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# A quantitative description of the insulin-induced ultrastructural changes in newborn rat hepatocytes

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**Summary.** The effects of insulin on the ultrastructure of newborn rat hepatocytes were systematically quantified at satisfactory statistical significance. Insulin prevented the normal postnatal increase in the total volume of lysosomes and the breakdown of glycogen inside these organelles. The lysosomal glycogen-hydrolysing enzyme, acid alpha 1,4 glucosidase was inhibited by the hormone. Insulin also prevented the normal postnatal increase in the total volume of peroxisomes, especially of the crystalloid core-devoid type. The hormone produced an increase in the area of cell membrane, due to the formation of many irregular folds of the cell surface. These results constitute good evidence for participation of lysosomes and peroxisomes in the overall glycogen degradation and or gluconeogenesis in the newborn rat hepatocytes.

**Key words:** Insulin, Ultrastructure of hepatocyte, Newborn rat

## Introduction

Carbohydrate metabolism is of particular importance to newborns. Hormones, such as insulin, glucagon or adrenalin exert an important role in the overall regulation of carbohydrate metabolism. Especially, insulin integrates hepatic metabolic function by affecting several metabolic processes including glycogen degradation (Dawes and Shelley, 1968).

Previous morphological studies showed that the administration of insulin in newborn rats induced definite changes in the ultrastructure of hepatocytes (Kotoulas et al., 1971; Kotoulas, 1981). However, no quantification of these findings has been attempted so far at satisfactory statistical significance. In this paper a systematic quantitative description of the insulin-induced ultrastructural changes in newborn rat hepatocytes is presented.

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## Materials and methods

### *Animals and handling of tissues*

Newborn rats were obtained from pregnant Wistar females. Five pregnant females were used and the average litter contained ten newborns. The newborns weighed 5.96 g ± 0.07. The weight is expressed as mean ± standard error. The sex of the newborn animals was not determined. After delivery the newborns were kept in an incubator at 36 °C. The animals were decapitated at birth and at 3 and 4 hours. No appreciable change in the weight of the animals at 4 hours was noted. Immediately before sacrificing, blood was obtained from the cervical veins for determining blood glucose. After decapitation, the liver was excised and processed for electron microscopy. Part of the liver was used for biochemical determinations.

### *Chemicals*

Crystalline zinc insulin solution (40 units/ml) was obtained from NOVO. For electron microscopy, all reagents, except araldite, were from Fisher Scientific Co. Araldite 502 was obtained from Ciba Co. Ltd. Glycogen, lot 87B-0020 and the reagents for deproteinizing blood and determining glucose were from Sigma Chemical Co.

### *Electron microscopy*

Liver tissue was fixed for 1 hour at 0 °C in 1% osmium tetroxide buffered with 0.1M phosphate buffer (pH 7.2). The tissue was dehydrated in a graded series of ethanol solutions, transferred to propylene oxide and then to a mixture of propylene oxide and resin. The embedding medium was prepared according to Mollenhauer (1964). Sections from the embedded tissue were cut with glass knives using an LKB microtome. Sections 1 μm thick were stained with toluidine blue and examined by light microscopy (Trump et al., 1961). Ultrathin sections of grey interference colour were placed 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). The sections were examined in a Philips 300 and partly in a Philips 75 electron microscope.

### *Morphometric analysis*

Morphometric analysis was performed on electron micrographs (Kotoulas and Phillips, 1971; Kotoulas et al., 1971). For this purpose, six normal animals killed at birth and six insulin-treated animals and an equal number of their controls killed after 4 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) was superimposed on the prints. The cross points of the lines of the lattice were used for planimetric work. The points enclosed within the area of a cytoplasmic component were counted and the fractional volume of this component was estimated according to the principles of stereology. The results were expressed as percentages 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. The horizontal lines of the lattice were used for measurement of the surface area of membranes. The intersections of the limiting membrane of a cell component with these lines were counted and the surface area of the membrane per unit of cytoplasmic volume was estimated (Weibel, 1969). The number of organelles per unit of cytoplsmic 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).

The volume of cytoplasm in $\mu m^3$/hepatic cell was determined from light micrographs taken from 1 µm-thick sections stained with toluidine blue and enlarged to a final magnification of 1,000. Firstly, the volume of hepatic cell cytoplasm/unit of volume of liver tissue was estimated. Secondly, the number of hepatic cell nuclei/unit of volume of liver tissue was estimated by the method reported 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.

