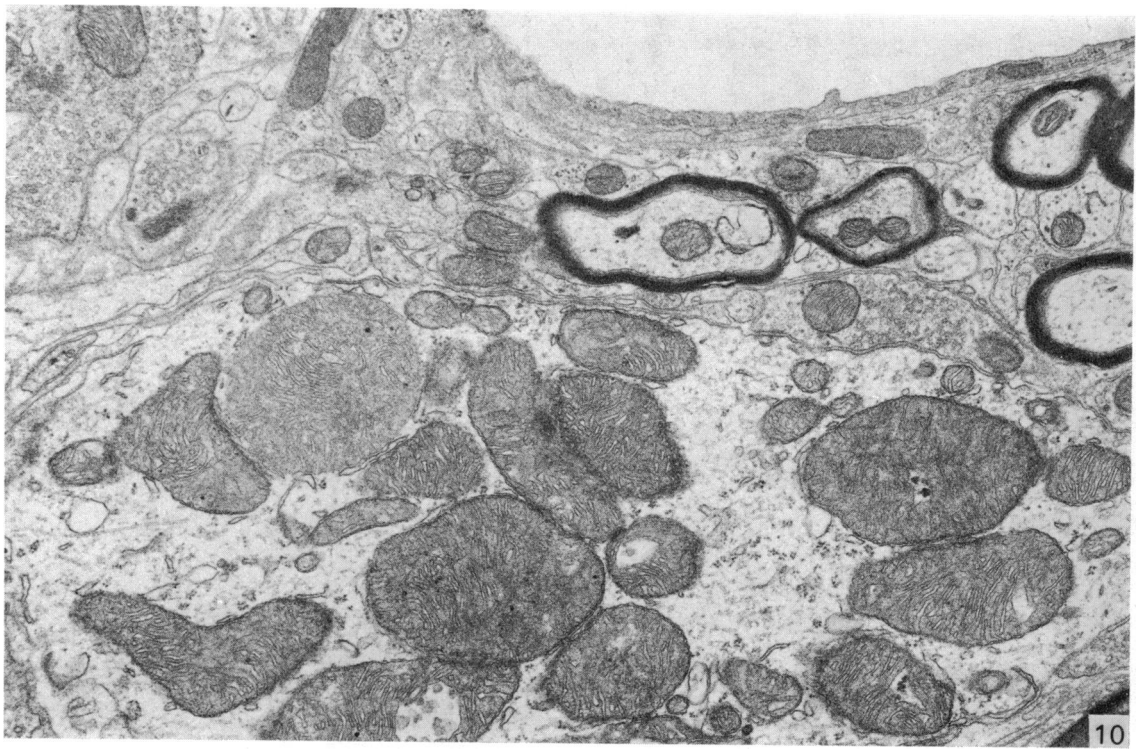
Fig. 9. In this field the two large accumulations of glycogen are found in sacs with multilayered walls, the inner aspect of which is irregular. These are presumed 'ex-mitochondrial sacs', one of which is partitioned. The outer wall at one pole of each sac (arrows) appears to be disrupted; it seems reasonable to infer that glycogen is being extruded at these sites. The proximity of packaged glycogen suggests that the extruded glycogen particles become membrane-bound. × 37100.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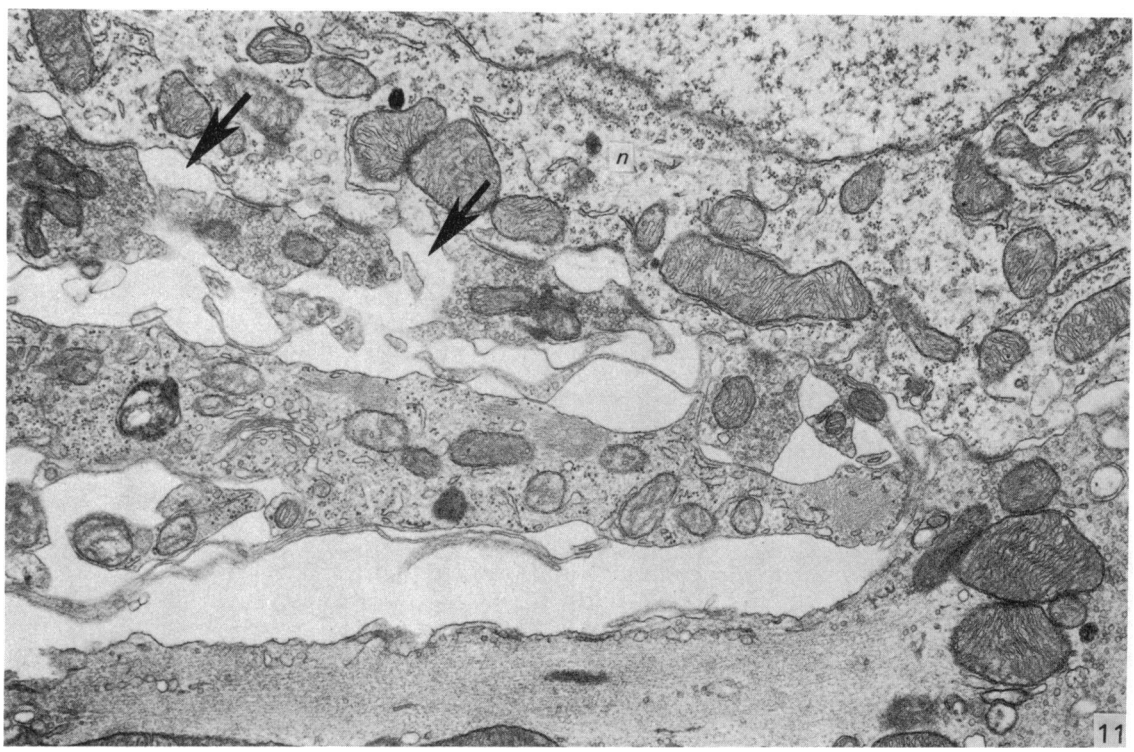
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cytoplasmic damage evidenced by membranous whorls and ER dilatations (Schutta & Johnson, 1971). These observations were regarded as evidence that the primary site of damage in bilirubin encephalopathy is in the general neuronal cytoplasm rather than in the mitochondria; and that mitochondrial abnormalities occur secondary to cytoplasmic disorganization. However, the circumstances prevailing after sulphadimethoxine has been administered may favour damage to different (cytoplasmic) elements. In the cochlear nuclei, the ultrastructural findings indicative of a mitochondrial disorder are both clear and numerous, although it should be borne in mind that our evidence in no way refutes biochemical changes in other cytoplasmic elements.

