The degradation of glycogen in the lysosomes of newborn rat hepatocytes: glycogen-, maltose- and isomaltose-hydrolyzing acid alpha glucosidase activities in liver

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Summary. The lysosomal glucosidase activities and glycogen degradation in newborn rat liver were studied by using biochemical assays, electron microscopy and quantitative morphometry. Glycogen-hydrolyzing, maltose-hydrolyzing and isomaltose-hydrolyzing activities were low at birth but increased afterwards. At the age of 6 hours they were markedly elevated. Actinomycin prevented the development of glucosidase activities indicating that these depend on protein synthesis. Parenteral glucose inhibited all three activities. This was apparently due to the abolition of normal postnatal hypoglycemia and the need for blood glucose. Cyclic AMP increased the glycogen-hydrolyzing but not the maltose-hydrolyzing activity. Propranolol inhibited the glycogen-hydrolyzing but not the maltose-hydrolyzing activity. The observations of this study provide further support for the hypothesis made by previous investigators that these activities are due to different enzymes.

Key words: Liver, Hepatocytes, Lysosomes, Glycogen, Alpha-glucosidase

Introduction

The lysosomes which are present in liver are known to contain hydrolases with specificities directed toward poly- and oligosaccharides. Previous investigators showed that the lysosomal enzyme activities of acid alpha 1,4 exopolyglucosidase (acid amyloglucosidase), acid alpha 1,4 glucosidase (acid maltase) and acid alpha 1,6 glucosidase (acid isomaltase) are able to catalyze the total hydrolysis of glycogen to glucose. These glucosidase activities are missing in the liver of patients with type II glycogenosis (Pompe's disease). Electron

micrographs of hepatocytes from such patients show that a large amount of undegraded glycogen is present inside the lysosomes (Hers, 1963; Lejeune et al., 1963; Rosenfeld, 1964; Illingworth-Brown et al., 1970; Jeffrey et al., 1970; Fuller et al., 1995). The presence of different enzymes having these activities was suggested by Lundquist (1985), Skoglund et al. (1987) and Kotoulas et al. (1991).

Inhibition of the glycogen-hydrolyzing activity of acid glucosidase (acid amyloglucosidase) in the rat liver and accumulation of undigested glycogen in the lysosomes of hepatocytes, were experimentally produced by a number of substances including glucose, ergotamine, propranolol, actinomycin D and insulin (Dallner et al., 1966; Kotoulas et al., 1971, 1991; Kotoulas, 1981, 1988; Skoglund et al., 1987; Kalamidas et al. 1994). Increase in this activity and accelerated mobilization of lysosomal glycogen, were produced by glucagon, cyclic AMP or caffeine (Kotoulas 1984, 1986; Kotoulas et al., 1991; Kalamidas et al., 1994).

In this paper, previous studies on the changes of glycogen-hydrolyzing activity of acid glucosidase (acid amyloglucosidase) in the newborn rat liver, were extended to include maltose-hydrolyzing activity (acid maltase) and isomaltose-hydrolyzing activity (acid isomaltase).

Materials and methods

Chemicals

Glycogen, Lot 126F-3846, Maltose, Lot 46F-0101, Isomaltose, Lot 127F-4011, Actinomycin D, Lot 62C-3400, Adenosine 3',5'-cyclic monophosphate (cAMP), Lot 29F-7030, and the reagents for determining glucose and cyclic AMP-dependent protein kinase were obtained from Sigma. Propranolol hydrochloride (Inderal), Lot PL29/5062 was from Imperial Chemical Industries. D-glucose was obtained from Serva. Reagents for electron microscopy were obtained as before (Kalamidas et al.,

1994).

Experimental Design

Newborn rats were obtained from pregnant Wistar females. Ten pregnant females were used and the average litter contained ten newborns. The sex of the newborn animals was not determined. After delivery the newborns were kept in an incubator at 36 °C and decapitated at various ages. After decapitation, the liver was excised and part of it was processed for electron microscopy. The rest was used for enzyme assays.

