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Hormonal regulation of rat liver cell development during the perinatal period

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HORMONAL REGULATION OF RAT LIVER CELL DEVELOPMENT DURING THE PERINATAL PERIOD

by

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ABSTRACT

During the first few days after birth rat liver undergoes several important structural and functional changes. Hepatocyte size decreases largely because glycogen, which is stored during late fetal life, is rapidly mobilized. However, the number of hepatocytes increases. Mitochondrial division parallels cell division, with the result that a constant number of mitochondria per hepatocyte is maintained for about 3 days after birth. Smooth endoplasmic reticulum (SER), which is essentially absent in the fetal liver, begins to form on the first postnatal day and reaches the adult level after about one week. Many hepatic enzymes increase in activity in the early neonatal period. Among these are: glucose-6-phosphatase, a microsomal enzyme of glycogen degradation; phosphoenolpyruvate carboxykinase, a soluble gluconeogenic enzyme, and NADPH-cytochrome c reductase, a microsomal enzyme of the mixed function oxidase system of drug metabolism. Cytochrome P-450, also involved in drug metabolism, is a component of the SER and increases in concentration as this organelle proliferates.

The mechanisms which initiate and regulate these developmental events are unknown, but it is suspected that hormones may be involved. Several investigators have shown that administration of certain hormones to fetal rats causes the premature appearance of hepatic enzymes, including those listed above. Thyroxine and glucagon were particularly effective in these studies, although not necessarily as inducers of the same enzymes. These hormones are also of interest because their serum concentrations undergo important increases near the time of

birth in rats.

The major purpose of this research was to examine the effects of thyroid hormones and glucagon on structural differentiation of perinatal liver using the technique of quantitative electron microscopy (morphometrics). The morphological studies were correlated with biochemical assays of glucose-6-phosphatase, NADPH-cytochrome c reductase, and cytochrome P-450.

Since thyroxine does not readily cross the placenta, it was administered directly to fetuses by injection through the maternal uterine wall. No changes in fetal liver ultrastructure were observed. It was suspected that this was due to technical problems, such as leakage of the hormone from the injection site.

The administration to pregnant rats of a thyroxine analog which does cross the placenta, 3,5-dimethyl-3'-isopropyl-L-thyronine (DIMIT), caused several striking structural and functional changes in 20-day fetuses. DIMIT-treated hepatocytes contained very little glycogen and were small in comparison with controls. The number of hepatocytes was increased in DIMIT-treated livers, and this effect was paralleled by an increase in the number of hepatocyte mitochondria. Microsomal NADPH-cytochrome c reductase and glucose-6-phosphatase activities were significantly increased, although the effect on the latter enzyme was inconsistent. All these changes were normal but premature, suggesting that fetal thyroid hormones may be the physiological regulators of their development. DIMIT did not induce formation of SER or cytochrome P-450.

Livers of 3-day old neonates from mothers treated with several

concentrations of propylthiouracil (PTU) showed no significant ultrastructural differences when compared with controls. The only functional effect of PTU was a decrease in glucose-6-phosphatase activity. It is suspected that even with PTU treatment enough fetal thyroid hormone remained in the circulation to trigger all those changes normally under thyroid control.

Since neither hyperthyroidism nor hypothyroidism produced any alteration in the amount of SER or cytochrome P-450, and since the thyroid effect on glucose-6-phosphatase was variable, the possibility that glucagon levels normally regulate these parameters was investigated. Glucagon injection into 20-day fetuses produced no discernible structural or functional changes. It was again felt that there were technical problems with these direct injection experiments, and that another method of studying glucagon effects should be found. Monolayer cell culture of fetal rat hepatocytes is planned for future studies of hormonal involvement in hepatocyte differentiation.

TABLE OF CONTENTS

LIST OF TABLES

INTRODUCTION

Structural development of rat hepatocytes during the perinatal period

Our present concept of the morphological development of normal rat liver is a synthesis of descriptions of many strains. According to these collected observations, the liver primordium of the rat is formed mainly from an endodermal diverticulum (Elias, 1955) which appears at about 10.5 to 11 days of gestation (Wood, 1965). Until approximately 3 days before birth, parenchymal cells are relatively small, up to 14 μm in diameter (Rohr et al., 1971; Greengard et al., 1972), and occupy no more than about one-half the total tissue volume (Dallner et al., 1966a; Rohr et al., 1971; Greengard et al., 1972). The rest of the liver is composed principally of hematopoietic tissue.

