Histol Histopath (1994) 9: 691-698

Histology and Histopathology

The breakdown of glycogen in the lysosomes of newborn rat hepatocytes: The effects of glucose, cyclic 3',5'-AMP and caffeine

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Summary. The effects of parenteral glucose, cyclic AMP and caffeine on the breakdown of glycogen in the lysosomes of newborn rat hepatocytes, were studied by using biochemical assays, electron microscopy and quantitative morphometry. Glucose prevented the normal postnatal increase in lysosomal volume, acid alpha 1,4 glucosidase activity and lysosomal glycogen breakdown. On the contrary, cyclic AMP and caffeine promoted this increase. There was a positive correlation between liver cyclic AMP concentration and acid glucosidase activity ($R = 0.84$, $p < 0,001$). Cyclic AMP also induced a change in the shape of lysosomes. The postulation that glucagon secreted after birth is the natural stimulus for the cyclic AMP-mediated postnatal increase in acid glucosidase activity and mobilization of the lysosomal glycogen in rat hepatocytes, is supported by these experimental findings.

Key words: Ultrastructure, Hepatocyte, Lysosomes, Glycogen, Glucose, Cyclic AMP, Caffeine

Introduction

Previous studies showed that the catabolism of lysosomal glycogen in rat hepatocytes was under hormonal control (Rosenfeld, 1964; Kotoulas and Phillips, 1971; Kotoulas et al., 1971; Pfeifer, 1971; Aronson, 1980). The glycogen-hydrolyzing activity of the enzyme acid alpha 1,4 glucosidase and the breakdown of glycogen in the lysosomes were induced by glucagon and inhibited by insulin in newborn rat hepatocytes. These hormones exerted their effect by causing alterations at the intracellular level of cyclic 3',5'-AMP (Kotoulas, 1981, 1984, 1986). In this paper the effects of parenteral glucose, cyclic AMP and caffeine were studied in newborn rat hepatocytes. Since glucose abolishes the normal postnatal hypoglycemia and secretion of glucagon, we decided to test the hypothesis that glucagon secreted after birth is the natural stimulus for the cyclic AMP-mediated postnatal increase in acid glucosidase activity and mobilization of lysosomal glycogen.

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Materials and methods

Chemicals

Glycogen (Cat No. 23540), Cyclic adenosine-3',5' phosphate (Cyclic AMP), D-glucose and caffeine were obtained from Serva. For electron microscopy, all reagents, except Araldite, were obtained from Fisher Scientific Co. Araldite 502 was obtained from Ciba Co. Ltd.

Experimental Design

Newborn rats were obtained from pregnant Wistar females. Eight 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. Immediately before sacrificing, blood was obtained from the cervical veins. 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 every two hours after birth up to the age of 12 hours. Another twelve normal animals were killed at the age of 6 hours. The rest of the animals were divided into three groups according to the agent used. Four glucose-treated animals and four controls from the same litters were killed at the age of 12 hours. Glucose was administered intraperitoneally 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). Control animals were injected with distilled water. Five cyclic AMP-treated animals and five

controls from the same litters were killed 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). This administration is known to result in a marked hyperglycemia (Kotoulas and Phillips, 1971). Control animals were injected with 0.9% NaCl. Four caffeine-treated animals and four controls from the same litters were killed at the age of 6 hours. Caffeine was administered intraperitoneally in 0.1 ml of a 0.25% solution of the compound in 0.9% NaCl. The animals were injected at birth and 3 hours after birth (a dose of 41.7mg/kg each time). Control animals were injected with 0.9% NaCl.

For the quantitative morphometric study on electron micrographs, three normal animals were killed at birth. Three cyclic AMP-treated and three caffeine-treated animals and an equal number of their controls were killed at the age of 6 hours.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 alpha 1,4 glucosidase was assayed in homogenates of liver tissue in a 10% dilution with distilled water. Usually 200 μl 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 (1%) as substrate. Incubation was carried out in 0.1M sodium acetate buffer (pH 4.7). Glucose was estimated by the method of Raabo and Terkildsen (1960) modified according to Sigma Technical Bulletin 510, using glucose oxidase, peroxidase and dianisidine. Protein was determined by the method of Lowry et al. (1951). Cyclic AMP was determined according to Amersham Bulletin TRK 432, using (8-^{3}H) cyclic adenosine-3',5' phosphate. The results were statistically evalued and correlated 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. Ultrathin sections, approximately 50 nm thick were 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; 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 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. 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 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 the 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). In preliminary studies the disector method for estimating number of organelles was used (Cruz-Orive and Weibel, 1990).