For the biochemical and electron microscopy studies, three normal animals were killed at birth and at the ages of 6 and 12 hours. The rest of the animals were divided into four groups according to the agent used. Seven actinomycin-treated animals and seven controls from the same litters were killed at the age of 12 hours. Actinomycin was administered intraperitoneally in 0.1 ml of a solution prepared according to Dallner et al. (1966). The animals were injected at 0 and 3 hours after birth (a dose of 0.8 mg/kg each time). Control animals were injected with carrier. Seven glucose-treated animals and seven controls from the same litters were killed at the age of 12 hours. Glucose was administered intra-peritoneally, in 0.07 ml of 20% solution of the compound, in distilled water. The animals were injected at birth and every two hours thereafter (a dose of 2.33 g/kg each time). Under these conditions the animals become markedly hyperglycemic (Kotoulas et al., 1971; Kalamidas et al., 1994). Control animals were injected with distilled water. Ten cyclic AMP-treated animals and ten controls from the same litters were killed at the age of 6 hours. Cyclic AMP was administered intra-peritoneally 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). This administration is known to result in a marked hyperglycemia (Kotoulas and Phillips, 1971). Control animals were injected with 0.9% NaCl. Three propranolol-treated animals and three normal controls from the same litters were killed 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).

For the quantitative morphometric study on electron micrographs, three normal animals were killed at birth and at the ages of 6 and 12 hours. Three cAMP-treated and three propranolol-treated animals and an equal number of their controls were killed at the age of 6 hours. Three actinomycin-treated and three glucose-treated animals and an equal number of their controls were killed at the age of 12 hours.

Biochemical methods

The glycogen-hydrolyzing activity of acid glucosidase was assayed in homogenates of liver tissue in a 10% dilution with ice-cold distilled water. The tissue was homogenized in a glass homogenizer. Usually, 100 μl of homogenate were used for the assay. The total enzyme activity was determined according to Hers (1963) and Lejeune et al. (1963) with glycogen (1%) as substrate. Incubation was carried out for 60 min in 0.1M sodium acetate buffer with 0.05% Triton-X100 at pH 4.7 and at 37 °C. The total maltose-hydrolyzing and isomaltose-hydrolyzing activities were determined in a similar way, with maltose (0.5%) and isomaltose (0.25%), respectively, as substrates (Jeffrey et al., 1970; Lundquist, 1985, 1986). The reactions were terminated by the addition of barium hydroxide and zinc sulfate and the reaction mixtures were deproteinized according to Somogyi (1945). Glucose was estimated by the method of Raabo and Terkildsen (1960), using glucose oxidase, peroxidase and dianisidine. Protein in the liver was determined by the method of Lowry et al. (1951). Enzyme activities 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 or isomaltose (Skoglund et al., 1987). The cAMP-dependent protein kinase activity was assayed in the homogenates according to Laks and Jungmann (1980) and Laks et al. (1981) in the presence of 10^{-6} M cAMP with protamine sulfate as substrate. The results were statistically evaluated according to Hill (1967).

Electron microscopy and morphometric analysis

Liver tissue was fixed for 1 hour at 0 °C in 1% osmium tetroxide buffered with 0.1M phosphate buffer at pH 7.2. The tissue was dehydrated in a graded series of ethanol solution, transferred to propylene oxide and then to a mixture of propylene oxide and resin (Kotoulas and Phillips, 1971). The embedding medium was prepared according to Mollenhauer (1964) using Araldite. Sections, 1 μm thick, were stained with toluidine blue and examined by light microscopy (Trump et al., 1961). Ultrathin sections, approximately 50 nm thick, were cut with glass knives using an LKB microtome. These sections were picked up on uncoated grids and stained at room temperature with a saturated aqueous solution of uranyl acetate for 10 minutes and Reynold's solution of lead citrate for another 10 minutes (Pease, 1964; Glauert, 1965).

