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Ultrastructural Study of the Purkinje Cells of Hamster Cerebellar Cortex after Chronic Alcohol Administration

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ULTRASTRUCTURAL STUDY
OF THE PURKINJE CELLS OF HAMSTER CEREBELLAR CORTEX
AFTER CHRONIC ALCOHOL ADMINISTRATION

by

Mohamed Hisham Atwa | Mostafa

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
Master of Science

May

1987

DEDICATION

To my father.

ACKNOWLEDGMENTS

I will always be indebted to my advisor, Dr. Faith LaVelle, for her unfailing support, guidance, enthusiasm and insight, which made the work possible.

I would also like to thank the other members of my Examination Committee, Dr. McNulty and Dr. Manteuffel, for their helpful and critical evaluation of this work.

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VITA

The author, Mohamed Hisham Atwa Mostafa, was born on March 28, 1957, in Zagazig, Egypt.

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He has been accepted into the Neuroscience Graduate Program at Loyola and is hopeful of receiving approval from the Mission Department, Cairo, Egypt, so that he may continue his graduate studies toward the Ph.D. degree.

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INTRODUCTION

From biochemical, physiological, and clinical studies the cerebellum is known to be affected by alcohol ingestion, but little is known about the accompanying changes that take place in the affected cerebellar cells. Functional changes in Purkinje cells after chronic alcohol consumption have been reported, but limited morphological data are available, especially concerning alteration of the cellular organelles which play important roles in the metabolic activity of the cells. The aim of this study was to identify and morphometrically characterize ultrastructural changes in mitochondria, lipofuscin granules, and endoplasmic reticulum within Purkinje cells of the cerebellar cortex after chronic alcohol administration in adult male hamsters. Consideration was also given to the possible reversibility of neuronal change after a period of withdrawal from alcohol.

Although the limited number of animals used in this study proved, for the most part, to be insufficient to overcome the variability of neuronal cytoplasmic fields that were chosen randomly and viewed at the ultrastructural level, results do nevertheless demonstrate a significant increase in mitochondrial diameter with exposure to alcohol and reveal other changes in value which may be meaningful. In addition, an unusual modification of rough endoplasmic reticulum into distinctive lamellar bodies is described.

GENERAL LITERATURE REVIEW

It is well accepted that alcohol has an effect on nerve cells, although contradictory results from different laboratories have indicated both cell activation and depression. In physiological studies on the rat cerebellum, acute exposure to relatively large dosages of ethanol have been shown to produce both an elevated (Seil et al., 1977; Rogers et al., 1980) and a decreased (Eidelberg et al., 1971; Mitra, 1977) firing rate of Purkinje cells. Using rat cerebellar slices, George and Chu (1984) have recorded a dose response relationship between ethanol and cerebellar Purkinje cell activity and have concluded that there is a progressive inhibitory effect of ethanol on these cells. Similarly, Yamada (1986) found that a low concentration of alcohol (.03%) applied directly to cerebellar slices did not affect the firing rate of rat Purkinje cells, while a high concentration (0.4%) completely inhibited their activity. However, at different concentrations between these two extremes, an initial inhibition of activity gradually disappeared, indicating the building of adaptive tolerance.

From the clinical aspect, it has been shown by Victor et al. (1959) in their work on 50 alcoholic patients that there is a definite type of cerebellar cortical degeneration, with severe loss of Purkinje cells in such areas as the flocculi and parts of the lingula. In similar patients, Ferrer et al. (1984) reported a significant

reduction of the dendritic arborization of Purkinje cells in the rostral part of the vermis, although not in the cerebellar hemispheres. Torvik and Torp (1986), in their work on 31 human alcoholics and 34 non-alcoholics reported significantly lower Purkinje cell population densities in the superior and middle segments of the vermis of alcoholics, and they concluded that about one half of all severe alcoholics have atrophy of the superior vermis.

In biochemically oriented studies, the effect of alcohol on protein synthesis in nerve cells has also been controversial. Tewari and Noble (1971), in their work on alcohol-treated mice, reported an increase in the brain ribosomes of alcohol-treated animals, but these ribosomes revealed a significantly reduced capacity for incorporating amino acid into proteins. Noble and Tewari (1973) showed that brain ribosomes of mice ingesting 10% alcohol for different relatively long periods revealed a significant decrease in incorporation of U-[¹⁴C] leucine in ribosomal protein. They concluded that the major defect was located in the pH5 enzyme fraction and to a lesser extent in the tRNA. They also reported that liver mitochondria, unlike brain mitochondria, showed a decreased ability to incorporate L-[¹⁴C] leucine into proteins, and that withdrawal from alcohol for two weeks was found to cause 40% recovery in the protein-synthesizing system. Noble and Tewari (1975), using mice and rats ingesting 10% alcohol, reported a greater inhibition of polypeptide synthesis in free than in membrane-bound polyribosomes. In vivo incorporation of (5-[³H])-orotic acid into RNA was more inhibited in polysomes than in

ribosomes, suggesting that ethanol affects messenger RNA. Tewari et al. (1980) reported significant inhibition of protein synthesis by ribosomes from alcohol-treated rats and concluded that the reduced ribosomal binding to stable messenger RNA may be a contributing factor in the inhibiting effect of alcohol on protein synthesis.

On the other hand, Kuriyama et al. (1971), using intraperitoneal injection of ethanol in mice, found that acute administration of ethanol inhibited protein synthesis, while chronic administration stimulated it. Lamar (1972) reported up to 40% reduction of [¹⁴C]-leucine incorporation into isolated ribosomes from rat brains after force-feeding animals with 5 g ethanol/K of body weight by stomach tube. In a different type of study, Rubin et al. (1970) found a decrease in amino acid incorporation in mitochondria isolated from chronically treated rats. Like many biochemists who have sought to identify a specific factor responsible for the noxious effects of alcohol, Wickramasinghe et al. (1986) have suggested that alcohol metabolism may generate one or more non-dialysable cytotoxic protein molecules which might play a role in the pathogenesis of alcohol-induced tissue damage.

The ultrastructural changes which accompany ethanol consumption have only recently been investigated in different populations of neurons, and statistical data for these changes are still limited. Volk (1980) fed rats 15% ethanol for 6 months and looked at the ultrastructural changes of their spinal ganglion cells. He reported an increase in lipofuscin, as well as the occurrence of paired helical

filaments which were interpreted by him as being induced by the interaction of ethanol with the biosynthesis of proteins. Working with the cerebral cortex of rats, Karwacka (1980) reported a marked decrease in the number of ribosomes, polysomes, and profiles of rough endoplasmic reticulum, all of which are involved in the synthesis of protein. He also noted proliferation of smooth endoplasmic reticulum and changes in the mitochondria, especially an elongation of their profiles and an increase in their number. His observations, however, lack any morphometric analysis to demonstrate their statistical significance. In a study that cross-referenced ultrastructural observations with biochemical analysis, Karwacka et al. (1980) noticed that although the configuration of isolated mitochondria was severely affected by chronic ethanol intake, the course of oxidative phosphorylation in the mitochondrial fraction of rat brain was not affected.

Dunmire and LaVelle (1983) recorded a significant increase in the preribosomal intranucleolar bodies of hamster facial neurons after chronic ethanol ingestion and related this to the alterations in protein synthesis and utilization reported by others for neurons. Also looking at the nucleus, Tavares and Paula-Barbosa (1981) have noted abnormal nuclear inclusions in granule and Purkinje cells of the rat cerebellum after treatment with alcohol.

Using the protocol that is being followed in the present study, Dunmire-Graff (1981) reported apparent mitochondrial swelling and dilated cisterns of endoplasmic reticulum in hamster neurons. In a

qualitative study of isolated mitochondria, Karwacka et al. (1980) described mitochondrial enlargement and disorientation of cristae in chronic alcoholic rats. Details of the few other studies done on neuronal organelles after exposure to alcohol will be reserved for the discussion.

The types of ultrastructural changes observed in nerve cells are similar to those reported for other cells of the body following ingestion of ethanol. Rubin and Lieber (1967) and Rubin et al. (1970), for example, in studies of human hepatocytes, noticed enlarged and elongated mitochondria, some of which contained small crystalline inclusions. RER decreased in amount, and the number of polyribosomes strikingly increased. Along with these changes, Rubin et al. found that, with the addition of ATP in vitro, mitochondrial swelling was abolished. Phillips et al. (1975), feeding alcohol to rats in a nutritionally adequate liquid diet, reported decreased RER and proliferation of smooth ER in hepatocytes. They also noticed that most of the mitochondria were normal in appearance after exposure to alcohol, although a small proportion of them were swollen. On the other hand, Kujawa and Karwacka (1980) found that intake of increasing concentrations of ethanol (2.5 to 10%) over a 4-week period caused enlarged hepatic mitochondria and a trend toward a decrease in their number. Interestingly, they reported that increasing the ethanol concentration to 42% and prolonging the period of exposure to 12 weeks did not markedly enhance the changes. Berman et al. (1983) reported that chronic alcohol exposure caused an increase in the volume and

surface densities of mitochondria of hepatocytes studied both in vivo and in vitro. In their work on isolated hepatic mitochondria of rats, Rubin and Cederbaum (1977) demonstrated the injurious biochemical effect of chronic alcohol exposure through depression of oxygen uptake and CO₂ production.

Similar organellar and functional changes after alcohol ingestion have been observed in other tissues. Darle et al. (1970) fed adult male rats different concentrations of alcohol for periods ranging from 14 to 53 weeks and looked at ultrastructural changes in the acinar cells of the pancreas. As early as 14 weeks, they reported local intracellular cytoplasmic degeneration with increased numbers of lipid droplets. They also reported mitochondrial swelling with fewer and markedly irregular cristae from 36 weeks of alcohol administration onward. Rubin et al. (1972) reported that when ethanol was isocalorically substituted for carbohydrate and accounted for 36% of total calories in the food of male rats and male human volunteers, the epithelium of the small intestine showed mitochondrial and endoplasmic changes, along with the appearance of irregular, dense, osmiophilic, whorled, myelin-like material in the cytoplasm. Similarly, such ultrastructural changes have been reported following stress factors other than alcohol. Vanneste and Bosch de Aguilar (1981) noted that aging can cause consistent mitochondrial changes in the form of giant mitochondria, as well as opaque ones with fewer cristae, in the spinal ganglion neurons of aging rats. Yamano et al. (1985) recorded that administration of cupric chloride in the brindled mouse causes

mitochondrial swelling and large lamellar bodies in Purkinje cells.

It is clear, then, that alcohol exerts a deleterious effect upon many types of cells, including neurons, and that two organelles most frequently affected are mitochondria and rough endoplasmic reticulum. The exact nature of the injury incurred by organelles responsible for the appropriate function, energetics, and synthesizing capabilities of cells awaits further study. The following report is one such study made at the ultrastructural, morphometric level.

MATERIALS AND METHODS

Sixteen adult male golden hamsters (Mesocricetus auratus) (initial weight 109-136 grams) were obtained from Harlen Laboratories, Indianapolis, Indiana. All animals were individually housed in plastic cages in the Loyola University Medical Center Animal Research Facility under the following conditions: $72^{\circ} \pm 2^{\circ}\text{F}$, 50% relative humidity, with 12 hour light/12 hour dark cycle. They received standard rat chow ad libitum. The animals were allowed one week for adjustment to these conditions before starting the experiment. They were then divided, according to their access to alcohol, into three different groups. The control group of six animals had unrestricted access to tap water as their only drinking fluid. The rest of the animals (10) were given unrestricted access to a 15% aqueous ethanol solution as their only drinking fluid. Half of these experimental animals (5) (group G_1E) were sacrificed after 7 weeks and the other half (5) (group G_2E) were removed from alcohol at 7 weeks and given access only to tap water as their drinking fluid for the following 4 weeks before being sacrificed. Of the six control animals, three were sacrificed at 7 weeks (G_1C) and three at 11 weeks (G_2C). The food and fluid intake of all animals was measured every three days and their body weight recorded every week. Differences in weight gain and in food and fluid intake between experimental and control animals were analyzed statistically, using Student's t test.

Just prior to sacrifice, the animals were weighed and anesthetized, using an intraperitoneal injection of sodium pentobarbital (Nembutal, 64.8 mg/cc) with a dose of 0.12 cc/100g body weight. The abdominal cavity of each anesthetized animal was rapidly opened and the chest cavity approached by cutting the diaphragm and subsequently reflecting the ventral thoracic walls. A blunt needle was advanced through a small incision in the left ventricle, to the aorta. Perfusion fixation was carried out using a gravity-directed method consisting of two suspended intravenous drip bottles connected to a common Y-type tubing, allowing for the sequential flow of saline and fixative solutions. The intracardiac needle was connected to the end of the Y-type tube. With the initiation of the perfusion, the right atrium was punctured to allow the escape of blood and perfusion solutions. To ensure a blood and clot free circulatory system, the animals were first perfused with a fast stream of normal saline wash solution. The resulting pale color of the liver and lungs, as well as the flow of fluids from the right atrium, served as indicators to start the fixative solution (2% formaldehyde-2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.4), which continued to flow until internal organs were firm and the animal became rigid. Both wash and fixative solutions were perfused at room temperature.