Neurons especially susceptible to bilirubin poisoning are the Purkinje cells in the cerebellum (Schutta & Johnson, 1967) and the large neurons of the cochlear nuclei, as noted in the present study. Since the biology of mitochondria is not uniform in all cells (Lehninger, 1965), differences in susceptibility to bilirubin may be due to peculiarities of mitochondrial biology. Different responses may also be related to cytoplasmic or regional pH differences (Silberberg, Johnson & Ritter, 1970).

Whether directly or indirectly, bilirubin certainly damages mitochondria *in vivo*. Our study has confirmed earlier observations that bilirubin causes mitochondria to enlarge (Odell, 1966; Schutta *et al.* 1970), become vacuolated, and accumulate glycogen. These glycogen deposits were the most striking feature seen in our material. Frequently, glycogen accumulations were also seen in smooth and rough ER. Although the presence of glycogen in organelles other than lysosomes is unusual (Ibrahim, 1975), several instances of intramitochondrial glycogen have been reported in neural and non-neural cells. Glycogen is a constituent of mitochondria in some reactive axonal endings after lesions (Lampert, 1967); in axons of old mice (Johnson, Mehler & Miquel, 1975); in pathologic muscle tissues (Hübner, Palussen & Kleinsasser, 1967; Hug & Schubert, 1970); retinal photoreceptor cells (Ishikawa & Pei, 1965; Ishikawa & Yamada, 1969); Warthin's tumour oncocytes (Tandler, Hutter & Erlandson, 1970); in riboflavin-deficient hepatic cells (Tandler, Erlandson & Wynder, 1968); and spermatozoa (Anderson & Personne, 1970).