Morphometric analysis was performed on electron micrographs as described before (Kotoulas and Phillips, 1971; Kotoulas et al., 1971). 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 13000. A lattice composed of horizontal and vertical lines at regular intervals (0.5 cm) 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 of a cytoplasmic component i.e. the volume of component per unit of cytoplasmic volume, is equal to the fraction of the points

enclosed within the area of this component in random cross sections of the tissue. In the case of lysosomes and lysosomal glycogen, the data of the micrographs from the same block were combined and therefore the means and standard errors were calculated from these combined data (Weibel, 1969). The fractional number of a cytoplasmic organelles i.e. the number of organelles per unit of cytoplasmic volume was estimated as described by Loud (1968). The mean tangent diameter of the organelle required for this estimation was found from the volume-to-surface ratio. To estimate this ratio, every other segment between two cross points on the horizontal lines of the lattice was used. The intersections of these segments with the organelle boundaries and the cross points of the lattice overlying the organelle were counted. Then, the volume-to-surface ratio was calculated according to the formula of Chalkley and Cornfield (Weibel, 1969).

The length-to-diameter ratio (λ) of organelles was determined according to Weibel (1969, 1979) using a graph. Lysosomes were assimilated to the ellipsoids. The value of the shape coefficient (ß) required for this determination, was derived from the formula of Knight, Weibel and Gomez as were the estimates of fractional volume, fractional number and number of transections per unit area of tissue section (Weibel, 1969). The size distribution coefficient (k) was taken as 1.07 (Weibel and Gómez, 1962; Weibel, 1969).

The volume of cytoplasm in μm^3/hepatic cell was determined from light photographs (one photograph from each of five animals of each group) taken from 1

μm-thick sections stained with toluidine blue and enlarged to a final magnification of 1000. First, the volume of hepatic cell cytoplasm/unit of volume of liver tissue was estimated. Second, the number of hepatic cell nuclei/unit of volume of liver tissue was estimated as described by Loud (1968). Then, the ratio of these two estimates was obtained. This calculation gave the volume of hepatic cell cytoplasm/hepatic cell nucleus. Except for the presence of binucleated cells, this calculation would have given the average cytoplasmic volume/hepatic cell. Since binucleated cells have twice the volume of mononucleated cells, the estimated volume of the hepatic cell cytoplasm/hepatic cell nucleus is still approximately the same as the volume of cytoplasm/hepatic cell (Loud, 1968). The mean tangent diameter of the hepatic cell nucleus required for the second estimate was found from the volume-to-surface ratio as described above. The results of morphometric analysis were evaluated by Student's t-test.

Results

Biochemical results

Glycogen-hydrolyzing, maltose-hydrolyzing and isomaltose-hydrolyzing activities during the first 12 hours after birth, are shown in Table 1. All three activities were low at birth but showed an increase at the age of 6 hours. Glycogen- and isomaltose-hydrolyzing activities returned to a lower level at the age of 12 hours. No significant decrease in maltose-hydrolyzing activity was observed at this age. Glycogen- and maltose-hydrolyzing activities were suppressed by the administration of actinomycin as shown in Table 2. Preliminary observations showed that isomaltose-hydrolyzing activity was also suppressed by actinomycin. The results

Table 1. Glycogen-, maltose- and isomaltose-hydrolyzing activities of acid glucosidase during the first 12 hours after birth. Results are means ± standard deviations. Each value includes 3 observations (μmol glucose/hr/mg protein).

	AGE (hours after birth)		
	0	6	12
Glycogen-hydrolyzing activity	0.096±0.039	0.219±0.064	0.161±0.046
Maltose-hydrolyzing activity	0.105±0.040	0.258±0.072	0.228±0.071
Isomaltose-hydrolyzing activity	0.027±0.015	0.084±0.041	0.038±0.019

Table 2. Glycogen- and maltose-hydrolyzing activities of acid glucosidase, 12 hours after actinomycin treatment of newborn rats. Results are means ± standard deviations. Each value includes 7 observations (μmol glucose/hr/mg protein).

