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Studies on the breakdown of glycogen in the lysosomes: The effects of hydrocortisone

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Summary. The effects of hydrocortisone on newborn rat liver were studied by using biochemical assays, electron microscopy and quantitative morphometry. Hydrocortisone increased the number of lysosomes in the hepatocytes. Most of the lysosomes represented glycogen-containing autophagic vacuoles. The glucocorticoid also increased the activity of the liver glycogen-hydrolyzing acid glucosidase and the breakdown of glycogen inside lysosomes. The activity of the liver acid mannose 6-phosphatase was decreased. This may be related to the stimulation of autophagic mechanisms in the newborn rat hepatocytes.

Key words: Hydrocortisone, Lysosomes, Glycogen, Lysosomal enzymes, Autophagy

Introduction

The breakdown of glycogen in the lysosomes of newborn rat hepatocytes is under hormonal control. Adrenalin, glucagon and insulin regulate this process through alterations in the tissue levels of cyclic AMP. These alterations modify the lysosomal glycogen-hydrolysing activity of glucosidase. The action of cyclic AMP could be mediated by cyclic AMP-dependent protein kinase (Rosenfeld, 1964; Kotoulas and Phillips, 1971; Kotoulas et al., 1971; Kotoulas, 1986; Skoglund et al., 1987; Montminy, 1997; Kalamidas and Kotoulas, 1999).

Very little is known about the role of glucocorticoids in the lysosomal glycogen degradation. Cortisone increases the number of lysosomes in the hepatocytes and the activities of acid α-1,4- and α-1,6-glucosidase in liver. This glucocorticoid also stabilizes the lysosomal membranes and prevents the intracytoplasmic lysosomal migration induced by various agents e.g. estrogen (Rosenfeld and Sayenko, 1963; Wiener et al., 1968; Bourne et al., 1971, 1973; Szego, 1972; Rosenfeld, 1975). In this paper the effects of hydrocortisone administration on newborn rat hepatocytes and liver glycogen-hydrolyzing activity of acid glucosidase, were studied. Since mannose 6-phosphate dcphosphorylation competence of lysosomes may be important for the regulation of the lysosomal function (Einstein and Gabel, 1991; Kalamidas et al., 1994; Hille-Rehfeld, 1995; Mukherjee et al., 1997), the activity of the liver enzyme acid mannose 6-phosphatase was also determined.

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Materials and methods

Chemicals

Hydrocortisone sodium succinate (Solu-Cortef), Lot 13-0834-01 was obtained from Upjohn Co. Actinomycin D, Lot 62C-34000; DL-Ethionine, Lot 116B-0320; Glycogen, Lot 126F-3846; D-Mannose 6-phosphate disodium salt, Cat. No. M-6876 and the reagents for determining glucose and inorganic phosphorus were obtained from Sigma. Reagents for electron microscopy were obtained as before (Kalamidas et al., 1994).

Experimental design

Newborn rats were obtained from pregnant Wistar females. Five pregnant females were used and the average litter contained ten newborns. The newborn animals weighed 5.91±0.08 g (Mean ± standard error). The sex of the newborns was not determined. After delivery, the newborns were kept in an incubator at 36 °C and decapitated at the age of 6 hours. Immediately before sacrificing, blood was obtained from the cervical veins. After decapitation, the liver was excised and part of the right lobe was processed for electron microscopy. The rest of the liver was used for enzyme assays.

Four hydrocortisone-treated animals and four controls from the same litters were killed at the age of 6 hours. Hydrocortisone was administered subcutaneously in 0.1 ml of a 1.2% solution of the compound in 0.9% NaCl. The animals were injected at birth (a dose of 200 mg/kg). Equal number of controls were injected with 0.9% NaCl. Four hydrocortisone-treated animals were also injected intraperitoneally with actinomycin in 0.1

ml of a solution prepared according to Dallner et al. (1966). The animals were injected with actinomycin at birth and 3 hours after birth (a dose of 0.8 mg/kg each time). Controls were injected with carrier only (Kotoulas, 1988). The animals were killed at the age of 6 hours. Four hydrocortisone-treated animals, were also injected intraperitoneally with ethionine in 0.1 ml of a 0.6% solution of the compound in 0.9% NaCl. The animals were injected with ethionine at birth (a dose of 100 mg/kg). Controls were injected with carrier only. The animals were killed at the age of 6 hours.

