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An electron microscopic and biochemical study of the effects of cyclic 3', 5'-AMP, ergotamine or propranolol on the lysosomes of newborn rat hepatocytes

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Summary. The effects of cyclic 3', 5'-AMP, ergotamine or propranolol on newborn rat liver were studied by using biochemical assays, electron microscopy and quantitative morphometry. Cyclic AMP enhanced the normal postnatal rise in the glycogen-hydrolysing activity of acid alpha 1, 4 glucosidase but had no effect on the maltose-hydrolysing activity of the enzyme. The results suggest that these activities may be due to different enzymes. Propranolol prevented the postnatal increase in the glycogen-hydrolysing activity of acid glucosidase and the breakdown of lysosomal glycogen, indicating that these phenomena represent beta-adrenergic functions in newborn rats. Ergotamine also inhibited the postnatal increase in this activity and the lysosomal glycogen mobilization. A reasonable explanation for these results is that ergotamine interferes with the action or formation of cyclic AMP.

Key words: Cyclic 3', 5'- AMP, Ergotamine, Propranolol, Lysosomes, Newborn rat, Hepatocytes, Autophagocytosis

Introduction

Very little is known about regulation of the catabolism of glycogen inside lysosomes. The lysosomal enzyme acid alpha 1, 4 glucosidase, which is capable of hydrolysing glycogen or maltose may control this process (Hers, 1963; Rosenfeld, 1964; Kotoulas, 1981). Lundquist (1986) differentiated between a glycogen-hydrolysing and a maltose-hydrolysing lysosomal enzyme activity (Skoglund et al., 1987).

Glycogen-hydrolysing activity is apparently under hormonal control. Adrenalin or glucagon increases this activity and accelerates the lysosomal glycogen breakdown while insulin has the opposite effect (Rosenfeld, 1964; Kotoulas, 1981, 1984). Cyclic AMP was found to increase the glycogen-hydrolysing activity and it was suggested that hormones exert their effect by causing alterations at the intracellular level of the nucleotide (Kotoulas, 1986).

In this paper the previous studies with cyclic AMP are further extended and the effects of ergotamine, a known antagonist of cyclic AMP, and propranolol, a beta-adrenergic antagonist, on liver acid glucosidase activity and on hepatic lysosomal glycogen breakdown in newborn rats are presented.

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Materials and methods

Animals and handling of tissues. Newborn rats were obtained from ten pregnant Wistar females. The average litter contained newborns. The sex of the newborns was not determined. The animals were sacrificed by decapitation at birth or kept at 35° C and sacrificed at the age of 6 hours. Part of the liver was processed for electron microscopy and the rest was used for biochemical assays.

Chemicals. Ergotamine tartrate, Lot 34F-0290, and Maltose, Lot 46F-0101, were obtained from Sigma. Propranolol hydrochloride (Inderal), Lot PL 29/5062, 269085, 0.1% solution, was from Imperial Chemical Industries and Dichloroisoproterenol HCL, Lot 367-154-D-60, from Eli Lilly and Co. Cyclic AMP, glycogen, reagents for determining glucose and for electron microscopy were obtained as before (Kotoulas, 1986).

Biochemical methods. The glycogen-hydrolysing activity of acid alpha 1, 4 glucosidase was assayed in homogenates of liver tissue in a 10% dilution with distilled water. Usually 200 ml of homogenate were used for the assay. The total activity of the enzyme was determined according to Hers (1963) and Lejeune et al.

(1963) with glycogen as substrate. Incubation was carried out in 0.1 M sodium acetate buffer (pH 5). The total maltose-hydrolysing activity was determined in a similar way with maltose as substrate. Activites were expressed as micromoles of glucose formed per hour per mg of protein. Correction was made for the fact that two glucose molecules are formed for each cleaved linkage in maltose (Scoglund et al., 1987). Glucose was estimated by the method of Raabo and Terkildsen, modified according to Sigma Technical Bulletin 510, using glucose oxidase, peroxidase and dianisidine (Raabo and Terkildsen, 1960). Glycogen and protein in the liver were determined as before (Kotoulas and Phillips, 1971).