### *Biochemical methods*

The acid alpha 1,4 glucosidase activity assay was performed on homogenates of liver tissue in a 10% dilution with distilled water. The determination of total activity of the enzyme was performed according to Hers (1963) and Lejeune et al. (1963) with glycogen as substrate (Kotoulas et al., 1991). The activity was expressed as µM glucose released per hour per mg protein. Glucose was estimated by the method of Raabo and Terkildsen, modified according to Sigma Technical Bulletin No. 510, using glucose oxidase, peroxidase and dianisidine. Glycogen and protein in the liver were determined as before (Kotoulas and Phillips, 1971). The blood was deproteinized by the method of Somogyi (1945).

### *Experimental design*

Six normal animals were killed at birth. Six insulin-treated animals and six controls from the same litters were killed after 4 hours. For the biochemical determinations six insulin-treated animals and six controls were also sacrificed at 3 hours. Insulin was administered subcutaneously in 0.1 ml of the original solution (40 units/ml). The treated animals were injected at 0 and 3 hours after birth (a dose of 666.7 units/kg each time). Control animals were injected with distilled water.

## Results

### *Biochemical results*

A preliminary experiment showed that a single insulin injection at birth did not significantly inhibit the mobilization of liver glycogen. The concentration of glycogen at 4 hours in the three insulin-treated animals; i.e., 0.381 ± 0.133 mg/mg protein, was not significantly higher than in the three controls; i.e., 0.258 ± 0.126 mg/mg protein. The results were expressed as means ± standard deviations ($p > 0.05$). The treated animals were hypoglycemic. They showed a significantly lower blood glucose level, i.e., 33.0 ± 11.2 mg/100 ml blood, compared to that of the controls; i.e., 52.3 ± 17.1 mg/100 ml blood. The results were expressed as means ± standard deviations ($p < 0.05$).

The inhibition of the postnatal mobilization of glycogen by two injections of insulin, at 0 and 3 hours, was studied in a more definitive experiment. The results of this experiment indicated that after the administration of the hormone, the concentration of glycogen 4 hours after birth did not decrease to the level of the controls; i.e. 0.246 ± 0.121 mg/mg protein, but remained significantly higher; i.e., 0.537 ± 0.181 mg/mg protein. The insulin-treated animals were deeply hypoglycemic

**Table 1.** Blood glucose and liver glycogen in control and insulin-treated rats.

| | BLOOD GLUCOSE (mg/100 ml blood) | | | LIVER GLYCOGEN (mg/mg of protein) | | |
|---|---|---|---|---|---|---|
| HOURS AFTER BIRTH | 0 | 3 | 4 | 0 | 3 | 4 |
| CONTROL | 54.1±13.6 | 39.6±12.2 | 52.3±13.1 | 0.739±0.206 | 0.461±0.157 | 0.246±0.121 |
| INSULIN | | 26±10.0* | <12.5** | | 0.541±0.176* | 0.537±0.181** |
| P | | <0.05 | <0.05 | | >0.05 | **<0.05** |

Results are means ± standard deviations. Number of observations= 6. *: only one injection of insulin was given, at birth; **: two injections of insulin were given, at birth, and at 3 hours.

**Table 2.** Acid alpha 1,4 glucosidase activity in control and insulin-treated rats (μM/h/mg of protein).

| HOURS AFTER BIRTH | 0 | 3 | 4 |
|---|---|---|---|
| CONTROL | 0.085±0.048 | 0.117±0.056 | 0.236±0.087 |
| INSULIN | | 0.093±0.050* | 0.090±0.050** |
| P | | >0.05 | <0.05 |

Results are means ± standard deviations. Number of observations= 6. *: only one injection of insulin was given, at birth; **: two injections of insulin were given, at birth, and at 3 hours.

**Table 3.** Components of rat hepatocyte at birth (% of cytoplasmic volume).

| Glycogen in hyaloplasm | Lysosomes | Glycogen in lysosomes | Mitochondria | Peroxisomes | Gogi complex |
|---|---|---|---|---|---|
| 50.0±3.9 | 0.30±0.07 | 0.13±0.02 | 9.53±0.80 | 0.42±0.05 | 0.16±0.04 |

Volumes are means ± standard errors. The results were computed from a total of 60 micrographs and an area of 9,900 $\mu m^2$

and their blood glucose level at 4 hours was less than 12.5 mg/100 ml blood while the level of controls was 52.3 ± 13.1 mg/ml blood. The treated animals were hypoglycemic also at 3 hours after the first injection at birth (Table 1).