For the quantitative morphometric study on electron micrographs, three hydrocortisone-treated animals and an equal number of their controls were killed at the age of 6 hours.

Biochemical methods

The glycogen-hydrolyzing activity of acid glucosidase (acid α-1,4-exopolyglucosidase, acid amyloglucosidase) was assayed in homogenates of liver tissue (Rosenfeld, 1964; Lundquist, 1985, 1986). The tissue was homogenized in a glass homogenizer and diluted 1:10 with ice cold distilled water. Usually, 200 μl of homogenate were used for the assay. The total enzyme activity was determined according to Hers (1963) and Lejeune et al. (1963) with 1% glycogen as substrate. Incubation was carried out for 60 min in 0.1M sodium acetate buffer with 0.05% Triton X-100 at pH 4.7 and 37 °C. The reaction was terminated by the addition of barium hydroxide and zinc sulphate and the reaction mixture was deproteinized according to Somogyi (1945). Glucose was estimated by the method of Raabo and Terkildsen (1960), using glucose oxidase, peroxidase and o-dianisidine. Protein in the liver was determined by the method of Lowry et al. (1951). Enzyme activity was expressed as micromoles of glucose formed per hour per mg of protein. The acid mannose 6-phosphatase activity was determined in the same homogenates, essentially according to Arion and Nordlie (1964) and Nordlie and Arion (1964), with 7mM mannose 6-phosphate as substrate. Incubation was carried out for 60 min in 0.1M sodium acetate buffer with 0.05% Triton X-100, at pH 5.2 and 37 °C (Einstein and Gabel, 1991). Inorganic phosphorus was measured by the method of Fiske and Subbarow (1925). Enzyme activity was expressed as micromoles of inorganic phosphorus produced per hour per mg of protein. Glycogen was estimated as before (Kotoulas and Phillips, 1971). 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 graded series of ethanol solutions, 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 Reynolds'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; Kalamidas et al., 1994). 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 x41600. The data of the micrographs from the same block were combined and therefore the mean and standard errors were calculated from these combined data (Weibel, 1969). The fractional number and volume of organelles were estimated as described by Loud (1968). The mean tangent diameter of the organelle required for the estimation of fractional number was found from the volume-to-surface ratio as described before (Kotoulas et al., 1971; Kalamidas et al., 1994). The diameter-to-length ratio (ε) of organelles was determined by using a graph of the shape coefficient (ß) with respect to the axial ratio ε. Lysosomes were assimilated to the ellipsoids (Weibel and Gomez, 1962). The value of this coefficient, ß, was derived from the formula of Knight, Weibel and Gomez and the estimates of fractional volume, fractional number and number of transsections per unit area of tissue section. The size distribution coefficient, k, was taken as 1.07 (Weibel, 1969, 1979). The formfactor (Luers et al., 1993), was determined using the estimates of lysosomal contour length and lysosomal profile area. The number of intersections of lysosomal membrane profile with the test line, required for the estimation of contour length, was found according to Weibel (1969) and Kotoulas et al. (1971). The volume of cytoplasm in μm^3/hepatic cell was determined from light photographs (one photograph from each of five animals of each group) as described before (Kotoulas et al., 1971; Kalamidas et al., 1994). The results of morphometric analysis were evaluated by Student's t-test (Hill, 1967).

Results

Biochemical results

Blood glucose, liver glycogen and liver acid glucosidase activity in the hydrocortisone-treated animals and their controls, are shown in Table 1. The treated animals were markedly hyperglycemic. No statistically significant change in the liver glycogen was noted. The activity of acid glucosidase was significantly increased in the treated animals. Actinomycin or

Table 1. Blood glucose, liver glycogen and liver acid glucosidase after hydrocortisone treatment of newborn rats. Results are mean ± standard deviations. Each value includes 4 observations.