The brain was then exposed, the cerebellum dissected out, and portions of the most lateral areas of the cerebellar hemispheres removed for preparation for electron microscopy in the following manner: tissue specimens were left in cold fixative (0°C) for 30

minutes, rinsed with cold sucrose-phosphate buffer, and postfixed for 90 minutes in osmium tetroxide (1.33% in phosphate buffer). They were then rinsed again with cold phosphate buffer, dehydrated in a graded ethanol series, and infiltrated with varying concentrations of propylene oxide and plastic (1:1, 1:3, and 100% plastic). The specimens were embedded in Epon 812 and Araldite 502 in a 37°C oven overnight and then for one week in a 60°C oven for complete polymerization.

Thick sections (1µm) were cut and stained with toluidine blue for purposes of orientation. After identification of the Purkinje cell layer, the blocks were retrimmed and ultrathin sections 600-900Å (showing silver to gray interference colors) were cut on a LKB88A ultramicrotome, using glass knives. These sections were mounted on uncoated, 200-mesh copper grids and stained with saturated aqueous (5%) uranyl acetate and Reynold's lead citrate. In order to randomize the counts and measurements to be made, the sections were coded and photographs taken without knowing whether the chosen cells belonged to control or experimental animals.

Ten Purkinje cells from each animal were photographed at a final magnification of 38,000X on an Hitachi HU11B electron microscope at an accelerating voltage of 75 KV. Each cell was represented by two pictures encompassing the widest areas of cytoplasm. The cytoplasmic organelles chosen for study were mitochondria, lipofuscin granules, and rough endoplasmic reticulum, including lamellar body modifications.

The volume density (V_v) of mitochondria (mitochondrial volume per unit volume of cytoplasm) was estimated using a double lattice test system containing 108 coarse points on a 22 cm x 16 cm grid, and using the formula $V_v = P_i/P_t = \mu\text{m}^3/\mu\text{m}^3$ where P_i = test points resting on mitochondria and P_t = test points on total cytoplasm. (See Weibel, 1973, for details of stereological techniques used throughout.) The surface density (S_v) of mitochondria and of rough endoplasmic reticulum (surface area of the component per unit volume of cytoplasm) was estimated using a coherent multipurpose test system and the formula $S_v = 2 I_i/L_t = \mu\text{m}^2/\mu\text{m}^3$ where I_i = intersections of membranes of mitochondria or of rough endoplasmic reticulum with test lines, and L_t = total line length within the cytoplasm. Lamellar bodies were divided into two groups: those with 4 cisterns or less, and those with more than 4 cisterns. Both groups were directly counted per area of cytoplasm ($\#/A$). In addition, the number per cytoplasmic area ($\#/A$), the individual profile area (SA), and the maximum diameter (MxD) of mitochondria and lipofuscin granules were determined using the computerized image analyzer Videoplan (Carl Zeiss). All mean values were subjected to Student's t test for statistical analysis.

RESULTS

Effects of Alcohol on Animal Weights and Daily Intake

Alcohol exposure at the levels used in this study resulted in neither sickness nor death and produced very few overt behavioral changes, other than transient hyperexcitability and a slight temporary loss of motor coordination. Animals that were returned to water after the 7-week period of alcohol consumption showed no adverse withdrawal symptoms.

While on the alcohol regimen (weeks 1-7), animals gained, on average, significantly more weight per day (0.7g) than their counterpart controls (0.5g) (Table 1). The rate of gain slowed with time in both sets of animals, however, over the span of the 7-week experiment (Table 2). For all animals that remained for an extra 4 weeks and drank only water, whether as controls or recovering after 7 weeks on alcohol, daily weight gain was reduced and the differences between the two groups became negligible.

The amount of solid food intake per kilogram of body weight was significantly less in animals receiving alcohol (40.5g/day), as compared with the controls (53.1g/day) (Table 3). When the absolute daily amounts of food eaten were compared, this same significant disparity was observed (5.4g vs. 7.0g). On the other hand, the absolute amount of fluid drunk was significantly more in experimental animals (25.9 ml/day) than in the controls (22.8 ml/day) (Table 4).

TABLE 1

Gain in body weight per week and per day per total experimental period in both control and experimental animals

In the first 7 weeks, experimental animals received a 15% alcohol solution as their only drinking fluid; during the following 4 weeks they were removed from alcohol and returned to water, which was the only drinking fluid for the controls throughout the study.

Weight Gain (in grams)

<u>Time Period</u>	<u>Control Animals</u>	<u>Experimental Animals</u>	<u>P Value of Difference</u>
First 7 Weeks	3.1/wk \pm 0.6	5.0/wk \pm 0.5	0.001
	0.5/day \pm 0.3	0.7/day \pm 0.4	
Following 4 Weeks	0.2/wk \pm 0.4	0.2/wk \pm 0.9	0.130
	0.0/day \pm 0.0	0.0/day \pm 0.0	

First 7 weeks, N = 6 controls
10 experimentals

Following 4 weeks, N = 3 controls
5 experimentals

Statistical analysis: Student's t test.
Mean initial body weight = 119 \pm 7.2 grams.

TABLE 2

Details of gain in body weight per week in control and experimental animals.

In weeks 1 through 7, experimental animals received a 15% alcohol solution as their only drinking fluid; during weeks 8 through 11 they were removed from alcohol and returned to water, which was the only drinking fluid for the controls throughout the study.

Weight Gain/Week (in grams)						
Week #	Control Animals			Experimental Animals		
	Mean	±	S.D.	Mean	±	S.D.
1	6.6	±	1.9	8.5	±	1.4
2	5.2	±	1.4	7.4	±	2.2
3	3.3	±	1.2	5.0	±	1.5
4	1.7	±	0.8	3.9	±	1.3
5	1.7	±	2.4	3.7	±	1.2
6	1.8	±	1.5	3.7	±	2.1
7	1.5	±	1.0	3.1	±	2.2
8	0.5	±	0.7	0.4	±	0.9
9	0.0	±	0.0	-0.6	±	1.3
10	0.0	±	0.0	0.6	±	0.9
11	0.0	±	0.0	0.2	±	0.4

N = as in Table 1.

Mean initial body weight = 119 ± 7.2 grams.

Animals = approximately 90 days old at beginning of experiment.

TABLE 3

Average daily consumption of food (rat chow)
by adult hamsters during periods of alcohol intake
(first 7 weeks) or recovery from alcohol (following 4 weeks).

Food Intake/Kg Body Weight/Day (in grams)					
Time Period	Control Animals		Experimental Animals		P Value of Difference
	Mean	S.D.	Mean	S.D.	
First 7 Weeks	53.1	± 11.8	40.5	± 10.2	0.0001
Following 4 Weeks	44.4	± 6.5	32.3	± 6.0	0.001
Food Intake/Day (in grams)					
First 7 Weeks	7.0	± 1.0	5.4	± 0.9	0.001
Following 4 Weeks	5.1	± 0.8	5.7	± 0.9	0.265
First 7 weeks, N = 6 controls 10 experimentals					
Following 4 weeks, N = 3 controls 5 experimentals					
Statistical analysis: Student's t test					

TABLE 4

Average daily consumption of fluid (water or 15% ethanol in water) by adult hamsters during periods of alcohol intake (first 7 weeks) and recovery from alcohol (following 4 weeks).

The intake of alcohol by experimental animals during the first 7 weeks was the equivalent of 16g per kilogram of body weight per day.

Fluid Intake/Day (in milliliters)

<u>Time Period</u>	<u>Control Animals</u>		<u>Experimental Animals</u>		<u>P Value of Difference</u>
	<u>Mean</u>	<u>S.D.</u>	<u>Mean</u>	<u>S.D.</u>	
First 7 Weeks	22.8 ±	1.7	25.9 ±	1.8	0.013
Following 4 Weeks	22.3 ±	1.8	17.1 ±	1.1	0.0001

First 7 weeks, N = 6 controls
10 experimentals
Following 4 weeks, N = 3 controls
5 experimentals
Statistical analysis: Student's t test.

The standardized amount of alcohol consumed by the experimental animals was determined to be 16 g per K of body weight per day.

During the last 4 weeks of the experiment, when the experimental animals were removed from alcohol and were allowed to "recover" with water as their drinking fluid, they experienced a marked decline in daily fluid intake (to 17.1 ml/day). Control animals remained quite constant, at 22.3 ml water per day. During this same interval of time, the absolute amount of food consumed became slightly higher in experimental animals and lower in the controls, but when the amounts of food consumed were computed per K of body weight and compared, the experimental group was still found to be eating significantly less than the control group (32.3g vs. 44.4g) (Table 3).

Description of Neuronal Ultrastructure

Purkinje cells are easily identified because of their large size (25-40 μm in diameter) and their focal position in the architecture of the cerebellar cortex as a one-cell-thick layer at the interface between the molecular and granular layers. As in other animals, the somata of hamster Purkinje cells are typically crowded with organelles, reflecting the high metabolic activity of these cells.

As seen in this study, mitochondrial profiles were typically ovoid and rather small (Fig. 1A), although some were more elongate and irregular in shape (Figs. 1B and 2). In the alcohol-exposed animals, mitochondria appeared to be somewhat more pleomorphic than normal.

Elongated mitochondria were more frequently encountered (Fig. 3B) than in the control or recovery groups. Occasionally, enlarged mitochondria with electron-dense deposits within the matrix were noticed in alcohol-ingesting animals (Fig. 3A) and not in other groups. The orientation of the cristae inside the mitochondria was primarily transverse, with no special differences between the three groups of animals. Translucent matrix and irregularly dispersed cristae were seen only in the occasional, swollen mitochondria of alcohol-affected cells (Fig. 8). As described below, there was frequently a very close positional relation between the mitochondrion and endoplasmic reticulum (Fig. 4A).

The cytoplasm of the Purkinje cells of all animals was densely populated with polyribosomal clusters (see Figs. 3-5). Rough endoplasmic reticulum was scattered throughout the cytoplasm (Fig. 4A,B) but only occasionally was arranged in the stacks of parallel cisterns typical of clumped Nissl bodies (Fig. 5A,B). Both wide (Fig. 5A) and narrow (Fig. 5B) cisterns of rough endoplasmic reticulum were noticed in control animals and in those ingesting alcohol and recovering after alcohol withdrawal.

One feature of nearly all Purkinje cells was a distinctive modification of rough endoplasmic reticulum to form "lamellar bodies". When two cisterns or more lay close together, their inner membranes became degranulated and the cytoplasm between adjacent membranes became relatively dense (Fig. 6A,B) with an intercisternal space of 120-150Å. The number of cisterns forming such lamellar bodies varied

from 2 to 10 and were typically stacked in parallel, flattened, regularly spaced and very clearly defined layers (Figs. 2 and 6A). Ribosomes were attached to the outermost membranes of the bodies, and to cisternal extensions that continued laterally from the bodies, usually from the outermost cisterns (Fig. 6A) but occasionally from intermediate ones. Individual cisterns of degranulated endoplasmic reticulum were often widened at their lateral extremes (Fig. 6A). As already mentioned, there was a characteristically close positional relationship between lamellar bodies and mitochondria. Frequently the bodies were closely applied to the outer membrane of the mitochondria (Fig. 7A,B). In some instances, mitochondria were almost entirely encircled by lamellar bodies (Fig. 7B). Exposure to alcohol or recovery from it did not seem to affect the overt appearance of the lamellar bodies.

Subsurface cisterns with the characteristics of lamellar bodies were frequently observed lying at a distance of 220-250 Å from the plasma membrane of the cell (Figs. 7A and 8). Occasionally the cisternal membranes of more centrally placed lamellar bodies showed continuity with the outer membranes of the nuclear envelope (Fig. 9A). Aggregates of undulating tubules of endoplasmic reticulum were noticed in both experimental and control groups (Fig. 9B).

Membrane-bound vesicular inclusions were a constant feature of Purkinje cell cytoplasm, though they were not nearly so numerous as mitochondria. Ultrastructurally there were two types of these dense, cytoplasmic, membrane-bound inclusions. Some were primary lysosomes

with a uniformly dense matrix and smaller dimensions (Figs. 8 and 10A). Other inclusions were irregular in size and shape and fit the description of secondary lysosomes (Figs. 10A,B). The largest and densest of these are considered to be lipofuscin granules (Figs. 8 and 10B); they had an electron-dense matrix which obscured the limiting membrane, but were sometimes more lucent centrally and often contained scattered dense particles, peripherally located vacuoles, or concentric lamellar arrangements (Figs. 7B and 11). No differences could be noticed between the three groups of animals studied.