The glycogen-hydrolyzing activity of the enzyme acid alpha 1,4 glucosidase was determined in the insulin-treated animals and their controls. The results showed that the administration of insulin resulted in an inhibition of the normal postnatal increase in the activity of enzyme. This activity remained low; i.e., 0.090 ± 0.050 μM glucose/h/mg protein and did not reach the level of the controls; i.e., 0.236 ± 0.087 mM glucose/h/mg protein at 4 hours after birth (Table 2).

### *Morphological results*

Quantitative and qualitative changes of the cell components were studied. The term hyaloplasm refers to the part of the cytoplasm which is devoid of organelles. The term lysosomes includes lysosomes and related particles from autophagic vacuoles to residual bodies, unless otherwise specified (DeDuve and Wattiaux, 1966; Rabinowitz et al., 1992). The elements of the rough endoplasmic reticulum described as vesicles include the ribosome-coated and sparsely coated (mixed) vesicles (Phillips et al., 1967; Kotoulas et al., 1971).

At birth, vast stores of hyaloplasmic glycogen were present and the cell organelles were crowded in small glycogen-free areas. Lysosomes, including autophagic vacuoles, were small and rare. A large part; i.e, 43% of the total lysosomal volume, was occupied by glycogen. Golgi apparatus was not well developed and only a few zones were found. This organelle had a round, compact form with few vesicles and vacuoles. The content of the apparatus was of low density. Mitochondria were noticeable. Peroxisomes were rather rare. The crystalloid core-devoid peroxisomes were very few. About 20% of the total peroxisomal volume belonged to these organelles. The rough endoplasmic reticulum was mainly in the form of cisternae. The cisternal membrane was densely coated with ribosomes. The vesicles of rough endoplasmic reticulum were not frequent. Most of them were sparsely coated with ribosomes. The surface area of their limiting membrane was small. The smooth endoplasmic reticulum was negligible (Tables 3, 4).

The appearance of the control animals 4 hours after birth differed in no respect from normal animals of the same age (Kotoulas and Phillips, 1971; Kotoulas et al., 1971; Kotoulas, 1981). The stores of hyaloplasmic glycogen were reduced compared to those seen at birth. Many lysosomes appeared and their total volume increased. It was estimated that at least 85% of their total volume was occupied by lysosomes of the autophagic type. Lysosomes usually occurred in groups and were found near the junction of hyaloplasmic glycogen with glycogen-free areas. They were often related to the Golgi apparatus. Lysosomes were sometimes separated from the hyaloplasmic glycogen by a narrow glycogen-free zone. Autophagic vacuoles were well developed and usually limited by a single membrane. They contained some undigested glycogen and other cytoplasmic components as well as amorphous electron-dense material. Most of the engulfed glycogen was of the monoparticulate type. A small part; i.e., 13%, of the total lysosomal volume was occupied by glycogen. Golgi apparatus was more developed than at birth. Many Golgi zones were found dispersed in the cytoplasm with numerous vesicles and vacuoles. The content of the organelle was of a relatively high density. Mitochondria were noticeable. Irregular shapes, such as branched, were very rare (<0.3%). Many peroxisomes appeared and their total volume increased when compared to that seen at birth. The crystalloid core-devoid peroxisomes

were numerous. About 46% of the total perixosomal volume belonged to these organelles. The rough endoplasmic reticulum cisternae were rather scarce. The cisternal membrane was not densely coated with ribosomes. The vesicles were numerous. Most of them were sparsely coated with ribosomes. The surface area of the limiting membrane of vesicles increased when compared to that seen at birth. The smooth endoplasmic reticulum was negligible. The cell surface was rather smooth. A few pits invaginated to form small vesicles. Rare coated vesicles were seen near the cell surface (Figs. 1, 2, Tables 5, 6).

In insulin-treated animals, the stores of hyaloplasmic glycogen remained vast and the cellular organelles were crowded into the glycogen-free areas. A small number of lysosomes was noted. They usually occurred in groups at the margins of the glycogen areas and often in close proximity to the Golgi apparatus. They were sometimes separated from the hyaloplasmic glycogen by a narrow glycogen-free zone. The total lysosomal volume remained low. It was estimated that at least 85% of this volume was occupied by lysosomes of the autophagic type. The autophagic vacuoles were usually small, round and limited by a single membrane. These vacuoles were usually filled with undigested glycogen of the monoparticulate type but sometimes contained other cytoplasmic components or amorphous material. A large part; i.e., 40% of the total lysosomal volume was occupied by glycogen. Golgi apparatus was not well developed. The organelle remained round and compact and only a few zones with some vesicles and vacuoles were seen. The density of the organelle content was low. The total volume of mitochondria showed an increase. Since the estimated number of mitochondria per $\mu m^3$ of cytoplasm did not significantly change (control 0.074 ± 0.015; insulin 0.077 ± 0.010, $p > 0.5$, statistical sampling as in Table 5), the increase in the total mitochondrial volume was apparently due to the presence of enlarged mitochondria. No appreciable change was noted in the