	Glycogen-hydrolyzing activity	Maltose-hydrolyzing activity
Control	0.182±0.061	0.244±0.080
Actinomycin	0.098±0.044	0.112±0.054
p	< 0.05	< 0.05

Table 3. Glycogen-, maltose- and isomaltose-hydrolyzing activities of acid glucosidase, 12 hours after parenteral glucose treatment of newborn rats. Results are means ± standard deviations. Each value includes 7 observations (μmol glucose/hr/mg protein).

	Glycogen hydrolyzing activity	Maltose hydrolyzing activity	Isomaltose hydrolyzing activity
Control	0.169±0.055	0.219±0.068	0.047±0.018
Glucose	0.111±0.030	0.134±0.030	0.030±0.010
p	< 0.05	< 0.05	< 0.05

Table 4. Glycogen- and maltose-hydrolyzing activities of acid glucosidase, 6 hours after cyclic AMP treatment of newborn rats. Results are means ± standard deviations. Each value includes 10 observations (μmol glucose/hr/mg protein).

	Glycogen-hydrolyzing activity	Maltose-hydrolyzing activity
Control	0.238±0.075	0.283±0.090
Cyclic AMP	0.364±0.150	0.233±0.072
p	< 0.05	< 0.05

of parenteral glucose administration are shown in Table 3. None of the three enzymic activities reached the level of the controls but remained significantly lower.

Glycogen-hydrolyzing and maltose-hydrolyzing activities were determined after administering cyclic AMP (Table 4). The glycogen-hydrolyzing activity was significantly higher in the treated animals than in the controls. However, the maltose-hydrolyzing activity was lower in the treated animals. These activities were also determined after administering propranolol. The glycogen-hydrolyzing activity was lower in the treated animals than in the controls. No significant change in the maltose-hydrolyzing activity was noted in the treated animals (Table 5). Cyclic AMP-depended protein kinase activity was lower in the three propranolol-treated animals (0.017 ± 0.006 mM Pi/hr/mg protein)

than in the three controls (0.062 ± 0.018 mM Pi/hr/mg protein). The difference was statistically significant ($p < 0.05$).

Table 5. Glycogen- and maltose-hydrolyzing activities of acid glucosidase, 6 hours after propranolol treatment of newborn rats. Results are means ± standard deviations. Each value includes 3 observations (μmol glucose/hr/mg protein).