Morphometric Analysis of Ultrastructural Changes

Mitochondria (Table 5)

The maximum diameter (MxD) of mitochondria of alcohol-treated animals (G_1E) showed a statistically significant increase ($P < 0.05$) as compared to the control group (G_1C) at the end of 7 weeks. This significance was not approached ($P > 0.05$) when 11-week control animals (G_2C) were compared to the recovering group (G_2E), because of the down-trend in values of the recovering group toward the control level.

Values for surface area (SA) and volume density (Vv) of mitochondria tended to increase after 7 weeks of exposure to alcohol (G_1E), although not at a significant level ($P > 0.05$). Both values tended to revert to normal following 4 weeks of recovery (G_2E). Unlike the values for SA, MxD, and Vv, all of which increased marginally with alcohol exposure, the mitochondrial counts (#/A)

TABLE 5

Differences in mitochondrial counts and measurements between control animals (G_1C) and those receiving alcohol (G_1E) for 7 weeks; also between controls (G_2C) and those removed from alcohol (G_2E) for the subsequent 4 weeks of the experiment.

Parameter	Animal Group	Mean	S.D.	S.E.	P Value of Difference
SA	G_1C	0.126	0.011	0.006	0.124
	G_1E	0.148	0.027	0.012	
	G_2C	0.122	0.024	0.014	0.268
	G_2E	0.140	0.043	0.019	
MxD	G_1C	0.512	0.008	0.005	* 0.046
	G_1E	0.565	0.053	0.023	
	G_2C	0.517	0.034	0.020	0.304
	G_2E	0.544	0.081	0.036	
Vv	G_1C	0.143	0.005	0.003	0.150
	G_1E	0.160	0.025	0.011	
	G_2C	0.139	0.008	0.005	0.366
	G_2E	0.146	0.030	0.013	
#/A	G_1C	0.917	0.030	0.018	0.572
	G_1E	0.902	0.130	0.058	
	G_2C	0.986	0.170	0.098	0.301
	G_2E	0.884	0.091	0.041	
Sv	G_1C	1.709	0.042	0.024	0.226
	G_1E	1.815	0.219	0.098	
	G_2C	1.871	0.136	0.078	0.804
	G_2E	1.779	0.137	0.061	

N = 3 for each control group (G_1C , G_2C).

N = 5 for each experimental group (G_1E , G_2E).

P values were determined for G_1C vs. G_1E , and for G_2C vs. G_2E , for each parameter.

Statistical analysis of differences: Student's t-test.

* $P < 0.05$.

tended to show lower values for experimental animals than for controls, although not at a level of significance. No consistent trends were demonstrated by estimates of surface density of mitochondrial profiles per unit volume of cytoplasm (Sv).

Endoplasmic reticulum (Table 6)

Measurements of surface density (Sv) of the rough endoplasmic reticulum showed no consistent changes with alcohol exposure or after withdrawal of alcohol. The numbers of small (SLB) and large (LLB) lamellar bodies per unit area of cytoplasm (#/A) were not significantly changed from controls after 7 weeks of alcohol-ingestion (G_1E) or after the 4-week withdrawal period (G_2E), although there was a slight increase in values for the small bodies during the latter period ($P=0.079$). Over the entire 11 weeks, there was constancy in the number of small lamellar bodies in controls (G_1C and G_2C), but the number of large lamellar bodies showed a slight increase with time. It should be noted that in all animals there were up to 10-fold more small than large lamellar bodies.

Lipofuscin granules (Table 7)

The three parameters used to characterize possible change in the lipofuscin granules included surface area (SA), maximum diameter (MxD), and numbers of granules per unit area of cytoplasm (#/A). No comparisons of values between control and experimental groups of animals showed any statistical significance.

TABLE 6

Differences in endoplasmic reticulum counts and measurements between control animals (G_1C) and those receiving alcohol (G_1E) for 7 weeks; also between controls (G_2C) and those removed from alcohol (G_2E) for the subsequent 4 weeks of the experiment.

Parameter	Animal Group	Mean	S.D.	S.E.	P Value of Difference
Rough endoplasmic reticulum					
Sv	G_1C	2.398	0.416	0.240	0.687
	G_1E	2.263	0.348	0.156	
	G_2C	2.704	0.283	0.164	0.690
	G_2E	2.530	0.517	0.231	
Lamellar Bodies					
#(SLB)/A	G_1C	0.433	0.186	0.107	0.500
	G_1E	0.432	0.053	0.024	
	G_2C	0.415	0.073	0.042	0.079
	G_2E	0.529	0.107	0.048	
#(LLB)/A	G_1C	0.031	0.011	0.007	0.169
	G_1E	0.049	0.028	0.012	
	G_2C	0.041	0.018	0.010	0.426
	G_2E	0.045	0.029	0.013	

N = 3 for each control group (G_1C , G_2C).

N = 5 for each experimental group (G_1E , G_2E).

SLB = small lamellar bodies (4 cisterns or fewer).

LLB = large lamellar bodies (more than 4 cisterns).

P values were determined for G_1C vs. G_1E , and for G_2C vs. G_2E , for each parameter.

Statistical analysis of differences: Student's t test.

P > 0.05 throughout.

TABLE 7

Differences in lipofuscin counts and measurements between control animals (G_1C) and those receiving alcohol (G_1E) for 7 weeks; also between controls (G_2C) and those removed from alcohol (G_2E) for the subsequent 4 weeks of the experiment.

Parameter	Animal Group	Mean	S.D.	S.E.	P Value of Difference
SA	G_1C	0.097	0.015	0.009	0.520
	G_1E	0.096	0.018	0.008	
	G_2C	0.100	0.017	0.010	0.266
	G_2E	0.106	0.009	0.004	
MxD	G_1C	0.397	0.023	0.013	0.392
	G_1E	0.410	0.076	0.034	
	G_2C	0.390	0.036	0.021	0.180
	G_2E	0.410	0.022	0.010	
#/A	G_1C	0.126	0.015	0.009	0.254
	G_1E	0.139	0.030	0.013	
	G_2C	0.148	0.020	0.012	0.878
	G_2E	0.131	0.017	0.008	

N = 3 for each control group (G_1C , G_2C)

N = 5 for each experimental group (G_1E , G_2E)

P values were determined for G_1C vs. G_1E , and for G_2C vs. G_2E , for each parameter.

Statistical analysis of differences: Student's t test.

P > 0.10 throughout.

DISCUSSION

It has been shown by several investigators that golden hamsters, unlike most laboratory animals, prefer ethanol solutions to tap water (McCoy et al., 1981; DiBattista, 1986). A 15% concentration was shown by Arvola and Forsander (1961) to be optimal for maximum consumption by male adult hamsters and was also the concentration used by Dunmire-Graff (1981), whose protocol the present study has repeated. The consumption here of about 16 grams of ethanol per kilogram of body weight per day comes very close to the values reported by others (Dunmire-Graff, 1981; McCoy et al., 1981; DiBattista, 1986). Although the food intake was significantly lower in alcohol treated animals than in controls, the caloric intake was maintained through the ingestion of alcohol (see discussion by Dunmire-Graff, 1981). Since they gained significantly more weight than the control animals and maintained an apparently healthy physical appearance, with well-groomed coats and a normal level of activity, it can be assumed that undernutrition did not occur. It should be noted that different alcohol-induced morphological changes have been reported by others in the cerebellum, hippocampus, cerebral cortex and other parts of the brain in nutritionally controlled animals.

One sign of malnutrition that has been observed in male hamsters is regression of the testicles (Eskes, 1983). No evaluation of gonadal responses to alcohol was attempted in the present study, but there were no obvious signs of testicular regression. DiBattista's (1986)

evidence parallels ours that 15% ethanol ingestion does not cause overt testicular regression.

The magnitude of the increases in body weight reported in this study fall between values reported by other investigators. McCoy et al. (1981) and DiBattista (1986) found that hamsters ingesting 15% ethanol gained almost twice as much weight as control animals, while Dunmire-Graff (1981) reported no change in body weights throughout her experiments. According to DiBattista (1986), any weight gained is mainly due to fat deposited, especially in retroperitoneal and epididymal adipose tissue. It is worth noting that younger animals have less fat than more mature ones and that a difference in age at the time of study may determine the extent of body weight increase. In our study, the rate of increase in both control and experimental animals decreased gradually over the first several weeks, indicating that the animals were relatively young adults at the beginning of the experiment and required a few weeks to reach a stable, mature body weight. Initial body weights of our animals ranged from 109 to 136 grams; the normal range for "adult" hamsters is 125-150 grams.

The animals in the present study did not show any withdrawal signs when removed from ethanol, a fact which has been explained by Thurman et al. (1978) and DiBattista (1986) as being due to accelerated hepatic metabolism of ethanol in hamsters, as well as to general central nervous system insensitivity to the drug. In the study after which ours was patterned, Dunmire-Graff (1981) determined that the blood ethanol level in hamsters rose sharply during the dark phase of the

day-night cycle, reaching the highest mean level of 141 mg% at 0200 hours. After a sharp drop, the lowest mean level (23 mg%) was recorded at 1400 hours. Thus it appeared that adult hamsters do not maintain high blood ethanol levels for extended periods of time and that, therefore, their brains do not suffer prolonged exposure to high concentrations of ethanol.

Nevertheless, as already shown in the Literature Review section of this thesis, alcohol is known to adversely affect neural and other tissues. Cellular components, such as those involved in protein synthesis and therefore crucial to the regulation of normal cell activity, have been shown to undergo changes that are characteristic of stressed or faulty metabolic machinery. Despite numerous qualitative morphological studies and biochemical analyses of neurons subjected to acute or chronic exposure to alcohol, there have been very few reports concerning the morphometric analysis of subcellular changes in such cells.

Dunmire-Graff (1981) described apparent changes in several organelles of hamster Purkinje cells after alcohol intake, but did not quantitate these changes. Our aim has been to analyze morphometrically and statistically some of the changes in mitochondria, lipofuscin, and rough endoplasmic reticulum described by Dunmire-Graff (1981). The present study thus confirmed, statistically, her report of enlarged mitochondria after exposure to alcohol. In contrast, the present study did not confirm her suggestions of dispersed cisterns of rough endoplasmic reticulum and increased quantity and size of lipofuscin

granules.

Although maximum diameters were the only mitochondrial measurements that demonstrated statistical significance, our results suggest that surface area and volume density of mitochondria also tend to increase as a consequence of alcohol exposure, and revert toward normal values after alcohol withdrawal. Our figures do not substantiate the qualitative estimations of Karwacka (1980) that mitochondria also increase in number with alcohol. Mitochondria had longer profiles in our alcohol-treated neurons, but we rarely noticed the kind of markedly distorted or gigantic mitochondria Dunmire-Graff (1981) described. Since the gigantic, distorted mitochondria appeared so seldom in our photographs, they had little impact on our morphometric analysis. Tavares and Paula-Barbosa (1983b), who reported a morphometric trend toward increased mitochondrial size in rat Purkinje cells in the first month of alcohol ingestion, found that with additional time there was a significant swelling of mitochondria after 3 and 6 months' exposure to 20% alcohol. As noted in our Literature Review section, the swelling of mitochondria is a common response to noxious agents by many types of cells. Severely swollen mitochondria are thought to precede cell death.

The surface density of rough endoplasmic reticulum (RER) did not show any morphometric change in our study. The observations of dispersal of RER and freeing of ribosomes seen by Dunmire-Graff (1981) after alcohol exposure were primarily in large neurons of the facial motor nucleus, which normally have many more clumps of RER cisterns

(Nissl bodies) than do Purkinje cells. The wide cisternae which Dunmire-Graff ascribed mainly to alcohol-exposed cells could be seen in the present study in both normal and experimental animals. Tavares and Paula-Barbosa (1983b) likewise saw no changes in the endoplasmic reticulum of Purkinje cells, even after 6 months' exposure to 20% alcohol in rats. In our study the rough endoplasmic reticulum appeared primarily as random, isolated cisterns, while in all groups of animals there was a marked flooding of the cytoplasm with polyribosomes. There seemed visually to be an increase in polyribosomal density with both alcohol and recovery, but this is a feature that is difficult to subject to morphometric analysis. Such an increase of free polyribosomes with alcohol has been noted by Rubin and Lieber (1967) and by Dunmire-Graff (1981). An increase in polyribosomes with alcohol could be due to disturbances in protein synthesis in nerve cells, as has been reported by several investigators (see Literature Review). A disturbance in protein synthesis is thought by Tewari and Noble (1971) to be due to a releasing action by ethanol of membrane-bound ribosomes. It has been postulated that with alcohol there are defects in mRNA molecules and/or in the ribosomes themselves, thus leading to a consequent decrease in the capacity of ribosomes to bind with mRNA (Noble and Tewari, 1975).