By the 19th gestational day, with birth occurring on the morning of day 22 according to the pregnancy dating system of Kalter (1968), fetal rat hepatocytes contain a large quantity of free ribosomes and polysomes, and a small amount of intracellular membrane in flattened stacks with attached ribosomes (rough-surfaced endoplasmic reticulum, or RER; Dallner et al., 1966a; Rohr et al., 1971). The Golgi apparatus appears as large and highly developed as in adult rat liver (Dallner et al., 1966a) and is located in the perinuclear area (Chedid and Nair, 1974). Mitochondria occupy approximately 5% of the total liver volume (Rohr et al., 1971). Glycogen, microbodies, and lipid droplets are scarce, and lysosomes and endoplasmic reticulum (ER) without attached ribosomes (smooth-surfaced ER, or SER) are essentially absent (Dallner et al., 1966a; Rohr et al., 1971).

During the last 3 days before birth parenchymal cells increase

markedly in size, reaching by birth an average maximum diameter of 22 μm (Rohr et al., 1971; Greengard et al., 1972). This increase is largely due to a massive accumulation of glycogen in the cytoplasm (Peters et al., 1962; Dallner et al., 1966a; Rohr et al., 1971). The volume of liver tissue occupied by hepatocyte RER also increases sharply and free ribosomal areas decrease in an inversely proportional manner. Mitochondria increase greatly in size and somewhat in number per hepatocyte. Lysosomes appear and microbodies become gradually more numerous (Rohr et al., 1971). The Golgi apparatus is essentially unchanged morphologically; however, it gradually moves nearer the bile canaliculus, its adult location (Chedid and Nair, 1974). SER is still practically absent (Dallner et al., 1966a; Rohr et al., 1971). By the time of birth, hepatocytes occupy nearly 80% of the total liver tissue volume (Rohr et al., 1971; Greengard et al., 1972).

During the first 24 hours after birth, neonatal hepatocytes become depleted of most of their glycogen and, as a result, are greatly reduced in size (diameter of 19 μm; Peters et al., 1962; Dallner et al., 1966a; Rohr et al., 1971.). Mitochondria increase further in size, and microbodies and lysosomes continue to increase in number. There is little quantitative change in Golgi or RER, although the latter membrane system appears more dilated. A very few patches of tubular SER begin to appear (Rohr et al., 1971).

By the third postnatal day the quantity of SER is significantly increased and the membranes are located in those areas of cytoplasm where the few remaining patches of glycogen are also found (Dallner et al., 1966a; Rohr et al., 1971; Herzfeld et al., 1973). At this

time the liver is similar to that found in adult rats in its proportion of parenchymal (85%) to nonparenchymal (15%) volume (Rohr et al., 1971; Greengard et al., 1972). Mitotic activity of hepatocytes is markedly increased during the first 3 days after birth, as is mitochondrial division. The slow but steady increase in volume of liver tissue occupied by microbodies continues, while the volume fraction of lysosomes declines (Rohr et al., 1971). No further change in Golgi or RER is apparent. The cytoplasm accumulates a moderate number of lipid droplets (Dallner et al., 1966a).

At 8 days after birth, the neonatal rat liver is structurally very similar to adult liver in terms of proportion and size of hepatocytes and quantities of major hepatocyte organelles and inclusions (Dallner et al., 1966a; Rohr et al., 1971).

Functional development of rat hepatocytes during the perinatal period

As the liver of the rat undergoes its structural development, a wide variety of functions also appear. These have primarily been studied by following the accumulation of certain major enzymes. There are two particularly active perinatal periods of enzyme development, one occurring between days 16 and 20 of gestation, and the other taking place during the first few days after birth. The enzymes which increase in activity at these times have been grouped into "clusters" ("late fetal" and "neonatal") and reviewed by Greengard (1970).