**Table 4.** Components of rat hepatocyte at birth (membrane area in $\mu m^2/\mu m^3$ of cytoplasm).

| RER cisternae | RER vesicles | Cell membrane |
|---|---|---|
| 0.39±0.03 | 0.18±0.03 | 0.37±0.05 |

Areas are means ± standard errors. The results were computed from a total of 60 micrographs and an area of 9,900$\mu m^2$.

**Table 5.** Comparison of hepatocytes from control and insulin-treated newborn rats 4 hours after birth (% of cytoplasmic volume).

| | Glycogen in hyaloplasm | Lysosomes | Glycogen in lysosomes | Mitochondria | Peroxisomes | Golgi complex |
|---|---|---|---|---|---|---|
| CONTROL* | 15.3±2.4 | 1.02±0.20 | 0.13±0.03 | 11.00±1.36 | 0.85±0.20 | 0.43±0.08 |
| INSULIN** | 38.7±2.7 | 0.62±0.12 | 0.25±0.05 | 18.43±2.36 | 0.39±0.05 | 0.26±0.07 |
| P | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 | <0.05 |

Volumes are means ± standard errors. *: results computed from a total of 60 micrographs and an area of 10,.200 $\mu m^2$; **: results computed from a total of 60 micrographs and an area of 8,500$\mu m^2$.

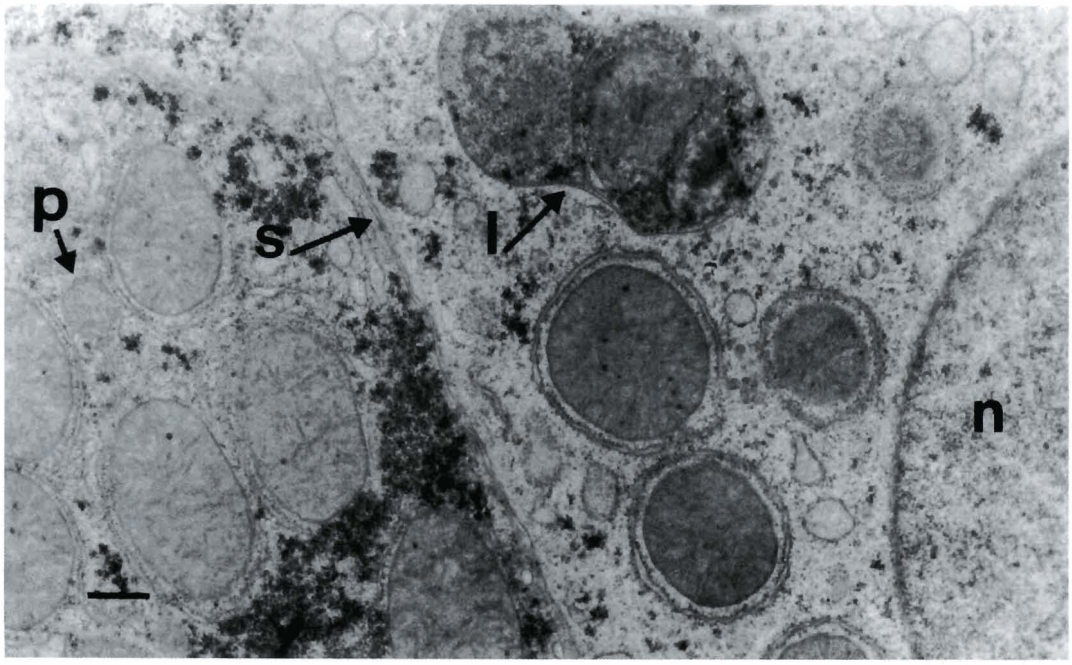


**Fig. 1.** Portions of three control rat hepatocytes 4 hours after birth. Glycogen (dark areas) is seen. A large lysosome-autophagic vacuole (l) is present. p= peroxisome, crystalloid core-devoid type. n= nucleus. s= cell surface. Bar, 0.5 µm.