In the present study, the paucity of stacked RER and the abundance of free polyribosomes in both normal and experimental neurons may be related to a curious modification of rough endoplasmic reticulum into large and small lamellar bodies, which were characteristic features of

the hamster Purkinje cells we studied. Such lamellar bodies were not reported in the detailed ultrastructural descriptions of rat cerebellar cortex by Palay and Chan-Palay (1974), nor can any such bodies be seen in their photographs, although subsurface cisternae of similar appearance were described in detail in Purkinje cells. Herndon, working also with rat Purkinje cells, did not notice lamellar bodies in his 1963 study, but did describe them in the same type of cells in another set of animals in 1964. In Purkinje cells of the hamster neither Takahashi and Wood (1970) nor Dunmire-Graff (1981) reported seeing lamellar bodies; upon close examination of available photographs of Dunmire-Graff's work, however, we have been able to identify very small profiles of these bodies. In the present study the lamellar bodies were seen extensively in both experimental and control animals. However, it should be stressed that certain cells were profusely filled with lamellar bodies while others were almost free of them. This difference may be due to different levels of metabolic activity of the cells at the time of fixation. Thus, lamellar bodies may not be a constant feature in either hamster or rat Purkinje cells, and their significance, when present, is unknown. Clearly, they are not a specific result of alcohol ingestion, since they were noticed regularly in control animals as well.

Perusal of the literature shows that the present observations are not isolated. Stacks of flattened, agranular, membranous organelles were first seen by Rosenbluth (1962) in neurons of the rat cerebral cortex and acoustic ganglion. Their first description as lamellar

bodies was given by Herndon (1964), who observed them in the Purkinje cells of rat cerebellar cortex. Since then lamellar bodies have been reported in several different cells and animals and in both normal and pathological cases (Table 8).

Lamellar bodies have been consistently described in close relation to rough endoplasmic reticulum (RER) (Herndon, 1964; LeBeux, 1972; Kishi, 1975; Spoerri and Glees, 1978; Buschmann, 1979; Bestetti and Rossi, 1980; Hervas and Lafarga, 1980; and Suarez, et al., 1983). Their cisternal membranes, however, differ from those of typical RER in that they are devoid of ribosomes except for the outermost membranes. In addition, there is a characteristic dense material in the narrow intercisternal spaces of lamellar bodies. Because continuity can be demonstrated between lamellar bodies and RER, the prevailing view is that lamellar bodies are derived from RER.

Kishi (1975), in his cytochemical study, suggested that lamellar bodies arise by close alignment of parallel cisterns of rough endoplasmic reticulum, with subsequent loss of ribosomes from their surface. This close alignment was evident in our material as well. Kishi further suggested that the binding of magnesium ions to the acidic polyions of intercisternal material results in a consequent drop of magnesium concentration in the membranes, which could cause the dissociation of ribosomes from the rough endoplasmic reticulum and the formation of lamellar bodies with Mg-rich intercisternal cytoplasm. On the other hand, Bestetti and Rossi (1980) have suggested that the intercisternal material could be modified ribosomes.

TABLE 8

Survey of animals and cells studied by
investigators describing cytoplasmic lamellar bodies

<u>Investigator</u>	<u>Year</u>	<u>Cells Studied</u>	<u>Animal Species</u>	<u>Condition</u>
Rosenbluth	1962	cerebral cortex and acoustic ganglion cells	male rat	normal
Herndon	1963	cerebellar Purkinje cells (subsurface cisterns only)	rat	normal
Herndon	1964	cerebellar Purkinje cells	rat	normal
Takahashi	1970	cerebellar Purkinje cells (subsurface cisterns only)	hamster	normal
LeBeux	1972	neurons of substantia nigra	rat, cat	normal
Kishi	1975	neurons of dorsal nucleus of vagus	rat	normal
Chandra and Stefani	1976	human lymphoma cells	human	in culture
Price	1976	arcuate neurons of hypothalamus	rat	castrated and mor- phine treated
Spoerri and Glees	1978	neurons of cerebral cortex	chicken	normal
Buschmann	1979	pyramidal cells of cerebral cortex	hamster	developing
Bestetti and Rossi	1980	different neurons from cerebellum and cerebral cortex	cattle, dog, goat	normal and patho- logical
Hervas and Lafarga	1980	neurons of para- ventricular nuclei	rat	normal
Suarez	1983	neurons of para- ventricular nuclei	hamster	normal

Along a somewhat different line of thinking, Suarez et al. (1983) observed lamellar bodies in continuity with subsurface cisterns, forming so-called LB-SSCs complexes just beneath the plasma membrane of the cell. Hervas and Lafarga (1980) reported that lamellar bodies were continuous with the perinuclear space via RER. The morphological continuity between lamellar bodies and both subsurface cisterns and perinuclear space was observed in the present study as well. Another observation of the current study, that of close positional association of lamellar bodies with mitochondria, also has support in the literature (LeBeux, 1972). It is of interest to note that the outermost membranes of the lamellar group are devoid of ribosomes whenever they lie close to the mitochondria.

The functional significance of the lamellar bodies was not addressed by Herndon (1964), who considered them to be artifacts resulting from anoxia of the brain due to certain preparative techniques. However, later authors have proposed that these bodies develop in response to a demand for unusually high metabolic activity in general (Bestetti and Rossi, 1980) or to increased demands of protein synthesis, such as in stage-specific protein synthesis during development (Buschmann, 1979). The continuity of lamellar bodies with subsurface cisterns and with the perinuclear space has been interpreted as providing a means of fast and direct communication from the plasma membrane to the nucleus of the cell (Suarez, 1983). McBurney et al. (1987) have recently suggested that subsurface cisternal membranes may play an important role in the maintenance of calcium ion homeostasis in

cell cytoplasm.

In some experimental investigations, so-called lamellar whorls, which are similar but not identical to lamellar bodies, have been shown to increase in number in response to testosterone deficiency following either castration or chronic morphine treatment (Price et al., 1976). This finding was projected to indicate that lamellar whorls might be a marker for hypothalamic neurons, which play a role in the LH-RH regulatory system.

If, in fact, lamellar bodies represent a response of rough endoplasmic reticulum to a heightened need for synthetic activity, our normal animals may have been undergoing some environmental or internal "stress" not experienced by Dunmire-Graff's (1981) animals. There may have been differences in their food or water supplies (e.g., higher or lower Mg or Ca content), although both groups of animals were housed and fed in the same research facility. They may have been studied at different times of year and thus were affected by different phases of a circannual rhythm. Our study extended from August to November; Dunmire-Graff's study extended throughout the entire year and over a period of two to three years. The differences may be sex-related; our hamsters were all males but Dunmire-Graff used both sexes, and her currently available photographs may be from female animals. Whatever the reasons for the phenomenon, the fact remains that in our "normal" neurons there was an extensive production of membranous lamellar bodies, possibly at the expense of the more commonly described stacks of RER cisterns. The internal lamellar membranes are degranulated but

not like classical smooth endoplasmic reticulum in appearance. The great numbers of narrow cisterns and compressed intercisternal spaces suggest that lamellar bodies may increase in thickness by replication of their inner membranes, once the original close alignment of two RER cisterns has formed the initial configuration. The true configuration of the more extensive lamellar bodies is impossible to determine without a study of serial sections.

One can speculate that no changes were found in RER disposition after alcohol-exposure in our Purkinje cells because the neurons had already responded to some other, unknown stress by converting much of their RER to lamellar bodies and thus producing an increased surface area of degranulated intracellular membrane. It may be, therefore, that little opportunity remained for further dispersion of RER by alcohol, nor for further freeing of membrane-bound ribosomes.

Although our studies of the influence of alcohol consumption on Purkinje lamellar bodies produced equivocal findings, it did seem that withdrawal from alcohol led to a slight increase in the number of small lamellar bodies. This may be due to special stresses during the recovery phase. Recent experimental studies have shown injurious effects on the central nervous system following either sudden or gradual withdrawal from chronic intake of alcohol. Riley and Walker (1978) fed adult mice an alcohol-containing liquid diet for 4 months, followed by a 2-month alcohol-free period, and reported a significant loss of dendritic spines on hippocampal pyramidal cells and dentate granule cells. Phillips and Cragg (1983) found that 9% alcohol intake

by mice for 4 months did not cause any change in number of synapses among the basal cells in field CA₁ of the hippocampus, although withdrawal from alcohol intake was found to decrease the number of cells in the same field. In 1984 the same investigators, working with mice for different periods of exposure to alcohol, with and without a subsequent period of recovery, reported a significant loss of Purkinje cells during withdrawal but not during alcohol consumption. No loss was noticed when the withdrawal was gradual. They suggested that sudden withdrawal from alcohol may cause more cellular damage than alcohol exposure.

On the other hand, some investigators believe that tissues may normalize after a period of withdrawal from alcohol. Thus, whereas 5 months' consumption of an ethanol liquid diet by rats caused a significant decrease in the thickness of the strata oriens and radiatum of the CA₁ field of the hippocampus, the thickness of the strata returned to control sizes after 2 months of withdrawal (McMullen et al., 1984).

On the biochemical level, Jarlstedt (1972) found that 15% alcohol for 8 months caused minor depression of protein synthesis in the cerebellum and in the cerebral cortex, while sudden withdrawal from alcohol for 24 hours caused a marked increase in protein synthesis in both structures. Hosein et al. (1980) showed that chronic alcohol feeding resulted in diminished specific activity of cytochrome C oxidase in rat hepatic mitochondria and a diminished capacity of mitochondria to oxidize short chain fatty acids. Both values returned

toward normal following alcohol withdrawal.

Our results indicate that certain organelles respond differently from others to withdrawal from alcohol. For example, the number of small lamellar bodies tended to increase only after withdrawal, while mitochondria were more adversely affected during exposure to alcohol and tended to normalize after a period of withdrawal.

Another area of uncertainty related to the effects of alcohol on nerve cells involves lipofuscin granules. Although the significance of accumulated lipofuscin granules is not fully understood, the prevailing view is that a high content of intraneuronal lipofuscin in normal neurons is consistently correlated with the aging process. The widely accepted notion is that they are derivatives of lysosomes and therefore involved in the breakdown of intracellular membranes (Tavares and Paula-Barbosa, 1983a). Sekhon and Maxwell (1974) described the morphological stages accompanying transformation of lysosomes into lipofuscin granules. Brunk and Ericsson (1972) found that all of the sequential stages of the transformation contained acid phosphatase, an essential enzyme in lysosomal activity. At the molecular level, Freund (1979) thought of lipofuscin as being the end product of oxidative degradation of unsaturated fatty acids of membrane lipids that are cross-linked with proteins. Since the accumulation of undigested end-products within lysosomal vesicles results in increased vesicle size, it follows that the larger end-stages of the process are the ones identified at light microscopic levels as "lipofuscin granules". It should be noted, however, that in ultrastructural studies investigators

routinely include all stages of lysosomes in their counts and measurements of "lipofuscin".

The increase or decrease of large amounts of lipofuscin pigments in neurons does not depend exclusively on aging. This deposit has been described in other experimental situations in which cell metabolism was disturbed by various factors. Tavares and Paula-Barbosa (1983a) found in rat Purkinje cells a significant increase in both the volume density and the number of lipofuscin granules per unit of surface area of cell cytoplasm after one month of 20% alcohol intake. These changes progressed with increased duration of ethanol exposure. Qualitative observations have yielded similar conclusions by Dunmire-Graff (1981), working on hamster Purkinje cells after 7 weeks' exposure to 15% alcohol, and by Volk (1980), looking at spinal ganglion cells after 6 months' ingestion by rats of 15% alcohol. On the other hand, Freund (1979), using fluorescence studies in lipid extracts of whole-brain homogenates, reported that small amounts of ethanol over a 5 month period resulted in no increase of brain lipofuscin granules in mice. In addition, he reported that high doses of vitamin E retarded the accumulation of brain lipofuscin with aging. Samorajski et al. (1978) found that 10% alcohol for 10 months, concomitant with adequate nutrition, unexpectedly reduced lipofuscin accumulation in neurons of the reticularis gigantocellularis. Our results showed that 15% alcohol for seven weeks produced no measurable change in either the number of lipofuscin granules per area of cytoplasm or in the surface area or maximum diameter of individual granules in hamsters. Additionally,

withdrawal did not affect any of these three variables. The discrepancy between results in various studies may be partly due to differences in cellular metabolism in different animal species, or, in some instances, to differences in the concentration of alcohol used or the total time of exposure to alcohol. In any event, in both this study and others (e.g., Tavares and Paula-Barbosa, 1983a), it has been observed that some cells accumulate more lipofuscin granules than others after exposure to alcohol, indicating cell-specific responses to alcohol toxicity. Perhaps a better estimate of total numbers of granules may be achieved at lower magnifications. Randomly chosen, restricted areas of cytoplasm can miss both widely dispersed single granules and aggregates of granules localized elsewhere within the cell. Because of the scarcity of morphometric studies related to lipofuscin, no definite conclusions concerning their response to ethanol can be reached as yet.