The diameter-to-length ratio of organelles was determined according to Weibel (1979) using a graph and estimates of fractional volume, fractional number and number of transsections per unit area of tissue section. Lysosomes were assimilated to the ellipsoids (Loud et al., 1965; Weibel, 1979).

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 resutls of morphometric analysis were evaluated by Student's t-test.

Results

Biochemical results

Blood glucose, liver acid glucosidase and liver cyclic AMP during the first 12 hours after birth are shown in Table 1. The blood glucose concentration fell at the age

Table 1. Blood glucose, liver acid glucosidase and liver cyclic AMP, during the first 12 hours after birth. Results are means±standard deviations. Each value includes 3 observations.

	AGE (hours after birth)						
	0	2	4	6	8	10	12
Blood glucose (mg/100ml)	53.2±14.0	37.6±12.2	46.1±13.8	56.1±14.8	53.7±13.9	54.6±13.6	51.8±13.6
Liver glucosidase (μM glucose/hr/mg protein)	0.099±0.046	0.105±0.051	0.199±0.081	0.203±0.080	0.172±0.073	0.170±0.073	0.165±0.070
Liver cyclic AMP (pM/g wet wt.)	38.7±8.9			107.6±20.4			70.1±15.7

Table 2. Blood glucose, liver acid glucosidase and liver cyclic AMP at the age of 12 hours, after parenteral glucose treatment of newborn rats. Results are means±standard deviations. Each value includes 4 observations.

	BLOOD GLUCOSE (mg/100ml)	ACID GLUCOSIDASE (μM glucose/hr/mg protein)	CYCLIC AMP (pM/g wet wt.)
Control	45.9±14.0	0.179±0.060	57.1±12.2
Glucose	350.8±114.2*	0.110±0.048	32.3±7.80
P	<0.05	<0.05	<0.05

*: at 6 hours, 380.5±210.0.

Table 3. Liver acid glucosidase and liver cyclic AMP at the age of 6 hours, after cyclic AMP treatment of newborn rats. Results are means±standard deviations. Each value includes 5 observations.

	ACID GLUCOSIDASE (μM glucose/hr/mg protein)	CYCLIC AMP (pM/g wet wt.)
Control	0.217±0.079	93.7±19.0
cAMP	0.308±0.090	148.8±33.1
P	<0.05	<0.05

Table 4. Liver acid glucosidase at the age of 6 hours, after caffeine treatment of newborn rats (μM glucose/hr/mg protein). Results are means±standard deviations. Each value includes 4 observations.

Control	0.199±0.082
Caffeine	0.289±0.088
P	<0.05

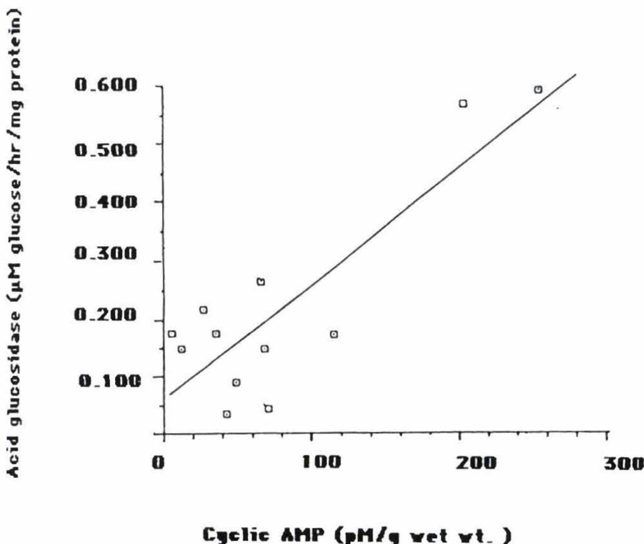

Fig. 1. Relationship between activity of liver acid glucosidase and concentration of liver cyclic AMP in newborn rats. The correlation coefficient, R= 0.84 (p< 0.001).

of 2 hours and rose again at the age of 6 hours. The liver enzyme activity was low at birth but it showed an abrupt increase at the ages of 4-6 hours. It returned to a lower level at the age of 8 hours. The liver cyclic AMP concentration was elevated at the age of 6 hours. A positive correlation existed between acid glucosidase and cyclic AMP levels in normal animals at the age of 6 hours (Fig. 1).