Among the most dramatic enzyme changes during the late fetal period are those associated with glycogen synthesis and deposition. Both total glycogen synthetase and the active (a or I) form of this enzyme increase from nearly zero to greater than adult levels between

16 days of gestation and birth (Jacquot and Kretchmer, 1964; Eisen et al., 1973; Devos and Hers, 1974; Watts and Gain, 1976). Phosphoglucomutase (Jacquot and Kretchmer, 1964) and uridine diphosphoglucose pyrophosphorylase (Isselbacher, 1957) also are involved in glycogen synthesis and belong to the late fetal cluster of enzymes.

The neonatal cluster includes enzymes of several pathways whose development is essential for adaptation to extrauterine life. Birth involves a change from an environment rich in glucose to one in which the diet consists of high fat-low carbohydrate maternal milk. To meet the need for glucose, glycogen is mobilized. Glucose-6--phosphatase is one enzyme of glycogen degradation which increases from very low levels to several times the adult activity within the first two days after birth (Dawkins, 1963; Dallner et al., 1966b). This enzyme is initially located on the RER, but with development becomes distributed approximately equally between the RER and SER (Dallner et al., 1966b; Leskes et al., 1971a,b). The active (a) form of glycogen phosphorylase, another enzyme of glycogen catabolism is also increased in activity during the first day after birth (Devos and Hers, 1974; Watts and Gain, 1976).

As liver glycogen is largely depleted within the first postnatal day there is a need for the rapid development of gluconeogenic capability. An important enzyme of this pathway is phosphoenolpyruvate carboxykinase, which has very low activity until delivery and then increases rapidly during the first 24 hours after birth (Ballard and Hanson, 1967; Pearce et al., 1974).

The oxidative degradation of amino acids also increases just after birth, probably as an additional means of meeting the need for

energy after glycogen has been depleted. The developmental pattern of tyrosine aminotransferase has been thoroughly studied. It is undetectable before birth and reaches greater than adult activity within 12 hours after delivery (Greengard and Dewey, 1967). Serine dehydratase undergoes a similar change (Greengard and Dewey, 1967).

A major function of adult liver involves the metabolism of endogenous circulating steroid hormones and exogenous drugs. This is accomplished by microsomal electron transport chains which use molecular oxygen and couple the hydroxylation of substrates with the oxidation of reduced nicotinamide adenine dinucleotide (NAD)-containing compounds (Brodie et al., 1958). The electron transport chain which oxidizes the reduced form of NAD-2'-phosphate (NADPH) has been studied in considerable detail. In this so-called "mixed function oxidase" system, the steroid or drug to be metabolized forms a complex with the oxidized form of a heme-containing cytochrome, cytochrome P-450. The latter compound is then reduced by an enzyme, NADPH-cytochrome c reductase. Molecular oxygen combines with reduced cytochrome P-450 to yield a hydroxylated substrate, oxidized cytochrome P-450, and water. Finally, the return of NADPH-cytochrome c reductase to its reduced form is coupled with the formation of $NADP^+$ from NADPH (Brodie et al., 1958; Omura and Sato, 1964).

Both cytochrome P-450 and NADPH-cytochrome c reductase are present in some form on fetal rat liver microsomes, but are considered here with the neonatal cluster since their greatest development occurs after birth. A protein precursor of cytochrome P-450 is detectable electrophoretically from about the 19th day of gestation onward

(Siekevitz, 1973); however, heme is not bound to the protein and, therefore, the cytochrome is not spectrophotometrically measurable until just after birth (Dallner et al., 1966b; Siekevitz, 1973). The concentration of cytochrome P-450 then increases rapidly and adult levels are nearly reached by the 8th postnatal day (Dallner et al., 1966b; Maines and Kappas, 1975). Cytochrome P-450 is thought to reside on the SER (Jones and Mills, 1974). NADPH-cytochrome c reductase activity is detectable as early as the 15th day of gestation and increases slightly until birth (Lang, 1965). There is a more rapid increase after birth. Adult levels of activity are probably not reached until somewhere between the 12th and 25th postnatal days (Lang, 1965; Greengard and Dewey, 1968), although one study reported a sudden surge to adult enzyme activity by the end of the first day after birth (Dallner et al., 1966b). NADPH-cytochrome c reductase, like glucose-6-phosphatase, appears first on the RER, but in adults is found on both the RER and SER (Dallner et al., 1966b).