It is important to consider the limitations of a morphometric study, such as the one reported here. For example, although we were unable to confirm Dunmire-Graff's (1981) visual observations that lipofuscin increases in amount and that stacked cisterns of RER are replaced by scattered fragments of RER after exposure to alcohol, her observations may nevertheless be generally correct. Our random analysis of individual organelles at high magnification may have inadvertently misrepresented the state of the cell.

Examination of blind-coded photographs taken from random areas of Purkinje cell cytoplasm revealed the presence of very few stacks of RER

in any cells. Even though the cells in this study presented more degranulated lamellar bodies than stacked RER, we know from light microscopy and from low power electron micrographs that Nissl bodies (stacked RER) do exist in moderate quantity in hamster Purkinje cells, and that they are often localized near the nucleus or toward the cell membrane. Unless they are selectively chosen for photographing, however, they may fail to appear in a study such as this. Lipofuscin granules also tend to exist in localized clusters and can be wholly missed in randomly photographed cell areas. No such problem is encountered in a study of mitochondria because they are scattered quite evenly throughout the cytoplasm and are well represented in every electron micrograph. Even so, extensive examination of photographs suggested that there are differences between particular areas of cytoplasm within a given cell. There also appear to be differences between individual Purkinje cells as a whole, i.e., differences in normal functional activity and differences in sensitivity to alcohol. Such natural variation makes the counting and measuring of small units seen at high magnification very difficult to assess at levels of statistical significance, unless the numbers of animals, cell samples, and photographs are very large.

SUMMARY AND CONCLUSIONS

Chronic ethanol administration in adult male hamsters has been followed by a morphometric characterization of ultrastructural changes in mitochondria, lipofuscin granules, and endoplasmic reticulum within Purkinje cells of the cerebellar cortex. The possible reversibility of neuronal change after withdrawal from alcohol has also been considered. The results of this study have led to the following conclusions:

- 1) Mitochondrial maximum diameter increased significantly after exposure to alcohol and tended to normalize after subsequent withdrawal from alcohol. Such a response is typical of stressed cells with altered metabolic activity. Other parameters such as volume density and surface area revealed a tendency toward increased values with alcohol and normalization with withdrawal, although not to a level of statistical significance.
- 2) An unusual arrangement of degranulated endoplasmic reticulum into lamellar bodies of different sizes has been described for the first time in hamster Purkinje cells. It is apparently derived from rough endoplasmic reticulum; its functional significance is unknown. It changed very little with alcohol exposure.
- 3) Rough endoplasmic reticulum was primarily present as individual, irregularly dispersed cisterns, which showed no particular change in surface density with alcohol or

withdrawal. The commonly reported alcohol-induced response of dispersal of stacked cisterns of RER and freeing of membrane-bound ribosomes into the cytoplasm could perhaps not occur because there were very few stacks of RER to begin with, and the cytoplasm was already flooded with polyribosomes in the control cells.

- 4) Certain organellar features often ascribed to changes with alcohol exposure were found to be characteristic of both control and experimental neurons. For example, both wide and narrow cisterns of rough endoplasmic reticulum were seen in all cells, with no apparent relation between cisternal width and alcohol treatment.
- 5) The number of lipofuscin granules, along with the surface area and maximum diameter of individual granules, showed no consistent changes with alcohol or withdrawal. It may be that the relative insensitivity of hamsters to alcohol leads to little lysosomal breakdown of membranous organelles.
- 6) There was considerable variability in the disposition of organelles within different fields of cytoplasm seen at the ultrastructural level. Also, individual neurons differed considerably from each other in their organellar content. Because of this inherent variability, certain organelles, such as lipofuscin granules, appear so infrequently or in such restricted locales that they are difficult to analyze morphometrically at a level of statistical significance unless

the numbers of animals and tissue samples are very large. Furthermore, when minute fractions of cytoplasm are photographed for study in a random, unbiased fashion -- rather than selectively choosing organelles of a particular appearance to study, it becomes difficult to obtain enough morphometric information to demonstrate statistical significance. The results of this study, obtained with relatively high magnification electron microscopy, suggest that analyses of certain organelles might be better served by using lower magnification in order to visualize a larger field of cytoplasm and a broader spectrum of organelle dispersion.

FIGURE 1

Typical mitochondrial profiles (M) seen in both control and alcohol-exposed Purkinje cells of adult hamsters. Note clusters of free ribosomes (R) and fragments of rough endoplasmic reticulum (RER) scattered throughout the cytoplasm.

- A. Relatively small, ovoid mitochondrial profiles; fairly regular in shape. These are particularly predominant in normal cells and in those recovering from alcohol exposure.
- B. Pleomorphic mitochondrial profiles; more elongate and irregular in shape. These are more numerous after treatment with alcohol.

Magnification here and in all subsequent figures = 38,000X.

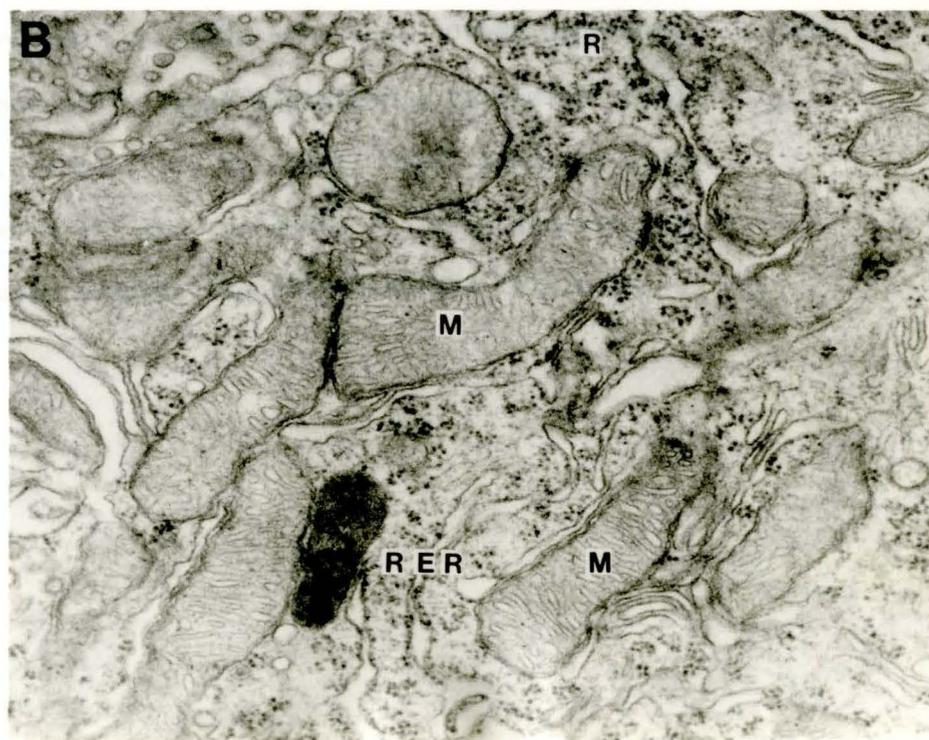
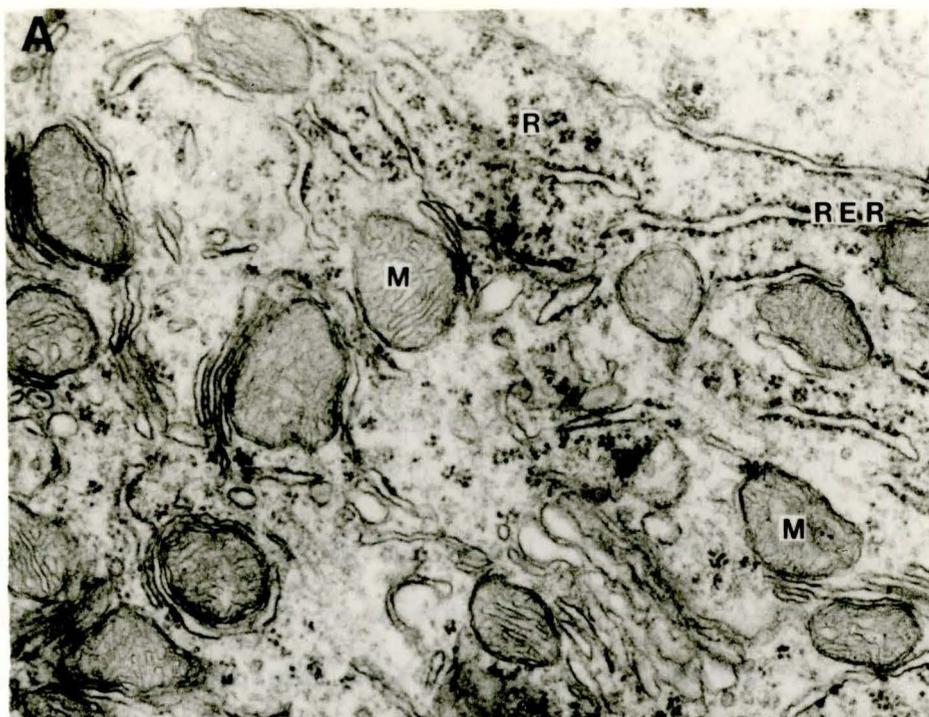


FIGURE 2

A typical field of normal Purkinje cell cytoplasm to show the crowded organelles and their considerable variety. Although greatly elongated mitochondrial profiles are more numerous, on average, in alcohol-exposed neurons, they are also seen in normal cytoplasm, as shown here (M). Other organelles include free ribosomes (R), rough endoplasmic reticulum (RER), Golgi bodies (G), large (LLB) and small (SLB) lamellar bodies, neurofilaments (F) and lysosomes (L). The nucleus (N), nuclear envelope (open arrow), and cell membrane (black arrow) are indicated.

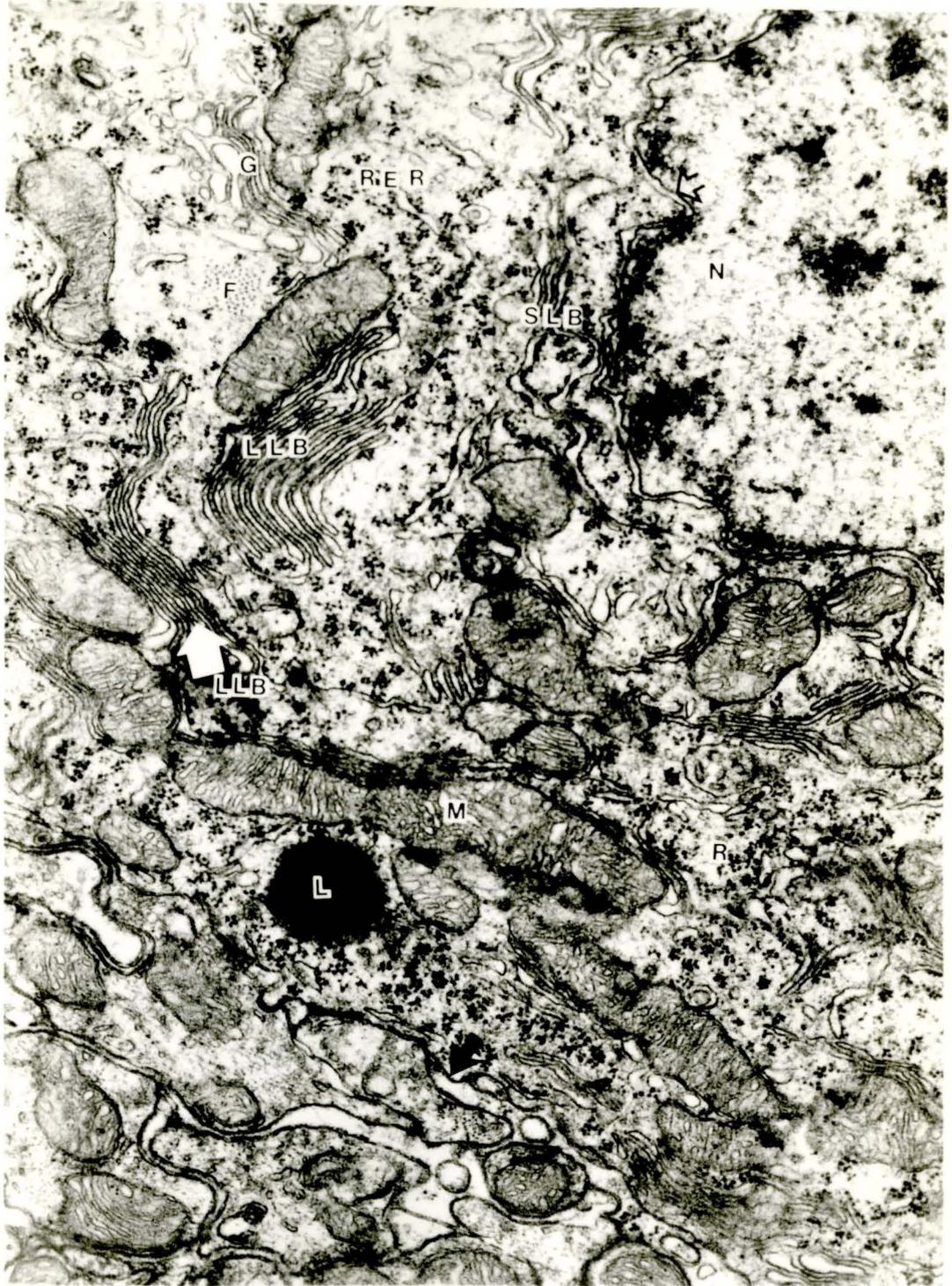


FIGURE 3

Two extreme mitochondrial configurations as occasionally seen in alcohol-treated Purkinje cells.