Electron microscopy and morphometric analysis. Liver tissue was fixed for 1 hour at 0° C in 1% osmium tetroxide buffered with 0.1 M phosphate buffer at pH 7.2 . Grey-coloured ultrathin sections were cut using an LKB microtome and stained with a saturated aqueous solution of uranyl acetate and Reynold's solution of lead citrate.

Morphometric analysis was performed on electron micrographs as described before (Kotoulas and Phillips, 1971). Three normal animals killed at birth, three cyclic AMP-treated, three ergotamine-treated and three propranolol-treated animals and an equal number of their controls killed at the age of 6 hours, were used. From each liver five blocks were prepared and from each block two randomly taken micrographs were used. For the morphometric work the pictures were enlarged to a final magnification of 13,000. A lattice composed of horizontal and vertical lines at regular intervals (0.5 cm) drawn on a transparent material was superimposed on the prints. The cross points of the lines of the lattice were used for planimetric work. According to the principles of stereology, the fractional volume occupied by a cytoplasmic component is equal to the fraction of the points enclosed within the area of the component in random cross sections of the tissue. The fractional volume of a component was expressed as a percentage of cytoplasmic volume. In the cases of lysosomes and lysosomal glycogen, the data from the micrographs of the same block were combined and therefore the means and standard errors were calculated from these combined data. Mitochondrial matrix granules were counted on micrographs enlarged to a final magnification of 41,600. The results were expressed as number of granules counted per μ^2 of cytoplasm. Since the sections of tissue were not very thin as compared with the dimensions of the granule, no attempt was made to estimate other parameters. The results were evaluated by Student's t-test (Weibel and Gómez, 1962; Weibel, 1969).

Experimental design. Five animals were killed at birth. The rest of the animals were divided into groups according to the agent used; cyclic AMP, ergotamine, propranolol or dichloroisoproterenol. Ten cyclic AMP-treated animals and ten controls from the same litters were sacrificed at the age of 6 hours. Cyclic AMP was administered intraperitoneally in 0.2 ml of a 0.3% solution of the compound in 0.9% NaCl. The animals were injected at birth and 3 hours after birth (a dose of 100 mg/kg each time). Ten ergotamine-treated animals and ten controls from the same litters were sacrificed at the age of 6 hours. Ergotamine was administered intraperitoneally in 0.1 ml of a 0.05% solution of the compound in 0.9% NaCl. The animals were injected at birth and every hour thereafter (a dose of 8.3 mg/kg each time). Ten propranolol-treated animals and ten normal controls from the same litters were sacrificed at the age of 6 hours. Propranolol was administered intraperitoneally in 0.1 ml of a 0.1% solution. The animals were injected at birth and 2 hours after birth (a dose of 16.7 mg/kg each time). Five dichloroisoproterenol-treated animals and five controls from the same litters were sacrificed at the age of 6 hours. Dichloroisoproterenol was administered intraperitoneally in 0.1 ml of a 0.1% solution of the compound in 0.9% NaCl. The animals were injected at birth and 3 hours after birth (a dose of 16.7 mg/kg each time).

Results

Biochemical results

Both glycogen-hydrolysing and maltose-hydrolysing activities of acid glucosidase were determined after administering cyclic AMP. The glycogen-hydrolysing activity was significantly higher in the treated animals, i.e., 0.364 ± 0.150 μM glucose/hour/mg protein, than in the controls, i.e., 0.238 ± 0.075 μM glucose/hour/mg protein, while no significant change was noted in the maltose-hydrolysing activity (Table 1).

Glycogen-hydrolysing activity was determined after administering ergotamine. In the treated animals, the activity of the enzyme did not reach the level of the controls, i.e., 0.223 ± 0.066 μM glucose/hour/mg protein, but remained significantly lower, i.e., 0.141 ± 0.040 μM glucose/hour/mg protein (Table 2). The concentration of glycogen in the liver of five ergotamine-treated animals was significantly higher, i.e., 0.455 ± 0.120 mg/mg protein, than in five controls, i.e., 0.210 ± 0.095 mg/mg protein. The results were means ± standard deviations ($p < 0.05$).