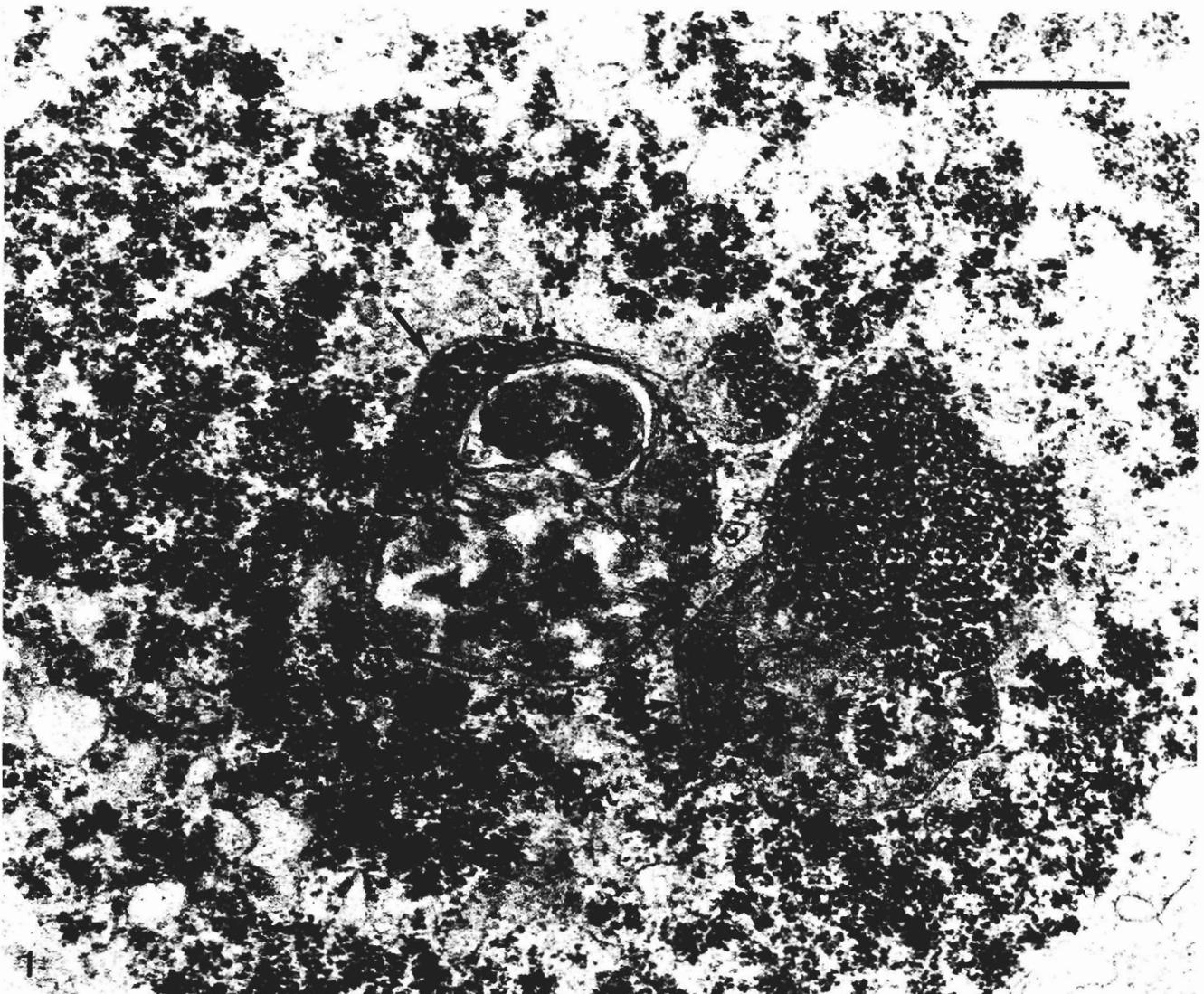|	Glycogen-hydrolyzing activity	Maltose-hydrolyzing activity
Control	0.212 ± 0.058	0.240 ± 0.077
Propranolol	0.123 ± 0.030	0.217 ± 0.062
p	< 0.05	> 0.05