The results of parenteral glucose administration are shown in Table 2. The newborn animals became hyperglycemic. The liver acid glucosidase activity and cyclic AMP concentration at the age of 12 hours, did not reach the level of the controls remaining significantly lower. Preliminary observations at the age of 6 hours, showed similar results. The results of cyclic AMP administration are shown in Table 3. This administration resulted in increased concentration of cyclic AMP in the liver. The activity of acid glucosidase was also increased. Similar results were obtained after the administration of caffeine (Table 4).

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 bith has been described before (Kotoulas and Phillips, 1971; Kotoulas et al., 1971). At birth, vast areas of hyaloplasmic glycogen were present. Lysosomes were small and rare. They were located at the margins of the hyaloplasmic glycogen areas. A large part of the lysosomal volume was occupied by undigested glycogen. The Golgi apparatus was

small and showed a compact form. At 4-6 hours the hyaloplasmic glycogen was reduced. Many lysosomes, usually of the autophagic type, appeared and their size was increased. They were located at the margins of the hyaloplasmic glycogen areas and contained moderate amounts of undigested glycogen. The Golgi apparatus was developed. At 9-12 hours the hyaloplasmic glycogen was totally depleted. The number and size of lysosomes were further increased and these organelles occurred in clumps. Most of them had the appearance of residual bodies. They included negligible amounts of glycogen. The Golgi apparatus was well developed and many zones were seen in the cytoplasm.