	BLOOD GLUCOSE (mg/100 ml)	LIVER GLYCOGEN (mg/mg protein)	LIVER ACID GLUCOSIDASE (μmoles glucose/hr/mg protein)
Control	49.0±15.2	0.315±0.093	0.202±0.050
Hydrocortisone	203.6±54.0	0.288±0.090	0.379±0.084
Hydrocortisone + actinomycin	132.0±43.6	0.317±0.100	0.078±0.038
Hydrocortisone + ethionine	104.3±38.2	0.325±0.100	0.109±0.040
p	< 0.05	> 0.05	< 0.05

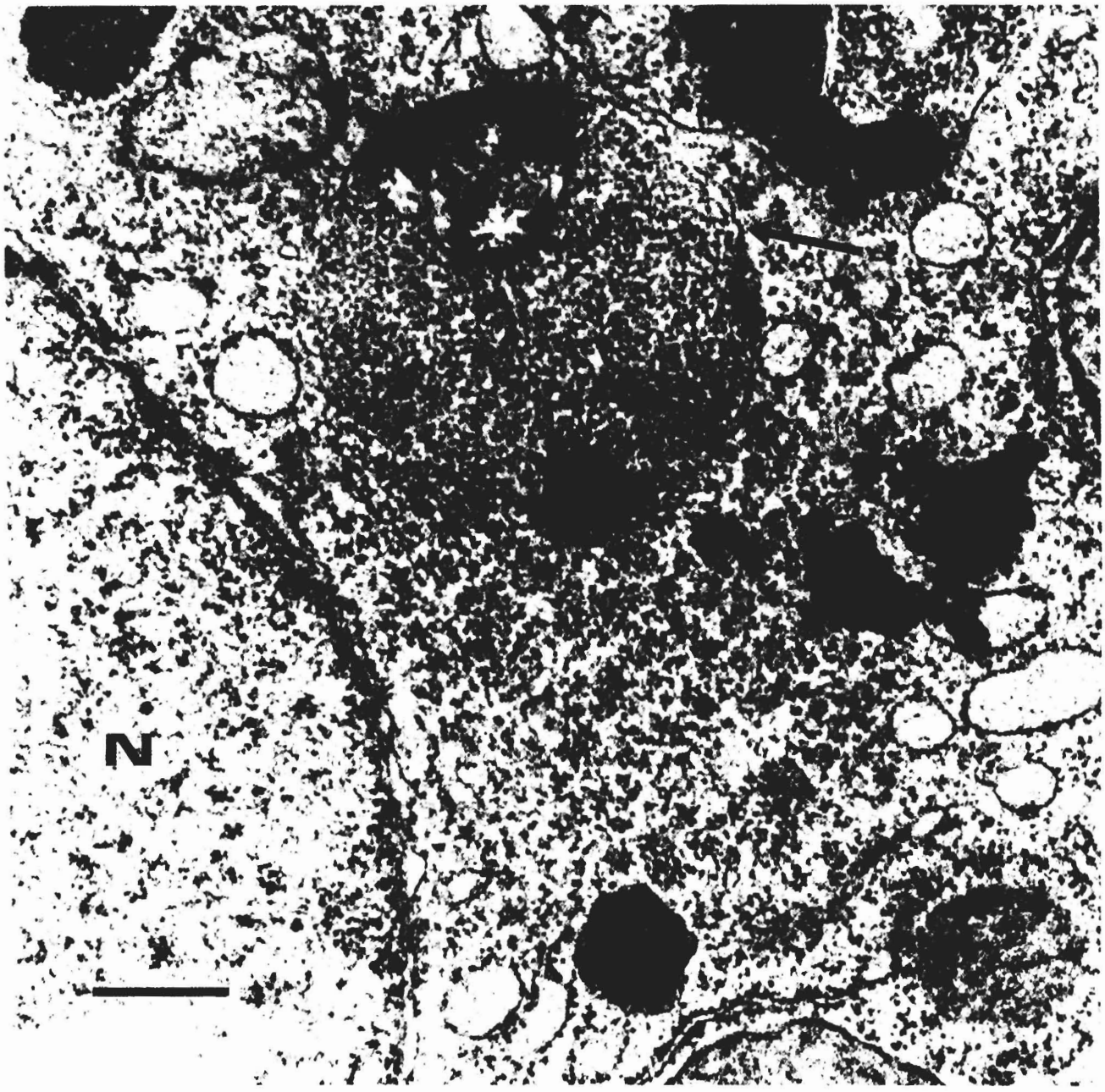

Fig. 1. Portion of a control rat hepatocyte at the age of 6 hours. A lysosome-autophagic vacuole (arrow), including undigested glycogen is seen. Moderate glycogen stores are seen in the hyaloplasm (dark particles). N: nucleus. Bar: 0.5 μm.

ethionine given simultaneously with hydrocortisone, resulted in a reduction of the glucocorticoid-induced hyperglycemia. Blood glucose level was not so high. The activity of acid glucosidase was low. No appreciable change in the liver glycogen was noted.