The sequence of steps purported to occur in normal glycogen metabolism does not provide a ready explanation for the intramitochondrial glycogen found in Gunn rat neurons. It has been demonstrated that bilirubin has an effect on membranes of brain mitochondria. A bilirubin-damaged 'leaky' membrane might allow the influx of cytoplasmic glycogen into the mitochondria. Conceivably, the mitochondria phagocytose glycogen from the cytoplasm, since it has been shown that mitochondria can take up ferritin, silica and saccharated iron oxide (see Tandler *et al.* 1970 for review). Alternatively the altered mitochondria may possess the enzymes necessary for glycogen synthesis. Indeed, Anderson & Personne (1970) have demonstrated in certain spermatozoa that mitochondria may contain not only glycogen but also the enzymes of glycogenesis and glycogenolysis.

Fig. 10. In bilirubin encephalopathy, neuronal mitochondria are often hypertrophied and, in this form, occur in groups as shown here. Irregularities in cristal geometry may be encountered in hypertrophied mitochondria, and presumed glycogen inclusions are quite common. ×20300.

Fig. 11. Microcavitation in the neuropil adjacent to a neuron (*n*) in the AVCN of a hyperbilirubinaemic rat aged 4 weeks. Axosomatic synapses remain but glial elements appear to be reduced. Microcavitation is evident in close relationship to the specialized contact sites (arrows). ×17900.

The glycogen deposits appeared in a variety of shapes, sizes and electron densities. The presence of this variety of forms probably reflects different macromolecular branching patterns and composition, pH, activators, inhibitors and other intracellular factors (Takeuchi *et al.* 1975).

Various mechanisms whereby bilirubin may exert its cytotoxicity have been considered. Although some doubts have arisen as to whether uncoupling of oxidative phosphorylation (depression of cellular respiration) is the primary biochemical event underlying bilirubin cytotoxicity, our studies show that mitochondrial alterations are among the earliest manifestations of bilirubin encephalopathy and are noted even without other apparent damage to the neuron. Ernster (1961) emphasized that brain function is very sensitive to agents acting on cellular respiration and that severe inhibition of mitochondrial respiration and phosphorylation is not necessary in order to interfere with central nervous system activity. As an extension of this point, the existence of a bilirubin concentration mechanism in the mitochondrion would allow bilirubin to produce mitochondrial effects *in vivo* even though it was argued that the general bilirubin concentration was inadequate to uncouple the oxidation cycle. Answering this doubt is important, even though difficult.

Brain cells may concentrate bilirubin by transferring it from albumin to cellular proteins, possibly to the very lipoprotein structures on which bilirubin then exerts its damaging effect. The evidence that there is an abnormal build-up of glycogen in relation to cristae supports the claim that a disorder inducing glycogenesis, and conceivably obstructing oxidative phosphorylation, is the biochemical mechanism underlying the structural and functional disorder. The possibility cannot be ruled out that the limiting membrane of the mitochondria may be leaky, allowing influx of either glycogen or its precursors. Certainly we have noted a disruption of the limiting membrane after the mitochondrion has been converted into a glycogen-containing sac. Previous morphological and biochemical findings appear compatible with the hypothesis that the primary effects of bilirubin may be on membrane function, and particularly on mitochondrial membranes.

SUMMARY

The cochlear nuclei of homozygous Gunn rats aged 2 days to 7 months were examined. Ultrastructural abnormalities were observed in all age groups studied, including 2 and 4 days old animals. Mitochondrial alterations are among the earliest manifestations of bilirubin encephalopathy (2 days). In mitochondria of large neurons, vacuoles were found which contained increasing (with age) collections of alpha and beta glycogen particles. Some of the larger 'ex-mitochondrial sacs' appear to have been caught at the point of disruption, with glycogen-filled vacuoles in close proximity. Dilated profiles of rough ER also contained glycogen particles. In the cytoplasm of the same large neurons, elaborate myelin figures surrounded tongues of cytoplasm, vacuoles and degenerative elements. Reconsideration of previous morphological and biochemical observations in the light of the present findings makes it appear very likely that bilirubin primarily affects membrane function, especially in mitochondria.

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