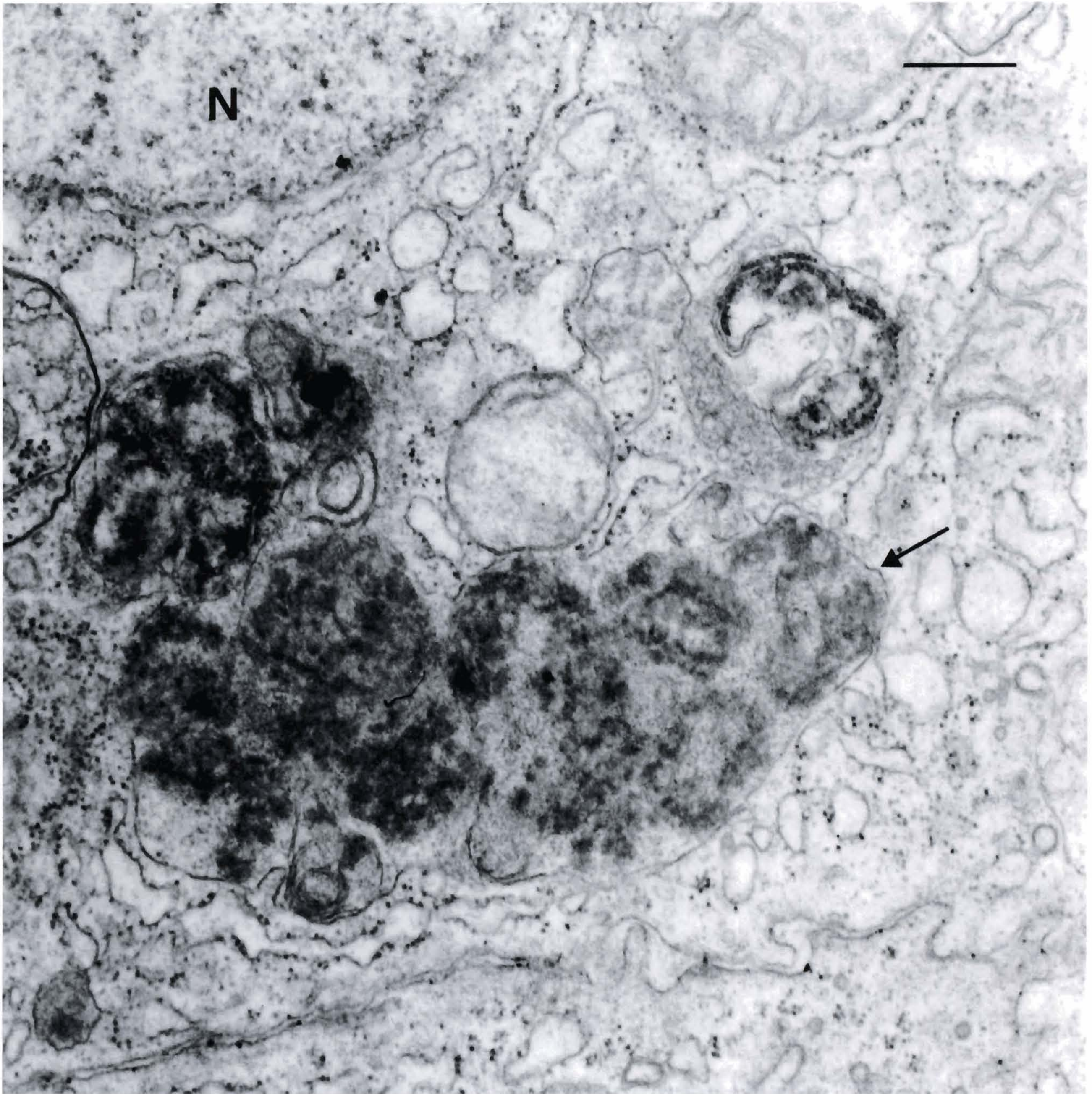

Fig. 2. Portions of two control rat hepatocytes at the age of 12 hours. A large lysosome-residual body (arrow) containing no glycogen is present. N: nucleus. Bar= 0.5 μm.

Group I. Glucose-administered

The control animals at the age of 12 hours in no respect differed from the normal animals of the same age. Glycogen was totally depleted. Many large lysosomes of the residual body type appeared. Their total fractional volume was 5.83±0.70. They usually occurred in irregular clumps with an estimated diameter-to-length ratio of 1:4. A negligible part i.e., <1% of the lysosomal volume was occupied by undigested