Glycogen-hydrolysing activity was also determined after administering propranolol. In the treated animals, the activity did not reach the level of the controls, i.e., 0.212 ± 0.058 μM glucose/hour/mg protein but was significantly lower, i.e., 0.123 ± 0.030 μM glucose/hour/mg protein (Table 3). No significant difference in the concentrations of liver glycogen between five propranolol-treated animals; i.e., 0.201 ± 0.104 mg/mg protein and five controls, i.e., 0.185 ± 0.086 mg/mg protein was noted. The results were means ± standard deviations ($p < 0.05$).

Liver glycogen was determined in dichloroisoproterenol-treated animals. No significant difference in

Table 1. Glycogen-hydrolysing and maltose-hydrolysing activities of acid glucosidase, 6 hours after cyclic AMP treatment of newborn rats (μM glucose/hr/mg protein)*

	Glycogen hydrolysing activity	Maltose hydrolysing activity
Control	0.238 ± 0.075 (10)	0.305 ± 0.116 (10)
Cyclic AMP	0.364 ± 0.150 (10)	0.259 ± 0.083 (10)
P	< 0.05	> 0.05

* Results are means ± standard deviations. Numbers in parentheses represent the number of observations.

Table 2. Glycogen-hydrolysing activity of acid glucosidase, 6 hours after ergotamine treatment of newborn rats (μM glucose/hr/mg protein)*

Control	0.223 ± 0.066 (10)	(At birth: 0.066 ± 0.021) (5)
Ergotamine	0.141 ± 0.040 (10)	
P	< 0.05	

* Results are means ± standard deviations. Numbers in parentheses represent the number of observations.

Table 3. Glycogen-hydrolysing activity of acid glucosidase, 6 hours after propranolol treatment of newborn rats (μm glucose/hr/mg protein)*

Control	0.212 ± 0.058 (10)
Propranolol	0.123 ± 0.030 (10)
P	< 0.05

* Results are means ± standard deviations. Numbers in parentheses represent the number of observations.

Table 4. Comparison of cytoplasmic components of the hepatocytes from control and ergotamine-treated newborn rats, 6 hours after birth*

Treatment	% of cytoplasmic volume		% of lysosomal volume occupied by glycogen	Mitochondrial matrix granules per μ^2 of cytoplasm
	Lysosomes	Glycogen in autophagic vacuoles		
Control**	1.49 ± 0.23	0.27 ± 0.07	18	< 0.04 (at birth 0.41 ± 0.05)
Ergotamine***	0.71 ± 0.14	0.24 ± 0.07	34	0.21 ± 0.04
P	< 0.05	> 0.5		< 0.05

* Results are means ± standard errors.
** Results computed from a total of 30 micrographs and an area of 5100 μm^2.
*** Results computed from a total of 30 micrographs and an area of 5300 μm^2.

the concentrations of liver glycogen between five dichloroisoproterenol-treated animals, i.e., 0.233 ± 0.098 mg/mg protein and five controls, i.e., 0.196 ± 0.084 was noted.

Morphologic results

Both qualitative and quantitative changes of cytoplasmic components of the rat hepatocytes were studied. The term lysosomes includes lysosomes and related particles, from autophagic vacuoles to residual bodies (DeDuve and Wattiaux, 1966).

Group I: Cyclic AMP administered. The appearance of the control animals at the ages of 0 and 6 hours and the cyclic AMP-treated animals at the age of 6 hours was as described before (Kotoulas, 1986). At birth, vast areas of hyaloplasmic glycogen were present. Lysosomes and autophagic vacuoles were rare and small. A large part, i.e., 50% of the total lysosomal volume was occupied by glycogen. Many mitochondrial matrix granules were noted. At the age of 6 hours the hyaloplasmic glycogen was reduced. The number and volume of lysosomes increased. The organelles occurred predominantly at the junction of glycogen and glycogen-free areas. Most of the lysosomal volume belonged to lysosomes of the autophagic type. A small part, i.e., 18% of the total lysosomal volume was occupied by glycogen. The number and size of mitochondrial matrix granules were drastically reduced. In the cyclic AMP-treated animals at the age of 6 hours the depletion of hyaloplasmic glycogen was advanced. Lysosomes were numerous and usually occurred at the margins of the stores of glycogen as in the controls. Most of the lysosomal volume belonged to lysosomes of the autophagic type. A very small part, i.e., 7% of the total lysosomal volume was occupied by glycogen. Rare mitochondrial matrix granules were noted.