In addition to enzymes involved in carbohydrate, amino acid, and drug metabolism, other enzymes undergo important developmental changes (both increases and decreases) during the perinatal period. Among these are components of the urea cycle (Greengard, 1970), heme synthesis (Woods, 1974), heme catabolism (Maines and Kappas, 1975), and cholesterol synthesis (Rodwell et al., 1976).

Control of the structural and functional development of liver

The factor(s) which initiate and regulate the morphological and enzymatic changes that characterize the perinatal rat liver are not established, nor is it certain that the structural and functional

aspects of development are controlled in the same way. For example, the birth process itself may be a stimulus for some biochemical events without initiating detectable morphological development. Rabbits, like rats, exhibit a rapid rise in glucose-6-phosphatase activity within 24 hours after birth at normal term. Prolongation of pregnancy in rabbits resulted in a delayed increase in the activity of this enzyme until after delivery (Dawkins, 1961). On the other hand, prolongation of pregnancy in rats did not appear to delay the morphological changes observed at the end of a normal gestation period. In particular, glycogen was markedly depleted and SER developed significantly, on schedule, when gestation was prolonged for three days (Thliveris, 1974). Conversely, premature delivery resulted in an early increase in glucose-6-phosphatase activity in rabbits (Dawkins, 1961) and in phosphoenolpyruvate carboxykinase activity in rats (Yeung and Oliver, 1967). The effects of premature delivery on hepatic morphology have not been studied.

Nutritional factors are also undoubtedly of major importance in regulating certain aspects of perinatal development. Glucose administration to rats at birth prevented both the depletion of liver glycogen and the usual increase in glucose-6-phosphatase activity (Dawkins, 1963). The effect of glucose on organelle differentiation in hepatocytes has not been investigated.

The birth event itself as well as nutritional status during the perinatal period are both intimately related to the animal's hormonal make-up. There is a large body of experimental evidence demonstrating that administration of one or more hormones at select times can

accelerate the normal course of hepatic enzyme development. For example, organ cultures of liver from rat fetuses on the 16th day of gestation contained extremely low quantities of glycogen and low levels of both total and active glycogen synthetase. The addition of hydrocortisone to the incubation medium promoted an increase in total glycogen synthetase, but no change in the active form of this enzyme or in glycogen content. Explants first pre-incubated in hydrocortisone and then exposed to insulin accumulated glycogen. They had no further change in total glycogen synthetase but exhibited a significant elevation in the active form of this enzyme (Eisen et al., 1973). In normal development glycogen is not deposited in the liver until at least the 19th or 20th day of gestation, even though the fetal pancreas secretes insulin from the 14th day onward (Clark and Rattner, 1973) and the liver has adequate insulin receptors for an activation of glycogen synthetase by the 16th day (Eisen et al., 1973). Specific receptors for glucocorticoids are present in the liver by the 16th gestational day (Feldman, 1974); however, fetal plasma glucocorticoid levels may not be adequate to produce an effect on glycogen synthetase until about the 19th day, when a peak concentration is reached (Martin et al., 1977). Thus, experimental data correlate well with the hormonal status of the animal during late gestation and suggest that corticosteroids and insulin are important regulators of hepatic development in the late fetal period, during which glycogen deposition is a major event.

Hormones other than glucocorticoids and insulin may predominate in the control of early neonatal development of the liver (see Table 1 for a summary of the experiments discussed below). Several enzymes

Table 1. Summary of the reported effects of hormonal treatment of fetal rats on hepatic events which normally occur in the early neonatal period.