The liver acid mannose 6-phosphatase activity was lower in four hydrocortisone-treated animals (8.3±1.6 μmoles P_i/hr/mg protein) than in four controls (12.7±2.1 μmoles P_i/hr/mg protein). The difference was statistically significant ($p<0.05$).

Morphological results

Both qualitative and quantitative changes were

Table 2. Comparison of hepatocytes from control and hydrocortisone-treated newborn rats.

TREATMENT	NUMBER OF LYSOSOMES	VOLUME OF LYSOSOMES	VOLUME OF LYSOSOMAL GLYCOGEN	% LYSOSOMAL VOLUME OCCUPIED BY GLYCOGEN
Control *	12.00 ± 2.20	1.15 ± 0.16	0.29 ± 0.04	25
Hydrocortisone**	18.70 ± 3.17	1.04 ± 0.16	0.17 ± 0.03	16
p	<0.05	>0.05	< 0.05	

Number of lysosomes is expressed as organelles/100 μm^3 of cytoplasm. Volume of lysosomes and lysosomal glycogen is expressed as μm^3/100 μm^3 of cytoplasm. Values are mean±standard errors. *: results computed from a total of 30 micrographs and a cytoplasmic area of 2120 μm^2. **: results computed from a total of 30 micrographs and a cytoplasmic area of 1770 μm^2.