Group II: Ergotamine administered. The hepatocytes of the controls at the age of 6 hours were as in Group I (Table 4, Fig. 1). In ergotamine-treated animals at the age of by 6 hours, the appearance of the hepatocytes was strikingly different from that of the controls, as reported previously (Kotoulas, 1986). The stores of hyaloplasmic glycogen remained large. The marked development of lysosomes, which was seen in the controls, was not observed in the ergotamine-treated animals. Most of the lysosomes, were of the autophagic type. The autophagic vacuoles were predominantly small and round and usually occurred at the margins of the areas of glycogen. A large part, i.e., 34% of the total lysosomal volume, was occupied by glycogen. Mitochondrial matrix granules were not as drastically reduced as in the controls (Table 4, Fig. 2).

Group III: Propranolol administered. Preliminary observations suggested that the administration of propranolol did not appreciably prevent the mobilization of hyaloplasmic glycogen. However, in the treated animals, a larger part, i.e., 30% of the total lysosomal volume was occupied by glycogen, as compared with the controls, i.e., 18%.

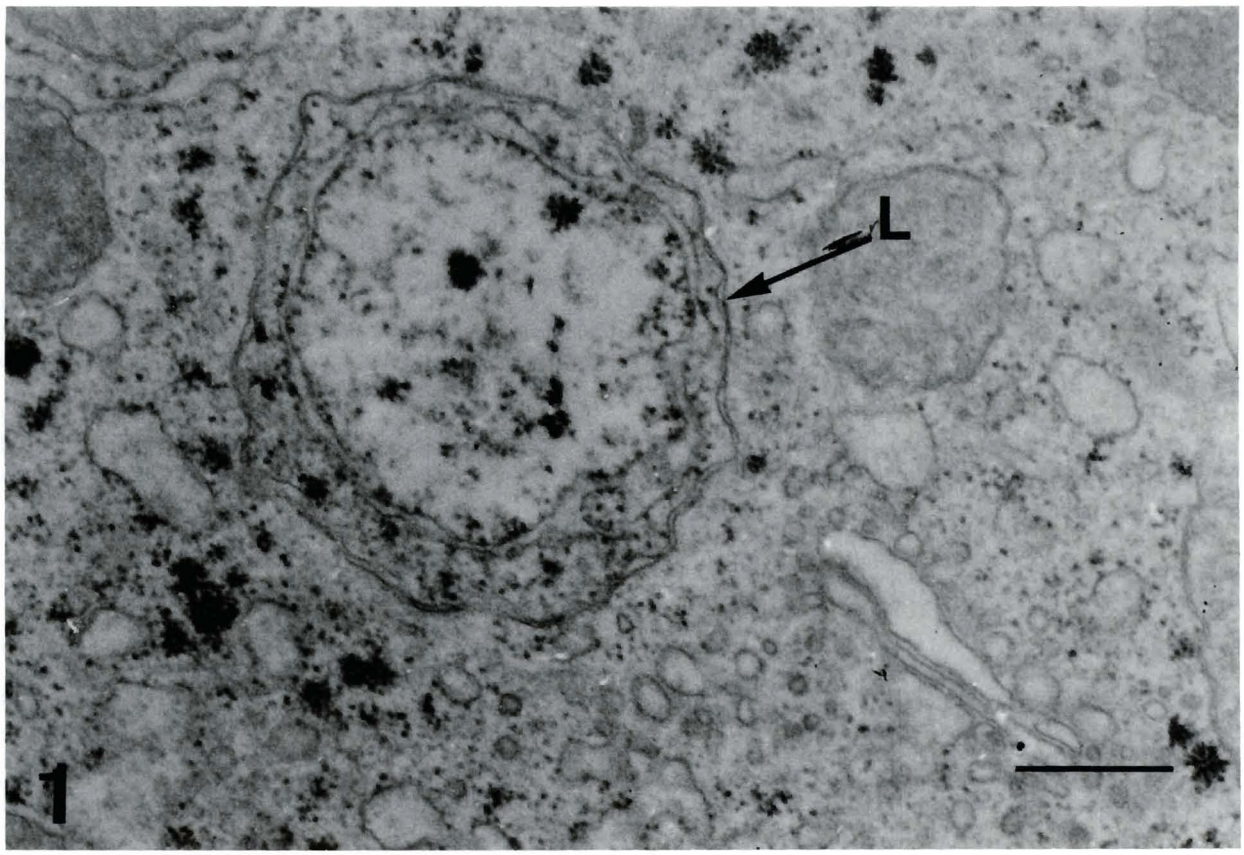

Fig. 1. Portion of a control rat hepatocyte at the age of 6 hours. A lysosome-autophagic vacuole containing a negligible amount of glycogen is seen (L). Bar, 0.05 μm.