- A. Enlarged mitochondrion (M) with electron-dense particles in the matrix. Compare its size with the more typical mitochondria in the surrounding cytoplasm. Cell membrane is indicated by arrow.
- B. Markedly elongate mitochondrion (M). Profiles of more typical length lie near it. N = nucleus; arrow indicates nuclear envelope.

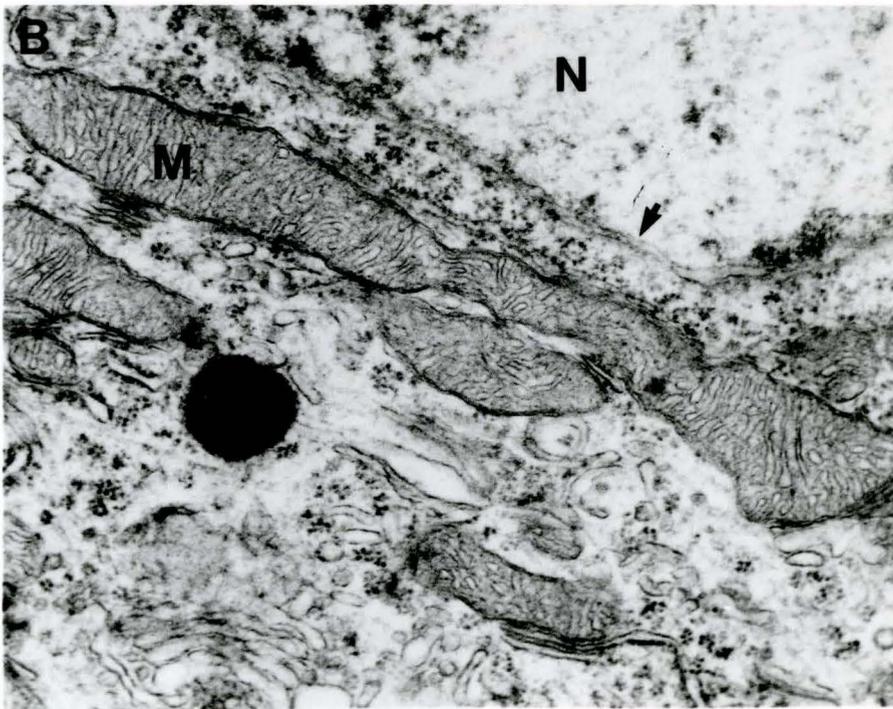
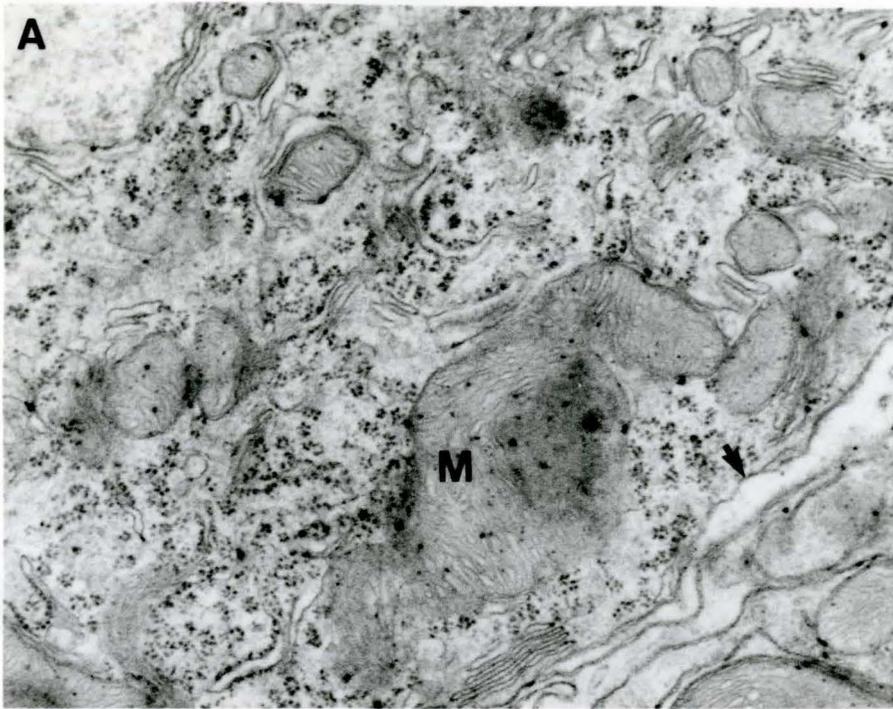


FIGURE 4

Rough endoplasmic reticulum (RER) and free ribosomes (R) profusely scattered throughout Purkinje cell cytoplasm. Such fields are found in both normal and alcohol-exposed neurons.

A. Close positional relation between mitochondria (M) and cisterns of rough endoplasmic reticulum. Arrows indicate two small lamellar bodies.

B. A more random scattering of rough endoplasmic reticulum and mitochondria (M) throughout the cytoplasm.

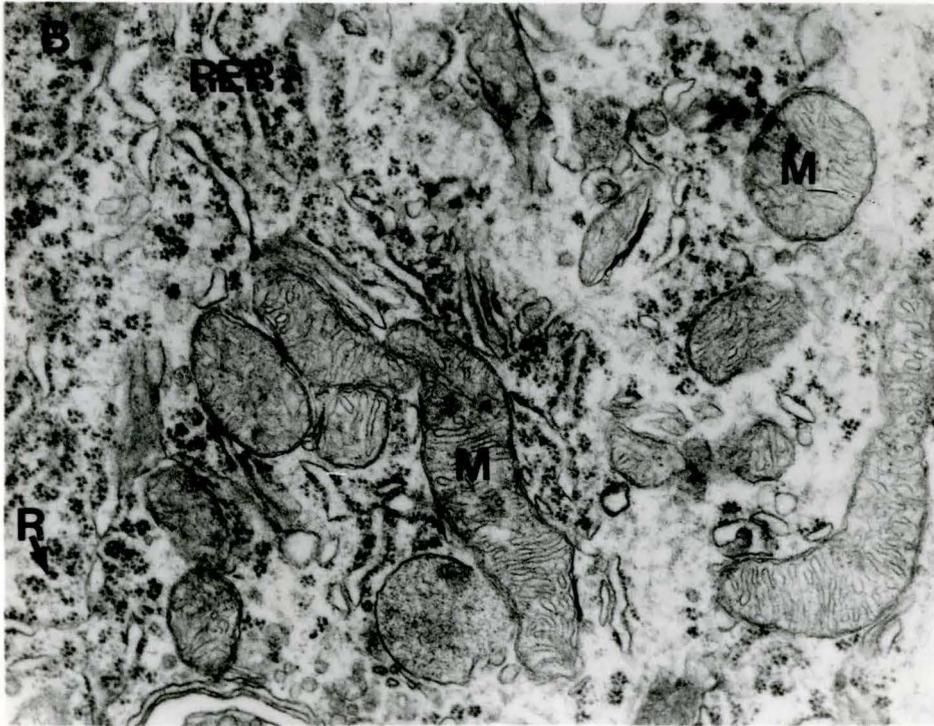
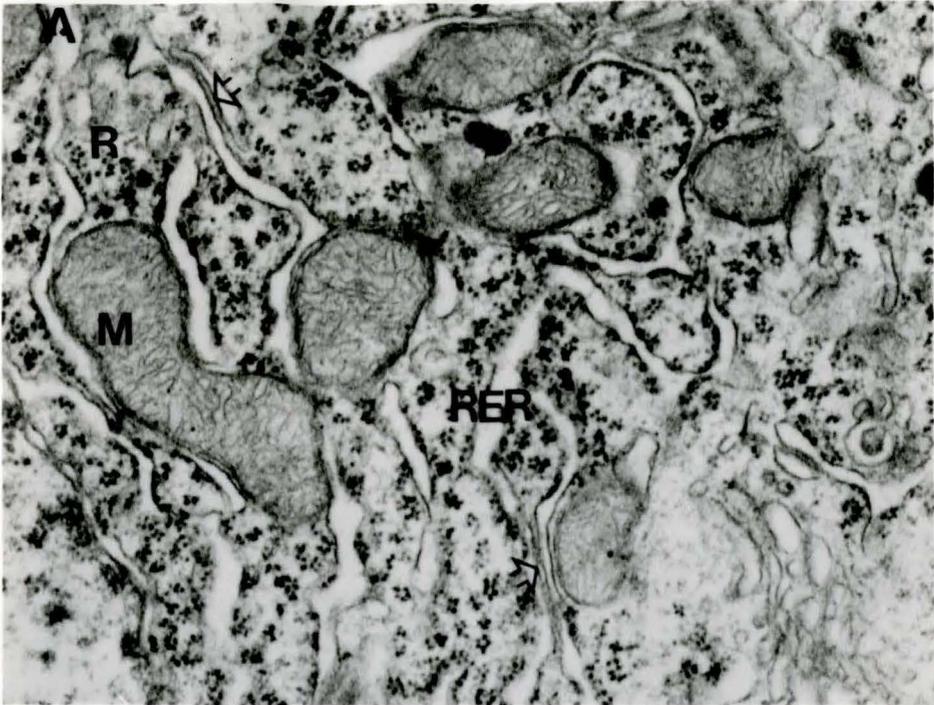


FIGURE 5

Parallel arrays of rough endoplasmic reticulum (RER), seen here with both wide and narrow cisterns in two control neurons.

- A. Wide cisterns. Arrow indicates nuclear envelope.
- B. Narrow cisterns. Cytoplasm is flooded with free ribosomes (R). Two secondary lysosomes, considered to be lipofuscin granules (L), lie in the center of the field.

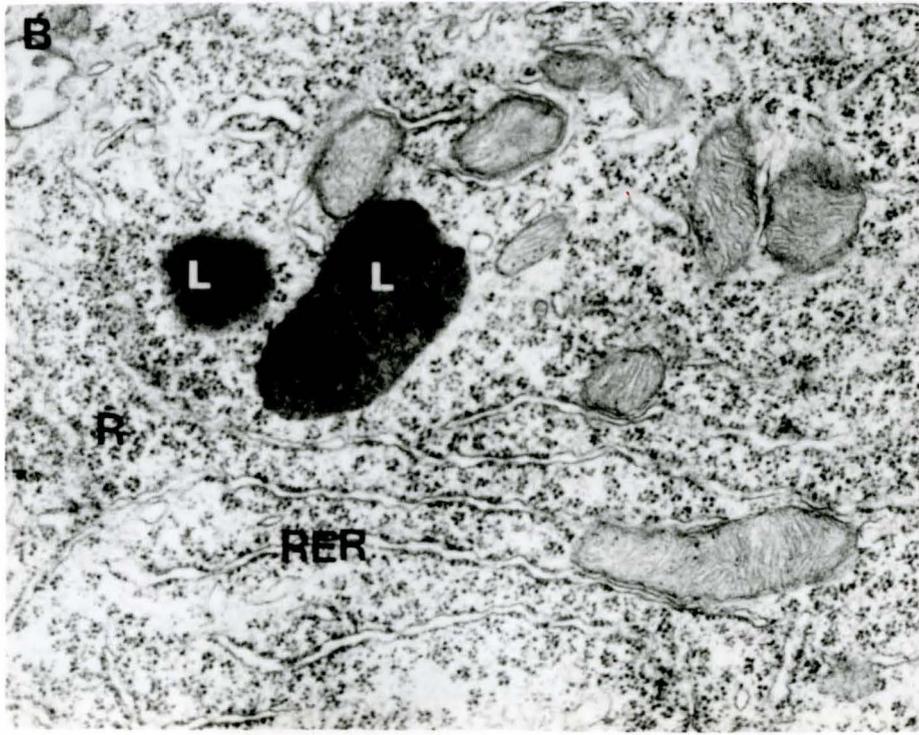
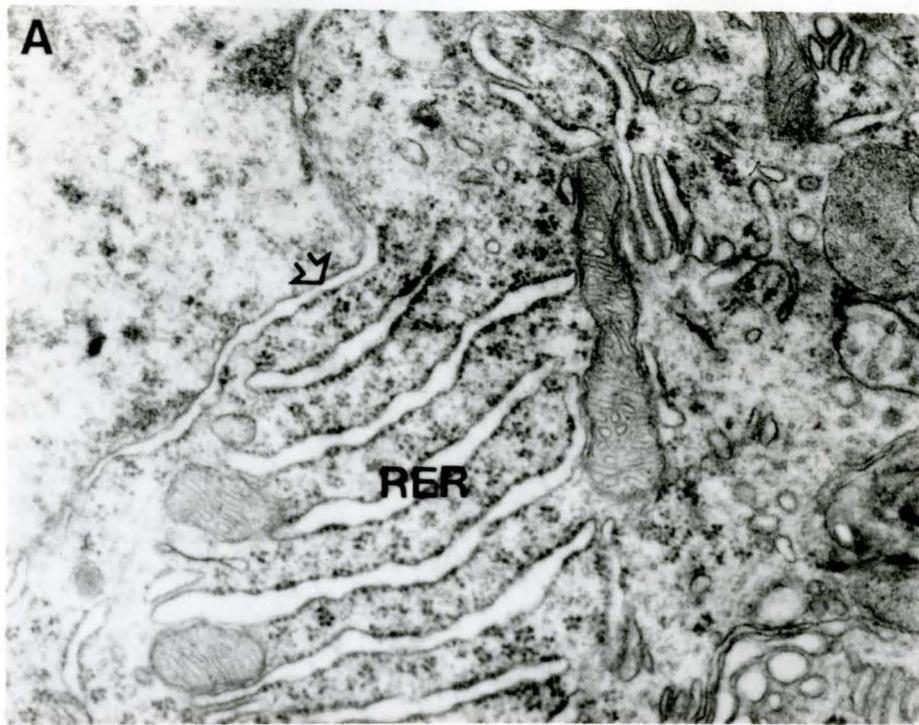