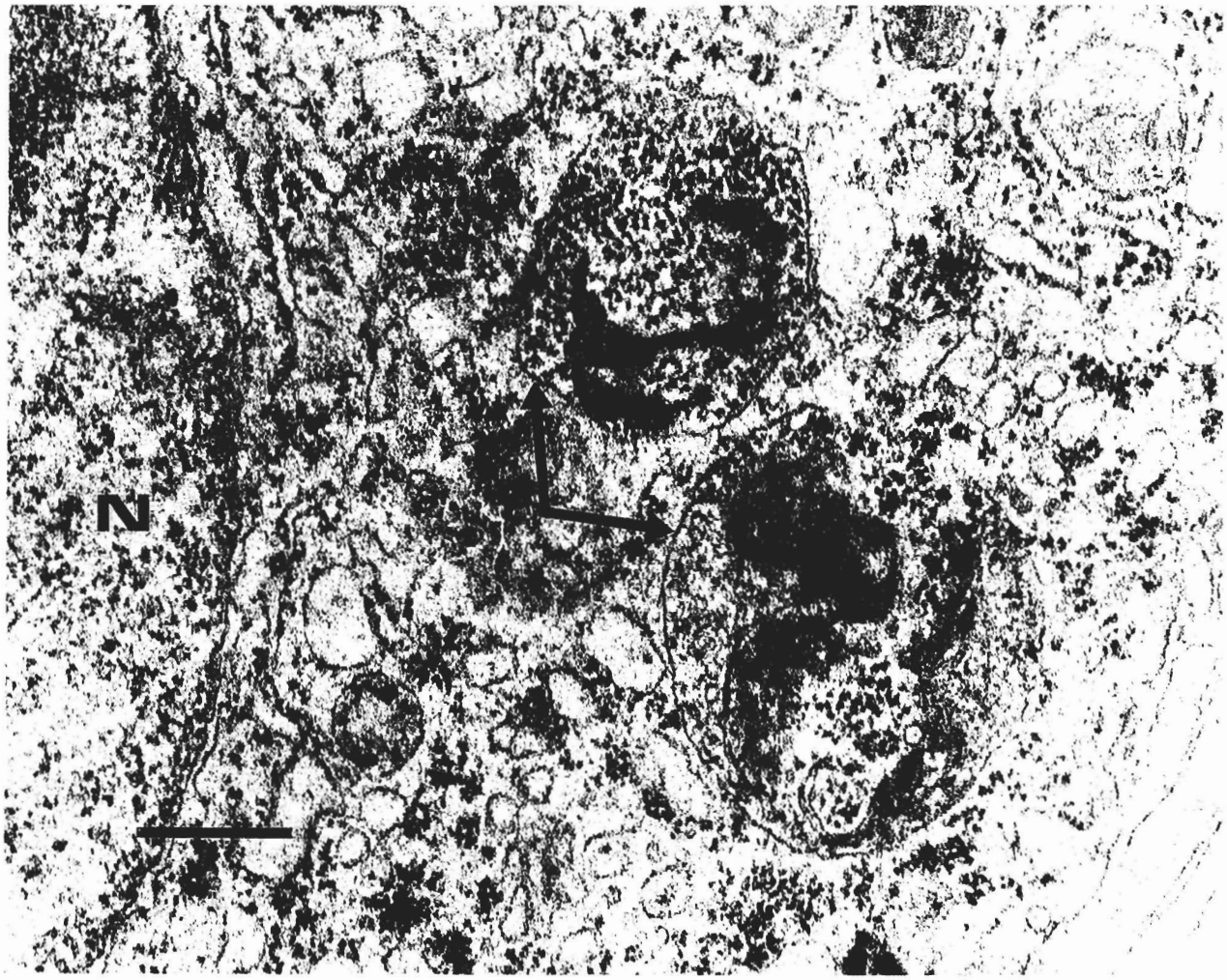

Fig. 2. Portion of a hydrocortisone-treated rat hepatocyte at the age of 6 hours. Two lysosomes-autophagic vacuoles (arrows) including relatively small quantities of glycogen are seen. Some glycogen stores are seen in the hyaloplasm (dark particles). N: nucleus. Bar: 0.5 μm.

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 at the age of 6 hours, has been described before (Kotoulas and Phillips, 1971; Kalamidas et al., 1994). Controls differed in no respect from normal animals. Moderate amounts of hyaloplasmic glycogen were present. Many large lysosomes appeared and most of them belonged to the autophagic type. Autophagic vacuoles occupied at least 85% of the total lysosomal volume. They were usually located at the margins of the hyaloplasmic glycogen areas. Few lysosomes had the appearance of residual bodies. The estimated diameter-to-length ratio (ε) of lysosomes was 1:1.7. Lysosomes contained moderate amounts of glycogen which occupied 25% of their total volume (Fig. 1, Table 2). The mean volume of cytoplasm per control hepatic cell was 4950 μm^3.

In the hydrocortisone-treated animals the hyaloplasmic glycogen showed no appreciable change. The number of lysosomes was increased. No increase in the total lysosomal volume was found. Most of the lysosomes belonged to the autophagic type. Autophagic vacuoles occupied at least 85% of the total lysosomal volume. They were usually located at the margins of hyaloplasmic glycogen areas. The estimated diameter-to-length ratio (ε) of lysosomes was 1:1.2. In preliminary studies, the formfactor determination (Luers et al., 1993) showed that the lysosomal profiles in the hydrocortisone-treated animals had a smaller deviation from the ideal circle than the controls. Lysosomes usually contained some glycogen which occupied only 16% of their total volume (Fig. 2, Table 2). The mean volume of cytoplasm per treated hepatic cell was 5150 μm^3.