Discussion

Previous studies suggest that the breakdown of lysosomal glycogen in newborn rat hepatocytes is due to the action of adrenalin or glucagon secreted after birth (Rosenfeld, 1964; Kotoulas and Phillips, 1971; Kotoulas et al., 1971). This action is mediated through an increase in the level of cyclic AMP which produces a rise in the activity of the lysosomal enzyme acid alpha 1,4 glucosidase. This enzyme hydrolytically degrades glycogen or maltose. The glycogen-hydrolysing activity of acid glucosidase is low at birth but increases during the first postnatal hours (Kotoulas, 1981, 1986). From the data presented in this and previous studies, it is apparent that exogenous cyclic AMP mimics the effect of hormones and enhances the normal postnatal rise in the glycogen-hydrolysing activity of the enzyme (Kotoulas, 1986). However, the maltose-hydrolysing activity may not respond to the same stimuli. Our observations showed that cyclic AMP administration resulted in no increase in this activity. This suggests that glycogen-hydrolysing and maltose-hydrolysing activities may be due to different enzymes as proposed by others (Lundquist, 1986; Skoglund et al., 1987).

There is an absence of knowledge of the intermediate steps distal to that of cyclic AMP, in the reaction sequence leading to the increase of glycogen-hydrolysing activity of the enzyme. The postnatal rise in this activity and the mobilization of glycogen content of the lysosomes were found to be dependent on protein synthesis (Kotoulas, 1988). The inductive effect of hormones and cyclic AMP may be mediated by activation of a cyclic AMP-dependent protein kinase.

The results of our study with propranolol, a beta-adrenergic antagonist, suggest that the postnatal increase in the glycogen-hydrolysing activity of acid glucosidase and the hydrolytic degradation of lysosomal glycogen in newborn rat hepatocytes represent a beta-adrenergic function. This is not the case for the phosphorolytic degradation of hyaloplasmic glycogen since propranolol and dichloroisoproterenol, another beta-adrenergic antagonist, did not prevent the postnatal mobilization of total liver glycogen in these animals. Moreover, the degradation of hyaloplasmic glycogen in rats was reported to be an alpha-adrenergic function. Functional beta-adrenergic receptors have been found before in rat hepatocytes but were not quantitatively important in the overall glycogen-mobilizing process of liver (Exton, 1979; Gilman et al., 1980). In our