FIGURE 6

Relation of lamellar bodies to rough endoplasmic reticulum. Although in this instance, Field A is taken from tissue recovering after exposure to alcohol and Field B is from an animal currently ingesting alcohol, such configurations are typical of nearly all Purkinje cells studied, whether control or experimental.

- A. Large lamellar body (LLB), showing characteristic closely stacked cisterns of degranulated endoplasmic reticulum, separated by narrow intercisternal spaces filled with relatively dense cytoplasm. Note that the outermost cisternal membranes are ribosome-studded (arrow), that ribosomes lie at the extreme ends of the cisterns, and that the cisterns are dilated at their ends. A branching of the otherwise parallel array of cisterns is seen at the lower right border of the body. Fragments of unrelated RER are scattered throughout the cytoplasm.
- B. A complex configuration of stacked cisterns of rough endoplasmic reticulum (RER) closely related to apparently incipient formation of the narrower cisterns and intercisternal spaces of small lamellar bodies (arrows). N = nucleus.

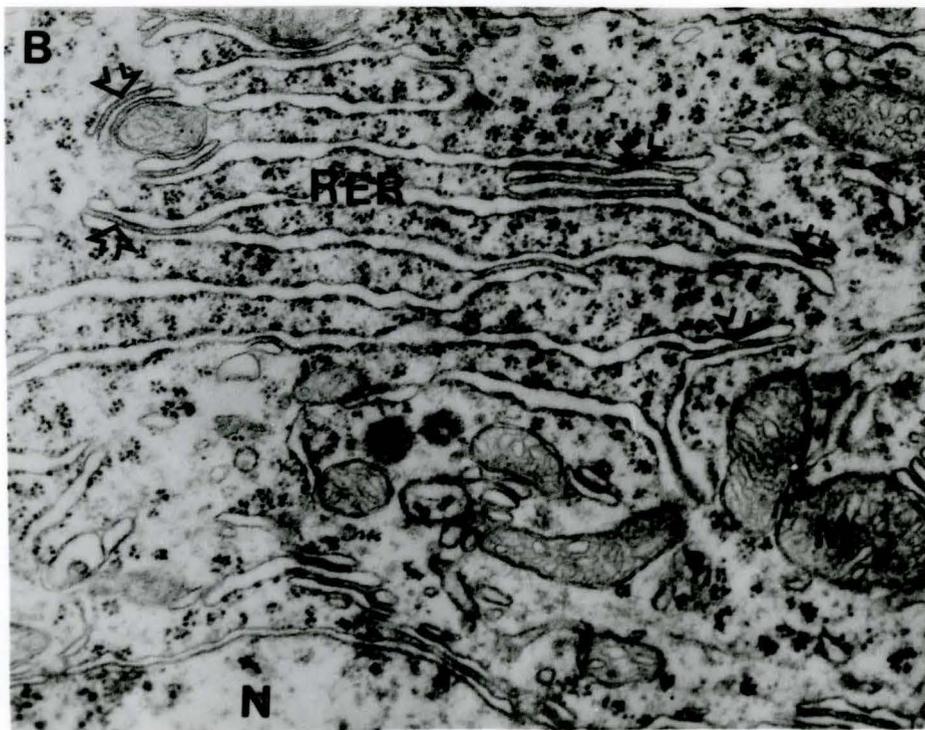
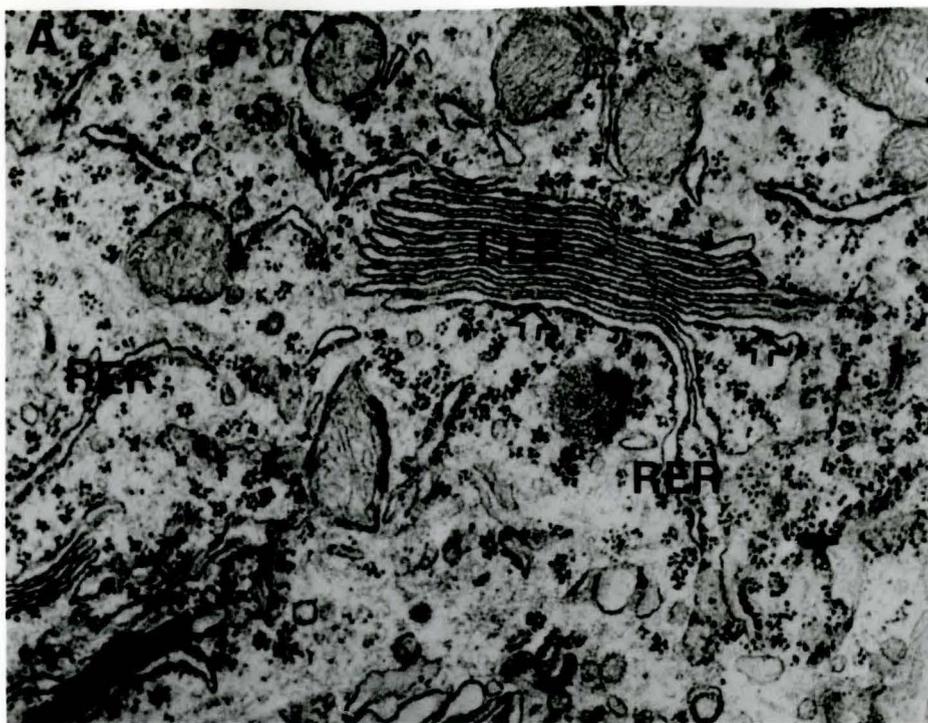


FIGURE 7

Lamellar bodies of various sizes, frequently showing close relationship to mitochondria (M). Both samples are from alcohol-treated animals, but the same types of configurations are characteristic of control cells and of cells recovering from exposure to alcohol.

- A. Many of the lamellar bodies shown here are "small" (SLB), i.e., they have four or fewer cisterns. Sometimes they merge with "larger" lamellar bodies (LLB) having more than four cisterns. Note their continuity also with rough endoplasmic reticulum (RER) and their frequent alignment next to mitochondrial membranes. At the lower left of the field are two sets of subsurface cisterns (SSC) lying immediately under the plasma membrane of the cell (arrow).
- B. The labeled mitochondrion (M) is almost completely encircled by lamellar configurations. Examples of both small (SLB) and large (LLB) lamellar bodies are seen in the field. For comparison of membrane arrangement and cisternal width, note the labeled Golgi body (G). Also seen in the field are two lysosomal (lipofuscin) bodies (L); the larger, darker one bears electron-dense particles, while the smaller one contains a concentric lamellar arrangement.

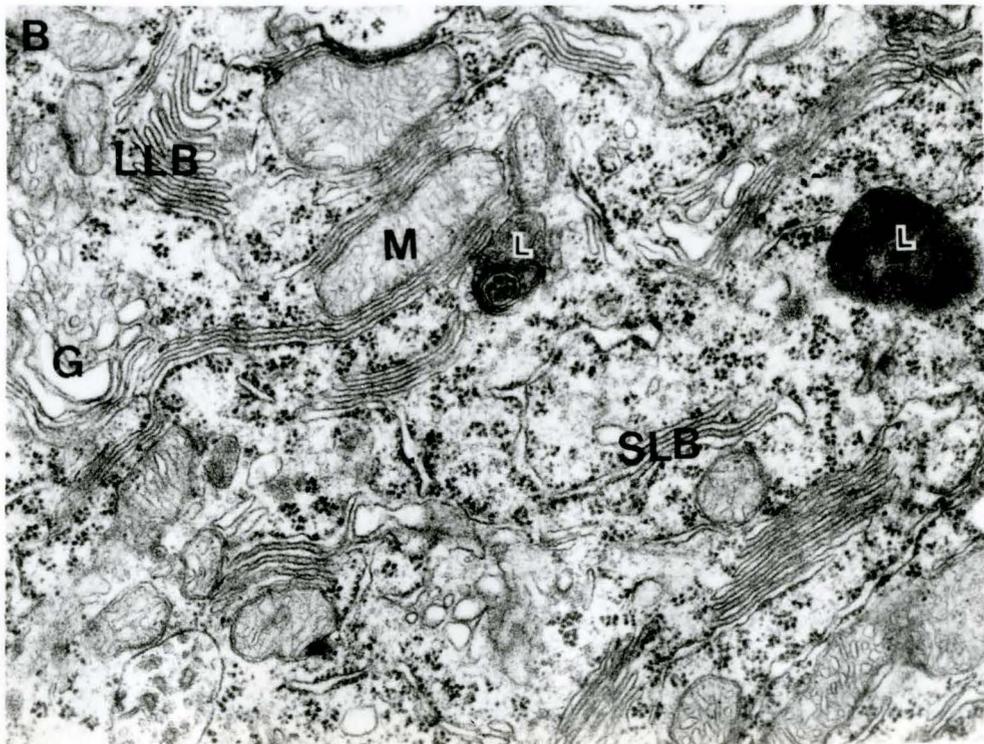
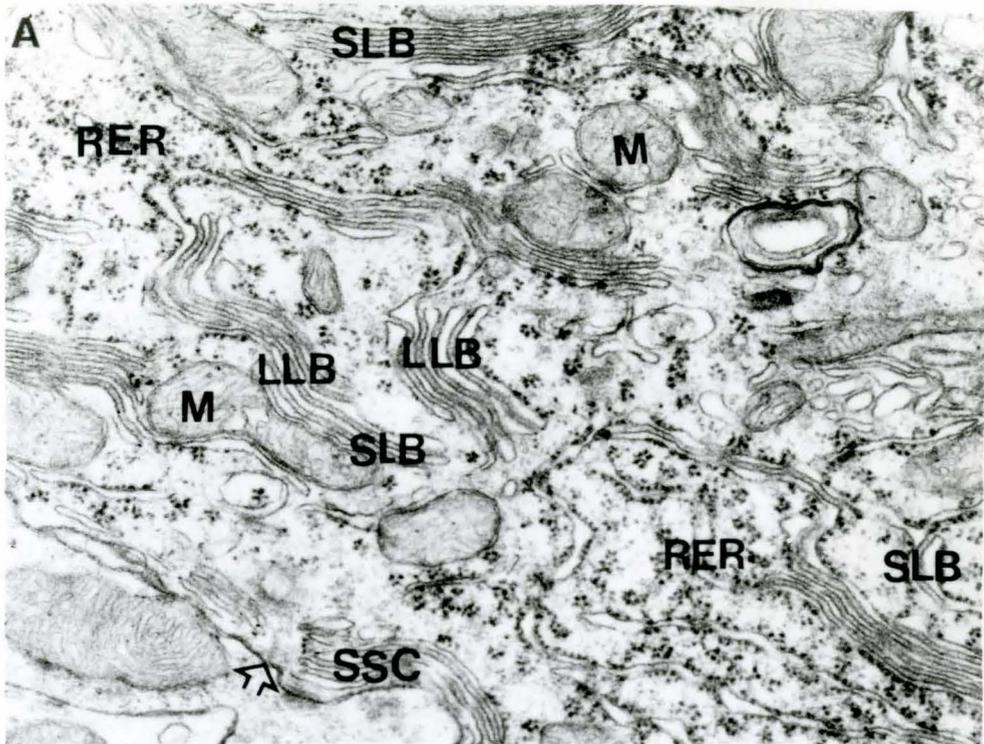


FIGURE 8

Field of Purkinje cell cytoplasm, showing a pale mitochondrion (M) with disrupted cristae; this type of abnormal mitochondrion is seen only in alcohol-exposed cells. Note the lamellar bodies almost completely encircling this organelle. Note also the presence of subsurface cisterns (SSC), which are essentially peripherally located lamellar bodies, close to the cell membrane (arrow). Cisterns of rough endoplasmic reticulum (RER) are typically wider than those of lamellar bodies (SLB). A small primary lysosome (L1) is in the upper right corner of the field; a larger, darker secondary lysosome (L2) lies below it; this latter body is reaching the end-stage of lysosomal activity and is therefore most correctly a lipofuscin granule. Two Golgi bodies are indicated (G).