shape of these organelles. The total volume of peroxisomes showed a 54% decrease. The estimated number of peroxisomes per $\mu m^3$ of cytoplasm decreased to a comparable extent (control 0.131 ± 0.015; insulin 0.065 ± 0.012, $p < 0.05$, statistical sampling as in Table 5). The crystalloid core-devoid peroxisomes were very few. Only 24% of the total peroxisomal volume belonged to these organelles. The rough endoplasmic reticulum remained mainly in the form of cisternae. The membrane of the cisternae was densely coated with ribosomes. The vesicles were not frequent and most of them were sparsely coated with ribosomes. The surface area of the limiting membrane of vesicles was small. The smooth endoplasmic reticulum was negligible. The cell surface profiles between adjoining cells and at the sinusoids were tortuous. Many folds projected from the surface and pits, sometimes coated, invaginated to form small vesicles. Coated vesicles were noticeable near the cell surface. A significant increase in the cell membrane area was noted (Figs. 3, 4, Tables 5, 6).

**Table 6.** Comparison of hepatocytes from control and insulin-treated newborn rats 4 hours after birth (membrane area in $\mu m^2/\mu m^3$ of cytoplasm).

| | RER cisternae | RER vesicles | Cell membrane |
|---|---|---|---|
| CONTROL* | 0.27±0.03 | 0.49±0.04 | 0.46±0.05 |
| INSULIN** | 0.36±0.03 | 0.23±0.03 | 0.76±0.07 |
| P | <0.05 | <0.05 | <0.05 |

Areas are means ± standard errors. *: results computed from a total of 60 micrographs and an area of 10,200$\mu m^2$; **: results computed from a total of 60 micrographs and an area of 8,500$\mu m^2$.

Since some of the data of this work could be explained on the basis of an absolute change in the cytoplasmic volume of hepatocytes of the treated animals, estimations of the cytoplasmic volume/hepatic cell were made by morphometric analysis. For this purpose, one light micrograph from each of five controls (a total of 235 control hepatic cell nuclei) and one light micrograph from each of five treated animals (a total of