Discussion

The results of this and previous studies by others show that hydrocortisone increases the activity of the glycogen-hydrolyzing acid glucosidase and enhances the breakdown of lysosomal glycogen (Bourne et al., 1971; Rosenfeld, 1975). This apparently represents a stimulation of a glycogen autophagy mechanism, induced by the glucocorticoid. Autophagy was found to be stimulated by gluconeogenesis-inducing agents (Ashford and Porter, 1962; Rosa, 1971). No significant change in the hepatic cell glycogen was noted in our newborn animals. This may be explained by the concurrent enhancement of gluconeogenesis and hyaloplasmic glycogen synthesis induced by the glucocorticoid (Wiener et al., 1968).

The mechanism by which the activity of acid glucosidase is increased, is unknown at present. Glucocorticoids modulate an insulin-sensitive α_1-adrenergic pathway in hepatocytes. Adrenalin which is secreted after birth, increases while insulin decreases the activity of acid glucosidase (Rosenfeld, 1964; Kotoulas, 1981; Garcia-Sainz and Hernandez-Sotomayor, 1985). Moreover, glucocorticoids reduce insulin binding to the hepatocytes (Olefsky et al., 1975; Kahn et al., 1978).

The experiments with protein synthesis inhibitors given simultaneously with hydrocortisone, suggested that the increase in glucosidase activity could be dependent on protein synthesis. These inhibitors decreased the activity of glucosidase and reduced the hydrocortisone-induced hyperglycemia. They had no appreciable effect on the hepatic glycogen. This may be due to the concurrent depression of gluconeogenesis and deposition of hepatic glycogen (Ashmore and Weber, 1968; Kotoulas, 1988; Hanson, 1997).

In the hydrocortisone-treated animals the number of lysosomes of the hepatocytes was increased. However, no increase in the total volume of these organelles could be detected. The lysosomes were not large and oval as in the control animals. These phenomena could not be explained by the results of this study (Swanson et al., 1987; Griffiths et al., 1990b, Rabinowitz et al., 1992; Araki et al., 1993; Jahraus et al., 1994; Kalamidas et al., 1994). Glucocorticoids are known to stabilize lysosomal membranes and prevent intracytoplasmic lysosomal migration. Thus, it is possible that hydrocortisone inhibits the normal fusion of lysosomes in these newborn animals (Wiener et al., 1968; Szego, 1972; Lang et al., 1998).

The inhibition of acid mannose 6-phosphatase activity produced by hydrocortisone is difficult to interpret. Cells may modify their lysosomal mannose 6-phosphate dephosphorylation competence and regulate lysosomal function (Griffiths et al., 1988, 1990a; Kornfeld and Mellman, 1989; Baron et al., 1990; Einstein and Gabel, 1991; Rabinowitz et al., 1992; Tjelle et al., 1996). The cation-independent mannose 6-phosphate receptor may operate in the formation of lysosomes efficient in acid mannose 6-phosphatase activity. The cation-dependent mannose 6-phosphate receptor may operate in the opposite direction and favor lysosomes deficient in this enzyme activity (Gabel et al., 1983; Einstein and Gabel, 1991). It may be that the hydrocortisone-induced stimulation of cellular glycogen degradation by autophagy in the newborn rat hepatocytes, is associated with the latter, phosphatase-deficient lysosomes. Phosphorylation-dephosphorylation mechanisms may play an important role in controlling autophagy (Seglen et al., 1990; Chou et al., 1994; Blommaart et al., 1997; Claus et al., 1998; Scott and Klionski 1998). This view is further supported by the results of certain preliminary experiments. Thus, the administration of the autophagy-inducing cyclic AMP (Kotoulas, 1986), resulted in decreased activity of the liver acid mannose 6-phosphatase (cyclic AMP, 7.2±1.1 μmoles Pi/hr/mg protein; controls, 10.7±2.0 μmoles P_i/hr/mg protein) while the administration of the autophagy-inhibiting propranolol (Kotoulas et al., 1991) which antagonizes the cyclic AMP, had the opposite effect, increasing the activity of this enzyme (propranolol, 14.8±2.3 μmoles P_i/ hr/ mg protein; controls, 11.6±2.1 μmoles P_i/hr/mg protein). Further experimentation is required to determine the biological

significance of these findings.

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