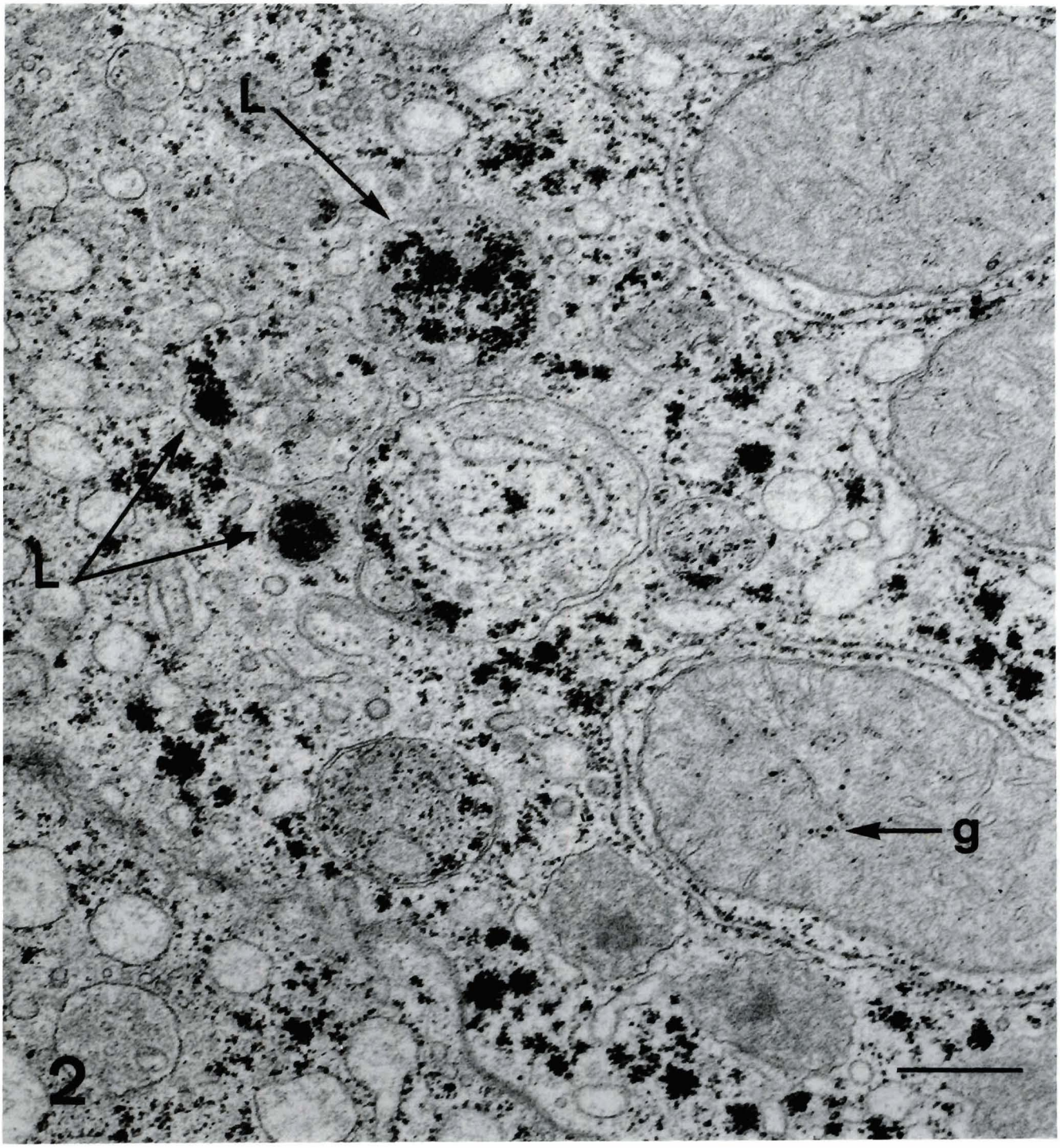

Fig. 2. Portions of two ergotamine-treated rat hepatocytes at the age of 6 hours. Lysosome-autophagic vacuoles containing moderate amounts of undigested glycogen are seen (L). Mitochondrial matrix granules (g) are noticeable. Bar, 0.5 μm.

experiments with beta-adrenergic antagonists, this was apparently due to the fact that the volume of the lysosomal glycogen, which is mobilized by a beta-adrenergic mechanism, is relatively small compared to the volume of hyaloplasmic glycogen (Kotoulas and Phillips, 1971). The lysosomal pathway for glycogen degradation may be important in cases where there is a demand for the massive liberation of free glucose (Rosenfeld, 1964; Kotoulas, 1986).

The administration of ergotamine, classed as an

alpha-adrenergic antagonist, resulted in inhibitions of the postnatal rise in the glycogen-hydrolysing activity of acid glucosidase and the degradation of lysosomal glycogen. A reasonable explanation for these results is that ergotamine acts by interfering with the action or formation of cyclic AMP. The inhibition of hyaloplasmic glycogen degradation, produced by the alkaloid, may be viewed as an alpha-adrenergic blocking effect (Moran, 1966; Sutherland et al., 1968; Exton, 1979). The inhibition of postnatal reduction in the size and number of the mitochondrial matrix granules observed in ergotamine-treated newborn rats supports the view that alpha-adrenergic blockage was obtained in these animals. These granules could be considered as a major intracellular storage site from which Ca^{2+} is mobilized during the alpha-adrenergic stimulation (Exton, 1979). Cyclic AMP, may be linked with the alpha-adrenergic mechanism, which is dependent on calcium, by modifying the cyclic AMP-dependent protein kinase and the probability of opening calcium channels (Rohrkasten et al., 1989).

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