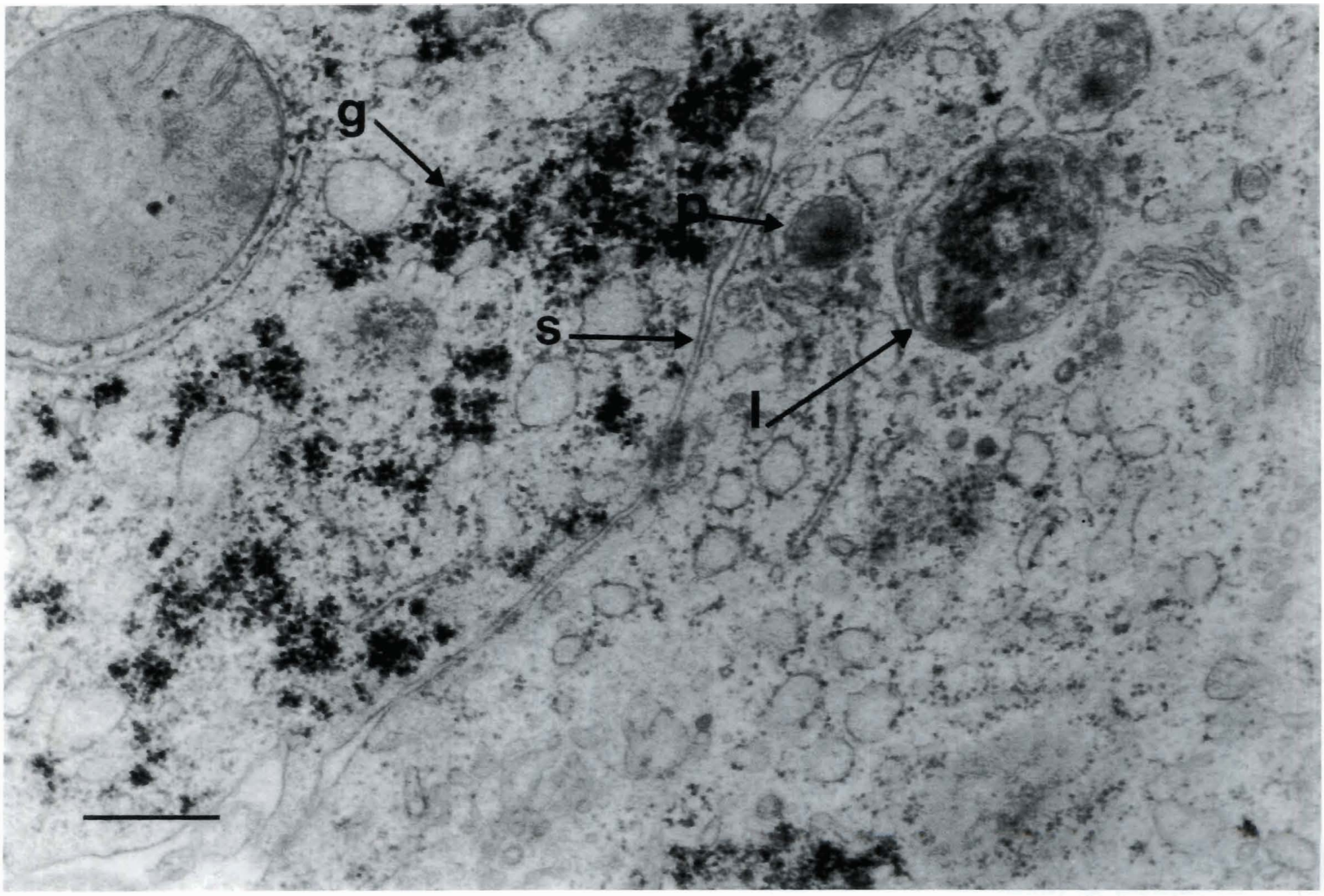


**Fig. 2.** Portions of two control rat hepatocytes 4 hours after birth. Moderate amounts of glycogen (g) are seen in the hyaloplasm. A lysosome-autophagic vacuole (l) containing a negligible amount of glycogen is present. p= peroxisome, crystalloid core-containing type. The surface (s) of both the adjoining cells is smooth. Bar, 0.5 µm.

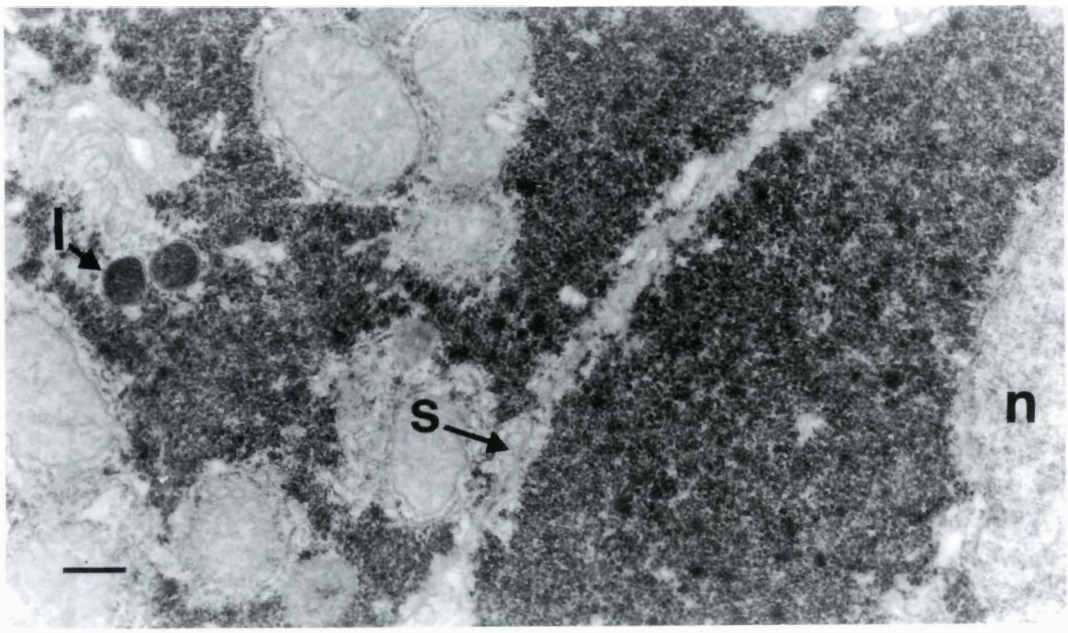


**Fig. 3.** Portions of two insulin-treated rat hepatocytes 4 hours after birth. Abundant glycogen stores (dark areas) are seen. Two small, round lysosome-autophagic vacuoles (l) filled with glycogen are present. The cell surface profiles (s) are tortuous. n= nucleus. Bar, 0.5 μm.