Fig. 1. Portion of a control rat hepatocyte at the age of 6 hours. Two lysosomes-autophagic vacuoles (arrows) including moderate amounts of undigested glycogen, are seen. Bar: 0.5 μm.
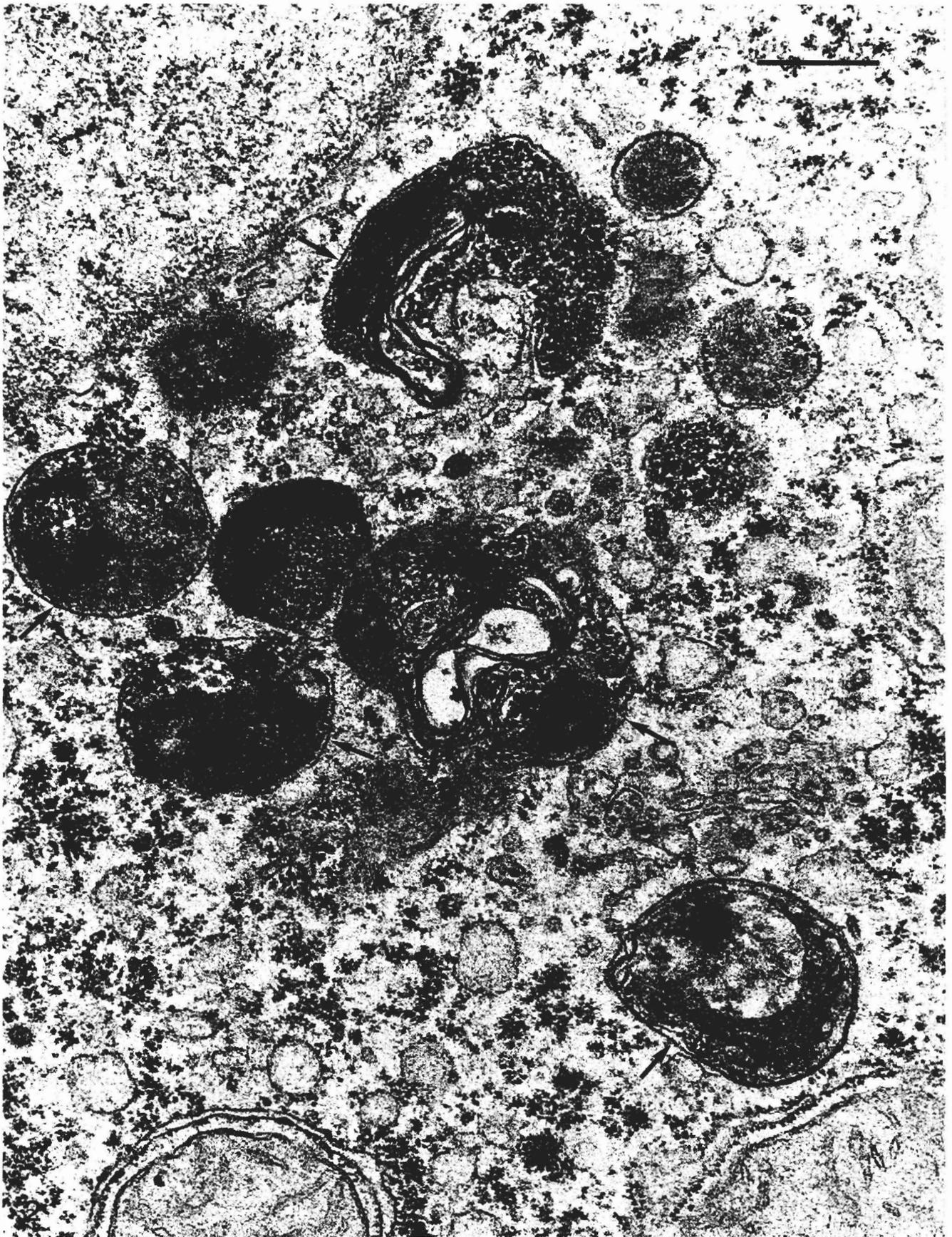
Fig. 2. Portion of a cAMP-treated rat hepatocyte at the age of 6 hours. Many lysosomes-autophagic vacuoles (arrows) including only small amounts of undigested glycogen, are seen. Bar: 0.5 μm.