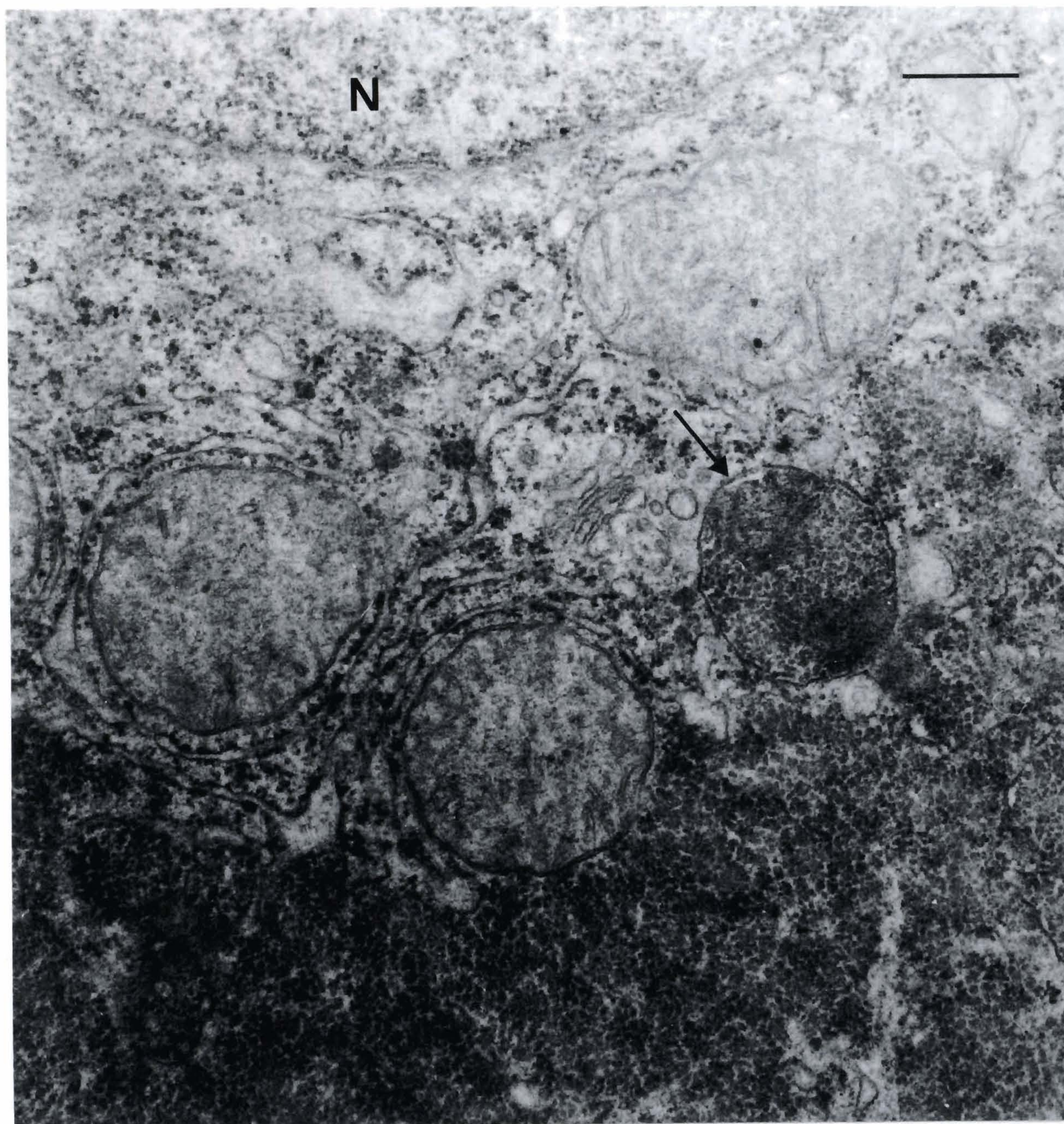

Fig. 3. Portion of glucose-treated hepatocyte at the age of 12 hours. A lysosome (arrow) containing undigested glycogen is present. Abundant glycogen stores are seen in the hyaloplasm (dark areas). N: nucleus. Bar= 0.5 μm.

Table 5. Comparison of hepatocytes from control and glucose-treated newborn rats, 12 hours after birth.

TREATMENT	% OF CYTOPLASMIC VOLUME*			% LYSOSOMAL VOLUME OCCUPIED BY GLYCOGEN
	Glycogen in hyaloplasm	Lysosomes	Glycogen in autophagic vacuoles	
Control**	<0.07	5.83±0.70	<0.04	<1
Glucose***	42.9±5.1 (at birth 48.7±3.0)	0.43±0.15 (at birth 0.30±0.08)	0.26±0.08 (at birth 0.16±0.03)	60 (at birth 53)
P	<0.05	<0.05	<0.05	

*: volumes of cytoplasmic components are means±standard errors; **: results computed from a total of 30 micrographs and an area of 4,960 μm^2; ***: results computed from a total of 30 micrographs and an area of 4,990 μm^2.

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

TREATMENT	% OF CYTOPLASMIC VOLUME*			% LYSOSOMAL VOLUME OCCUPIED BY GLYCOGEN
	Glycogen in hyaloplasm	Lysosomes	Glycogen in autophagic vacuoles	
Control**	15.1±3.2	1.38±0.12	0.24±0.05	17
Cyclic AMP***	8.9±1.3	2.00±0.18	0.12±0.03	6
P	<0.05	<0.05	<0.05	

*: volumes of cytoplasmic components are means±standard errors; **: results computed from a total of 30 micrographs and an area of 5,200 μm^2; ***: results computed from a total of 30 micrographs and an area of 5,150 μm^2.

glycogen. The Golgi apparatus was well developed. The mean volume of cytoplasm/control hepatic cell was 4420 μm^3 (Table 5, Fig. 2). In the glucose-treated animals the hyaloplasmic glycogen stores were preserved. The lysosomes were few, small and round and their total fractional volume was 0.43±0.15. The estimated diameter-to-length ratio of lysosomes was 1:1. A large part i.e., 60% of the lysosomal volume was occupied by undigested glycogen. The Golgi apparatus retained the compact form. The mean volume of cytoplasm/treated hepatic cell was 5450 μm^3. No appreciable difference in the nuclear size and shape between control and treated animals was noted (Table 5, Fig. 3).