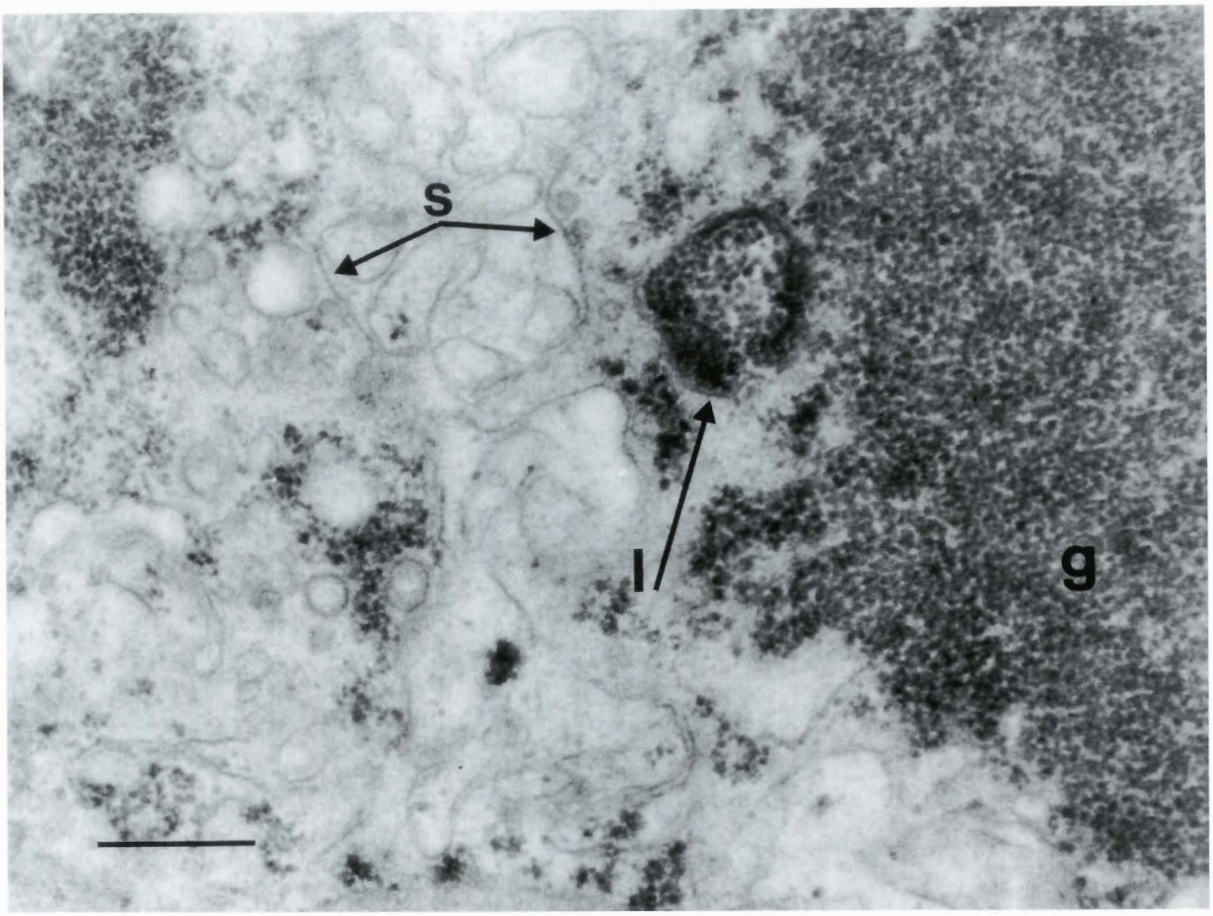


**Fig. 4.** Portions of two insulin-treated rat hepatocytes 4 hours after birth. Large amounts of glycogen (g) are seen in the hyaloplasm. A lysosome-autophagic vacuole (l) filled with undigested glycogen is present. Many folds project from the surface (s) of both the adjoining cells. Pits sometimes coated, invaginate to form small vesicles. Coated vesicles are noticed near the cell surface. Bar 0,5 μm.

208 treated hepatic cell nuclei) were used. The mean volume of cytoplasm/hepatic cell in the control animals was 4,950 $\mu m^3$ while in the insulin-treated animals it was 5,700 $\mu m^3$. This increase in the volume of cytoplasm (approximately 15%) could not explain the larger decrease observed in the fractional volume and membrane area of certain organelles in the treated animals.

## Discussion

The data presented in this and previous studies showed that the normal postnatal mobilization of hyaloplasmic glycogen in the rat hepatocytes was inhibited by the administration of insulin. The inhibition of glycogen mobilization in the hyaloplasm should be explained on the basis that the hormone inhibited the activity of the enzyme phosphorylase. The insulin-treated animals were deeply hypoglycemic. Insulin given alone, without simultaneously-administered glucose, does not promote synthesis of glycogen (Bishop and Larner, 1967; Hers and DeWulf, 1967; Kotoulas et al., 1971).