Morphological results

Both qualitative and quantitative changes were studied. The term lysosomes refers to lysosomes and related particles including autophagic vacuoles (DeDuve and Wattiaux, 1966; Dunn, 1990a,b). The appearance of normal rat hepatocytes during the first 12 hours after birth has been described before (Kotoulas and Phillips, 1971; Kotoulas et al., 1971; Iwamasa et al., 1982; Kalamidas et al., 1994). At birth abundant hyaloplasmic glycogen was present. Lysosomes were rare, small and spherical with an estimated length-to-diameter ratio of 1:1. They were located at the margins of the hyaloplasmic glycogen areas. A large part of the lysosomal volume was occupied by undigested glycogen (53%). At 6 hours the hyaloplasmic glycogen was reduced. Many large lysosomes, usually of the autophagic type, appeared. They were nearly spherical with an estimated length-to-diameter ratio of 1.4:1 and located at the margins of the hyaloplasmic glycogen areas. These lysosomes contained moderate amounts of undigested glycogen which occupied 17% of the lysosomal volume. At 12 hours the hyaloplasmic glycogen was totally depleted. The number and size of lysosomes were further increased. These organelles occurred in huge irregular clumps with an estimated length-to-diameter ratio of 4:1. Most of them had the appearance of residual bodies. They included negligible amounts of undigested glycogen. Control animals of all groups, in no respect differed from normal animals of the same age.

In the actinomycin-treated animals at the age of 12 hours, the hyaloplasmic glycogen was totally depleted. Lysosomes were few, round and filled with undigested glycogen. A large part, i.e. 48% of the lysosomal volume, was occupied by glycogen as reported before (Kotoulas, 1988). In the glucose-treated animals at the age of 12 hours, the abundant hyaloplasmic glycogen was preserved. The lysosomes remained few, small and spherical with an estimated length-to-diameter ratio 1:1. They were located at the margins of glycogen areas. These lysosomes included large amounts of undigested glycogen which occupied 65% of the lysosomal volume

(Table 6). The morphological changes observed in glucose-treated animals could not be attributed to the difference in the mean volume of hepatic cell cytoplasm between controls (4530 μm^3/hepatic cell) and treated animals (5200 μm^3/hepatic cell).

In the cyclic AMP-treated animals at the age of 6 hours, the hyaloplasmic glycogen was drastically reduced. The number and size of lysosomes increased as compared with the controls (Figs. 1, 2). Most of the lysosomes belonged to the autophagic type but some of them had the appearance of residual bodies. The shape of lysosomes was oval and the estimated length-to-diameter ratio was 3:1. Only small amounts of undigested glycogen remained in these organelles. Glycogen occupied 7% of their volume (Table 7). The morphological changes observed in cyclic AMP-treated animals could not be attributed to the difference in the mean volume of hepatic cell cytoplasm between controls (4750 μm^3/hepatic cell) and treated animals (4620 μm^3/hepatic cell). Preliminary observations suggested that in the propranolol-treated animals at the age of 6 hours, a larger part of the lysosomal volume was occupied by undigested glycogen as compared with the controls.

Discussion

The data presented in this and previous studies show that during the immediate postnatal period, the lysosomal glycogen in liver was progressively mobilized. At the age of 12 hours only a negligible part of the lysosomal volume was occupied by undigested polysaccharide (Kotoulas et al., 1971; Kalamidas et al., 1994). All three lysosomal glucosidase activities, namely glycogen-hydrolyzing, maltose-hydrolyzing and isomaltose-hydrolyzing, were low at birth but increased afterwards. At the age of 6 hours they were markedly elevated. Actinomycin prevented the postnatal development of these activities and the degradation of glycogen inside the lysosomes, indicating that the increase of activities represent de novo synthesis. Parenteral glucose also inhibited the postnatal

Table 6. Comparison of hepatocytes from control and glucose-treated newborn rats, 12 hours after birth.

TREATMENT	% OF CYTOPLASMIC VOLUME*		% LYSOSOMAL VOLUME OCCUPIED BY GLYCOGEN
	Lysosomes	Glycogen in autophagic vacuoles	
Control **	5.46±0.78	< 0.04	< 1
Glucose ***	0.40±0.17	0.26±0.08	65 (at birth 53)
p	< 0.05	< 0.05	

*: volumes of cytoplasmic components are means ± standard errors; **: results computed from a total of 30 micrographs and an area of 4850 μm^2; ***: results computed from a total of 30 micrographs and an area of 4800 μm^2.

Table 7. Comparison of hepatocytes from control and cyclic AMP-treated newborn rats, 6 hours after birth.