Group II. Cyclic AMP-administered

The control animals at the age of 6 hours in no respect differed from the normal animals of the same age. Glycogen stores were reduced. Many lysosomes appeared and their total fractional volume was 1.38±0.12. Most of them belonged to the autophagic vacuole type. The shape of lysosomes was nearly spherical and the estimated diameter-to-length ratio was 1:1.4. A moderate part i.e., 17% of the total lysosomal volume was occupied by undigested glycogen. The Golgi apparatus was developed. The mean volume of cytoplasm/control hepatic cell was 4810 μm^3 (Table 6). In the cyclic AMP-treated animals glycogen stores were markedly reduced. Lysosomes were numerous and their total fractional volume was increased i.e., 2.00±0.18. Most of them belonged to the autophagic vacuole type.

The shape of lysosomes was rather oval and the estimated diameter-to-length ratio was 1:3. A small part i.e., 6% of the total lysosomal volume was occupied by undigested glycogen. The Golgi apparatus was very well developed. The mean volume of cytoplasm/treated hepatic cell was 4600 μm^3. No appreciable difference in the nuclear size and shape between control and treated animals was noted (Table 6). Preliminary studies using the disector method for estimating fractional number of organelles showed similar results.

Group III. Caffeine-administered

Preliminary observations showed that caffeine administration resulted in changes similar to those of cyclic AMP.

Discussion

During the first twelve hours after birth there is a mobilization of the hyaloplasmic and lysosomal glycogen in liver. This represents a metabolic adaptation to the change from intrauterine to extrauterine life. The lysosomal pathway for glycogen degradation is important in cases where there is a demand for the massive liberation of free glucose (Dawkins, 1963; Rosenfeld, 1964; Kotoulas, 1986). The regulation of the breakdown of glycogen inside lysosomes is rather obscure. The glycogen-hydrolyzing activity of the lysosomal enzyme acid alpha 1,4 glucosidase may control this process (Kotoulas, 1986; Kotoulas et al., 1991). In previous studies the hypothesis was formulated that glucagon secreted after birth was the natural stimulus for the postnatal increase in acid glucosidase activity and mobilization of the lysosomal glycogen in rat hepatocytes (Kotoulas and Phillisp, 1971; Kotoulas, 1984, 1988). This is supported by our observations that postnatal development of lysosomes, increase in glucosidase activity and accelerated breakdown of the

lysosomal glycogen were subsequent to the postnatal hypoglycemia. Normal postnatal hypoglycemia is known to release glucagon (Dawkins, 1963). Parenteral glucose administration abolished hypoglycemia and prevented these changes. In this case, the lysosomes remained small and filled with undegraded glycogen. In the glucose-treated newborns, liver cylic AMP was found to be low. This is in accordance with the fact that glucagon action is mediated by cyclic AMP. Similar changes are observed after the administration of insulin. This hormone acts by counteracting the effect of glucagon to elevate cAMP (Pilkis et al., 1986; Maintas et al., 1993; Molero et al., 1994).

The administration of exogenous cyclic AMP resulted in elevated nucleotide concentration in the liver. This was accompanied by increased lysosomal volume, increased acid glucosidase activity and accelerated breakdown of lysosomal glycogen in the hepatocytes. Caffeine, which potentiates the effects of cyclic AMP, produced similar results (Northrop and Parks, 1964). Also, a positive correlation was found to exist between liver cyclic AMP and glucosidase activity in normal animals. 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 activity of the enzyme. The postnatal rise in this activity and the breakdown of glycogen inside lysosomes were found to be dependent on protein synthesis (Kotoulas, 1988). Also, cyclic AMP could influence the mechanism by which the acid hydrolase dephosphorylation competence of lysosomes is regulated (Einstein and Gabel, 1991; Glickman and Kornfeld, 1993; Meresse and Hoflack, 1993). The findings of this and previous studies suggest that the sequence of events after birth may be as follows. The normal postnatal fall in blood glucose produces a release of glucagon. This elevates the liver cyclic AMP concentration which increases the acid glucosidase activity. This change stimulates the breakdown of glycogen inside lysosomes (Dawkins, 1963; Exton and Park, 1968; Shelburne et al., 1973; Kotoulas, 1986).

The change in the shape of lysosomes observed in the cyclic AMP-treated animals cannot be explained by the data of this study. This phenomenon may be related to the formation of a lysosomal compartment analogous to the extended tubular compartment of the degradative pathway described in other cells (Swanson et al., 1987; Klausner et al., 1992; Rabinowitz et al., 1992).

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Accepted June 22, 1994