The normal postnatal increase in the total volume of lysosomes was also prevented by insulin. An inhibitory effect of this hormone on lysosomes has been observed before (Pfeifer, 1978). Since glucagon secretion after birth represents the physiological stimulus for the increase in lysosomal volume, this effect of insulin may be due to the fact that the hormone opposes the action of glucagon. Insulin probably acts by counteracting the effect of glucagon to elevate cAMP. Cyclic AMP has been shown to promote lysosomal development (Dawkins, 1963; Kotoulas and Phillips, 1971; Kotoulas, 1986; Pilkis et al., 1986). Most of the lysosomes in newborn rat hepatocytes belong to the autophagic type. Autophagocytosis has been considered as a morphological expression of gluconeogenesis. Hence, insulin action to the lysosomes may serve as part of the mechanism for the regulation of gluconeogenesis (Rosa, 1971; Pilkis et al., 1986).

The normal postnatal degradation of glycogen inside the lysosomes was also prevented by insulin. This undoubtedly resulted from the observed low levels of activity of the lysosomal glycogen-hydrolyzing enzyme acid alpha 1,4 glucosidase (Kotoulas, 1981, 1988; Skoglund et al., 1987). It has been shown that the activity of this enzyme and the hydrolytic degradation of lysosomal glycogen are under a cAMP-mediated hormonal control (Kotoulas et al., 1971, 1991; Pfeifer, 1971; Kotoulas, 1986). The inhibitory effect of insulin on the Golgi apparatus could be related to the effect of hormone on the lysosomal function. Lysosomal hydrolytic enzymes are known to be derived from the apparatus through a vesicle-mediated system of transport (Alcalde et al., 1992; Reaves and Banting, 1992). However, insulin could also exert a cAMP-independent effect on the Golgi apparatus (Einstein and Gabel, 1991; Lippincott-Schwartz et al., 1991.

The administration of insulin resulted in a decrease in the number of peroxisomes, mainly of the crystalloid core-devoid type. Peroxisomes are thought to provide a pathway for the formation of alpha keto acids which are the main building blocks for carbohydrate synthesis (DeDuve and Baudhuin, 1966). Since insulin inhibits gluconeogenesis, our results constitute evidence for the participation of peroxisomes in this metabolic process. These conclusions were supported by preliminary studies with glucagon which is known to promote gluconeogenesis. Three newborn rats injected with glucagon at 0 and 3 hours after birth, each time with a dose of 15 mg/kg, showed at 4 hours an increased total volume of the peroxisomes of hepatocytes; i.e., $1.12 \pm 0.14$ as compared to that of three controls; i.e., $0.78 \pm 0.12$. These results were means $\pm$ standard errors and the difference was statistically significant ($p < 0.05$). Most of the peroxisomes (60%) in the glucagon-treated animals belonged to the crystalloid core-devoid type.

The inhibited development of vesicles of the rough endoplasmic reticulum produced by insulin is difficult to interpret. A similar change was observed after the administration of glucose (Kotoulas et al., 1971). Since both insulin and glucose lead to the preservation of vast glycogen stores in the hyaloplasm, these changes may result from the rearrangement of rough endoplasmic reticulum imposed by the limited space that cell organelles could occupy (Jesequel et al., 1965).

The appearance of enlarged mitochondria in the insulin-treated newborn animals cannot be explained by the data of this study. Metabolic and or structural differences have been shown to exist between mitochondria of newborn and adult rat hepatocytes. Moreover, insulin specifically induces protein synthesis in developing cells (Walker, 1968; Bohme et al., 1992). The mitochondrial change noted in this work may thus represent a specific effect of insulin on the developing hepatocytes of the newborn rat.

The increased surface area of cell membrane, due to folds and pits observed in the insulin-treated animals, could facilitate the passive transport of glucose towards the cell interior (Ashmore and Weber, 1968). Insulin-induced irregular fluctuation of protrusion and withdrawal of the margins of cell surface has been observed before (Goshima et al., 1984). However, the regulation of glucose transport by this hormone may be more complex (Hudson et al., 1992).The cell membrane differentiations found in this study may be associated with the receptor-mediated pathway of internalization of insulin (Nielsen and Christensen, 1989; Doherty et al., 1990; Woodman et al., 1992).

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