TREATMENT	% OF CYTOPLASMIC VOLUME*		% LYSOSOMAL VOLUME OCCUPIED BY GLYCOGEN
	Lysosomes	Glycogen in autophagic vacuoles	
Control **	1.25±0.28	0.21±0.05	17
Cyclic AMP ***	2.13±0.30	0.14±0.03	7
p	< 0.05	< 0.05	

* : volumes of cytoplasmic components are means ± standard errors; **: results computed from a total of 30 micrographs and an area of 4970 μm^2; ***: results computed from a total of 30 micrographs and an area of 5200 μm^2.

development of glucosidase activities and the degradation of lysosomal glycogen. This should be explained on the basis of the abolition of normal postnatal hypoglycemia and need for blood glucose. The lysosomal pathway for glycogen degradation becomes important when there is a demand for the massive liberation of free glucose (Rosenfeld, 1964; Kotoulas, 1986; Liu et al., 1993; Kalamidas et al., 1994).

The administration of cAMP resulted in an increase in the glycogen-hydrolyzing activity and an accelerated degradation of lysosomal glycogen. However, this administration produced a decrease in the maltose-hydrolyzing activity. Propranolol, a beta-adrenergic antagonist, inhibited the glycogen-hydrolyzing but not the maltose-hydrolyzing activity. These findings support the hypothesis formulated by others that glycogen-hydrolyzing activity and maltose-hydrolyzing activity are due to different enzymes (Lundquist, 1986; Skoglund et al., 1987; Kotoulas et al., 1991). Moreover, they suggest that the postnatal increase in the glycogen-hydrolyzing activity of acid glucosidase in newborn rat liver, represent a beta-adrenergic function.

Previous studies constituted good evidence for active participation of lysosomes in the overall degradation of cellular glycogen in the newborn rat hepatocytes. This process is under hormonal control. Glucagon or adrenalin which is secreted after birth, produced an increase in the lysosomal glycogen-hydrolyzing activity and an acceleration of the degradation of lysosomal glycogen. Insulin (which opposes the action of glucagon) or glucose (which abolishes the postnatal hypoglycemia, the natural stimulus for glucagon secretion) inhibited this activity and the degradation of lysosomal glycogen (Rosenfeld, 1964: Kotoulas and Phillips, 1971; Kotoulas et al., 1971; Kotoulas, 1981, 1984; Skoglund et al., 1987; Maintas et al., 1993: Kalamidas et al., 1994). Hormones exert their action on the degradation of lysosomal glycogen and other aspects of autophagocytosis such as the changes in the volume and shape of lysosomes, through alterations in the cAMP level (Kotoulas, 1986; Kalamidas et al., 1994). The effect of cAMP on glycogen-hydrolyzing activity could be mediated by changes in the activity of cAMP-dependent protein kinase but cAMP could also act through a mechanism not involving this kinase activation (Van Dyke et al., 1996; Dremier et al., 1997). There is an absence of knowledge of the intermediate steps distal to those of cAMP and cAMP-dependent protein kinase, in the sequence of events leading to the increase in this lysosomal activity. It may well be that changes in the mannose-6-phosphate receptor and lysosomal mannose-6-phosphatase activity are of importance for the regulation of the glycogen-hydrolyzing activity (Einstein and Gabel, 1991; Gelato et al., 1993; Bresciani and Von Figura, 1996). The mechanism of regulation of maltose-hydrolyzing activity remains obscure. This process may not be mediated by cAMP.

The change in the shape and size of lysosomes

induced by cAMP, may represent the formation of a lysosomal compartment similar to the extended tubular compartment of the degradative pathway described by others (Swanson et al., 1987; Klausner et al., 1992; Rabinowitz et al., 1992; Desjardins et al., 1997). Lysosome fusion and formation of large organelles may also be controlled by a Ca^{2+}-dependent mechanism (Bakker et al., 1997).

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