FIGURE 9

Two special views of cytoplasmic membranes.

- A. Points of direct continuity (arrows) from outer membrane of nuclear envelope to a cistern of rough endoplasmic reticulum and thence to a small lamellar body (SLB). Other indicated structures include the nucleus (N), a mitochondrion (M), and a Golgi body (G). This cell is from an alcohol-treated animal.
- B. An aggregate of undulating tubules and circular profiles of endoplasmic reticulum. This cell is from a control animal.

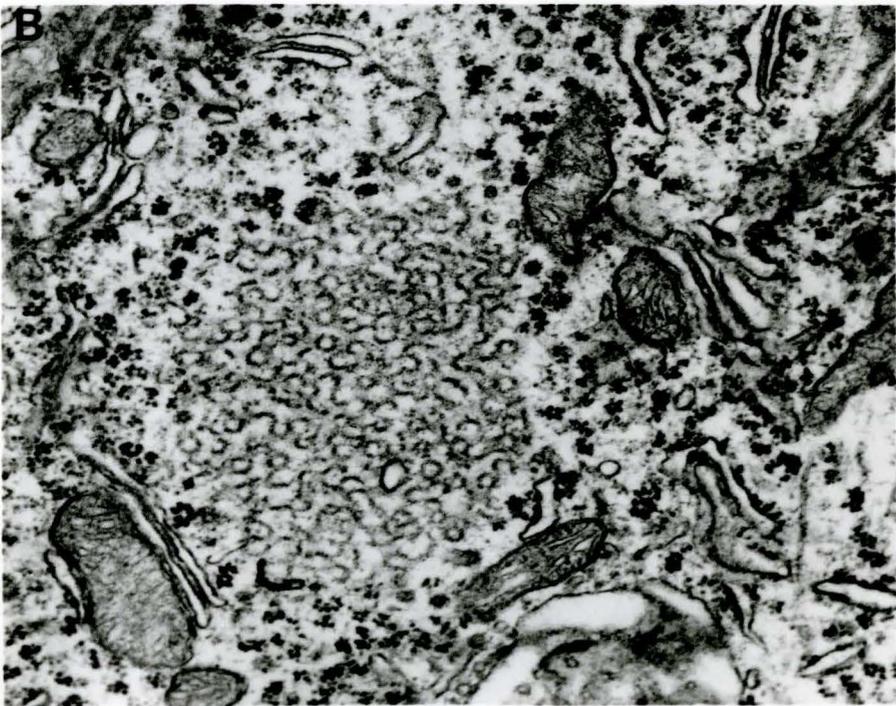
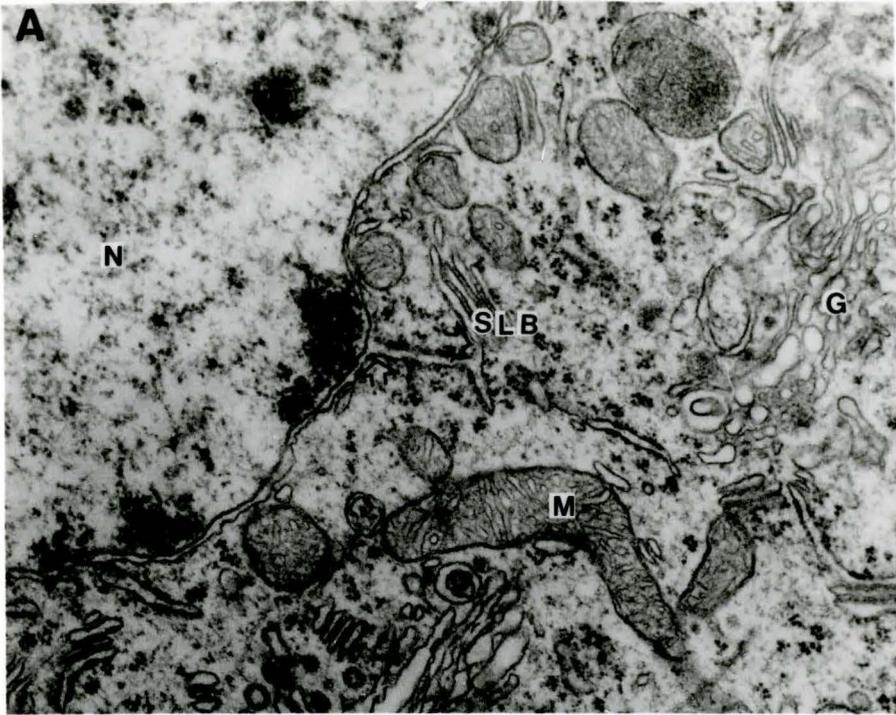


FIGURE 10

Electron-dense lysosomes seen at primary and secondary stages. Although only the end-stage lysosomes (residual bodies of light microscopy) are strictly-speaking lipofuscin granules, all stages were counted and measured as "lipofuscin" in this study (as in studies by other investigators). Field A is from a control animal, Field B from an alcohol-ingesting animal, but all stages of lysosomal autophagic activity are seen in all groups studied.

- A. Primary lysosome (L1) with clearly defined limiting membrane. The three denser bodies, one with scattered black particles (L2), are secondary lysosomes.
- B. Larger, more irregularly shaped end-products of lysosomal activity, i.e., lipofuscin granules proper (L). Dense particles can be seen in some of these; peripheral vacuoles are visible in the granule to the right.

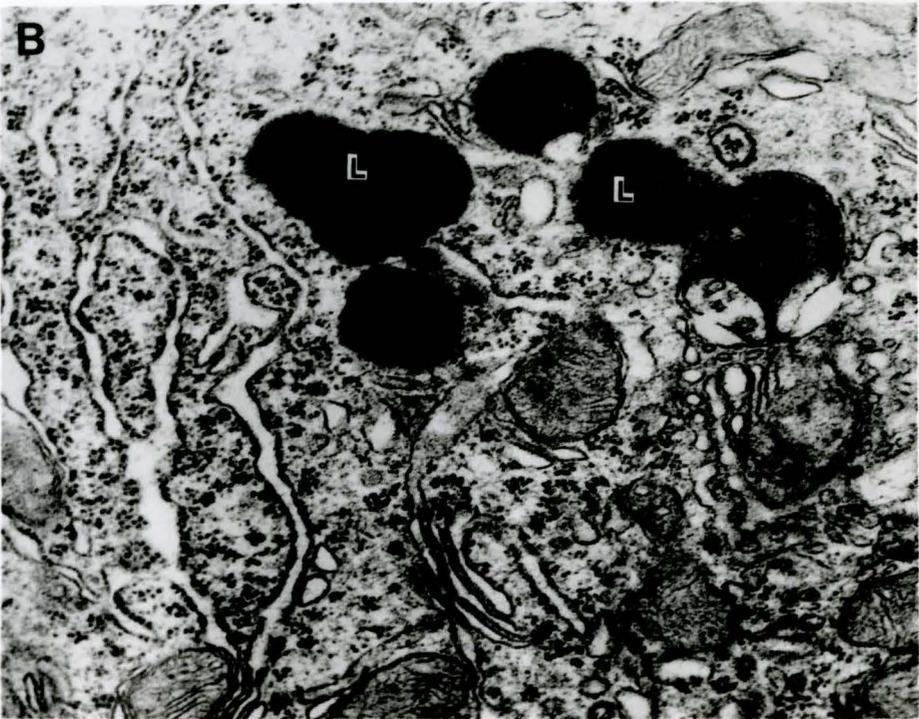
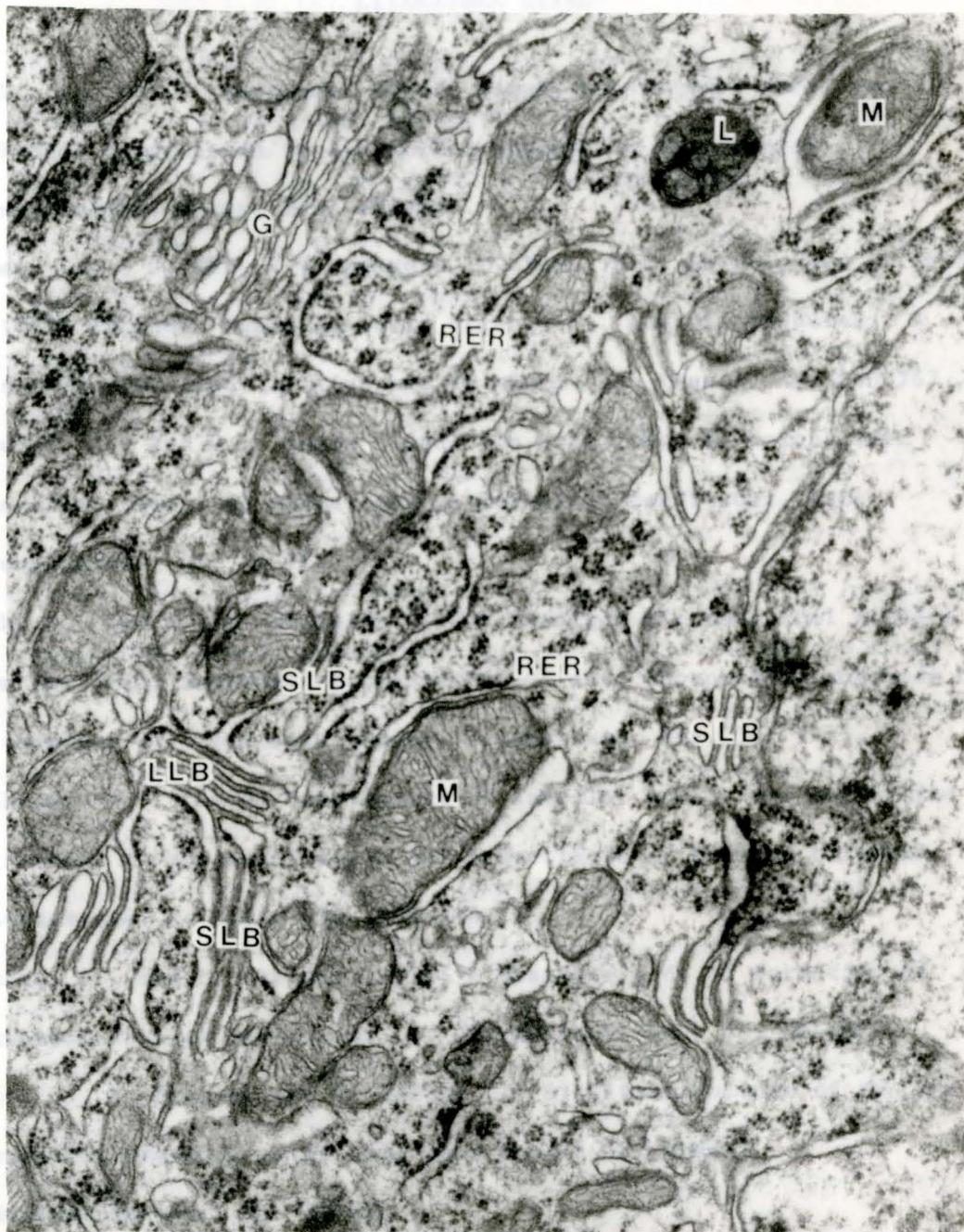


FIGURE 11

Typical complex field of Purkinje cell cytoplasm from an alcohol-exposed animal. Note the close relationship between mitochondria (M), rough endoplasmic reticulum (RER), and the many lamellar bodies (SLB, LLB). In the upper right corner of the field is a lipofuscin granule (L) containing a concentric lamellar arrangement. One Golgi body is indicated (G).



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Date June 9, 1987

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