Parameter	Normal neonatal change	Hormones producing premature change in fetus	Hormones producing no effect	References
Glucose-6-phosphatase activity	↑	thyroxine glucagon epinephrine	hydrocortisone insulin growth hormone	Greengard and Dewey, 1968; Greengard, 1969
Glucose-6-phosphatase activity	↑	glucagon	thyroxine	Boxer et al., 1974[a]
Phosphoenolpyruvate carboxykinase activity	↑	glucagon		Philippidis and Ballard, 1970
Tyrosine aminotransferase activity	↑	glucagon epinephrine	hydrocortisone thyroxine insulin growth hormone	Greengard and Dewey, 1968; Greengard, 1969
Serine dehydratase activity	↑	glucagon	hydrocortisone thyroxine insulin growth hormone	Greengard and Dewey, 1968; Greengard, 1969
NADPH-cytochrome c reductase activity	↑	thyroxine	glucagon hydrocortisone insulin growth hormone	Greengard and Dewey, 1968; Greengard, 1969

Table 1 (continued). Summary of the reported effects of hormonal treatment of fetal rats on hepatic events which normally occur in the early neonatal period.

Parameter	Normal neonatal change	Hormones producing premature change in fetus	Hormones producing no effect	References
Glycogen content	↓	glucagon		Chiu and Phillips, 1974
SER quantity	↑	glucagon		Chiu and Phillips, 1974
Hepatocyte volume	↓		glucagon thyroxine hydrocortisone	Greengard et al., 1972
Number of hepatocytes per unit volume of liver	↑		glucagon thyroxine hydrocortisone	Greengard et al., 1972

[a]Fetal liver in organ culture was used in this study. All other data reported in Table 1 are from in vivo experiments.

which normally increase after birth (i.e., enzymes of the neonatal cluster) can be induced prematurely in the fetus by hormones. When thyroxine was injected intraperitoneally through the maternal uterine wall into 20-day old rat fetuses, the fetal hepatic glucose-6--phosphatase activity more than doubled within 5 hours and increased further by 24 hours after the injection (Greengard and Dewey, 1968; Greengard, 1969). Glucagon and epinephrine had a similar effect, although stimulation was maximal 5 hours after injection. Hydrocortisone, insulin, and growth hormone failed to induce the enzyme (Greengard and Dewey, 1968; Greengard, 1969). In organ cultures of fetal rat liver glucagon, but not thyroxine, caused a stimulation of glucose-6-phosphatase activity (Boxer et al., 1974).

The gluconeogenic enzyme phosphoenolpyruvate carboxykinase could be induced in rat fetuses on the 21st day of gestation by glucagon injected intraperitoneally. Liver slices from these animals were then capable of an increased synthesis of glycogen from pyruvate (Philippidis and Ballard, 1970). Enzymes of protein degradation such as tyrosine aminotransferase and serine dehydratase also were evoked prenatally by glucagon, and tyrosine aminotransferase responded to epinephrine as well (Greengard and Dewey, 1968; Greengard, 1969). Neither of these two enzymes was induced by hydrocortisone, thyroxine, insulin, or growth hormone. In the adult rat, on the other hand, glucocorticoids are major regulators of several enzymes including tyrosine aminotransferase and glucose-6-phosphatase, while some hormones that are effective in the fetus diminish in importance (Greengard, 1969).

Enzymes of drug and steroid metabolism are also influenced by

hormones. Twenty-day old fetal rats injected intraperitoneally with thyroxine had a significantly enhanced NADPH-cytochrome c reductase activity. Glucagon, hydrocortisone, insulin, and growth hormone were all without effect (Greengard and Dewey, 1968; Greengard, 1969). Cytochrome P-450 has not been hormonally induced in fetal rats, but both NADPH-cytochrome c reductase activity and cytochrome P-450 content were decreased in adult rats injected with growth hormone (Wilson, 1973a,b). It has been suggested that the low capacity of fetal rat liver for drug metabolism is due to the high circulating levels of growth hormone which are present during late gestation and which decline shortly after birth (Wilson and Frohman, 1974). There is, however, no direct experimental evidence for this hypothesis.

In contrast with the sizeable accumulation of data supporting hormonal involvement in enzyme differentiation, there are few studies addressed to the investigation of hormone effects on structural development of the liver. In one qualitative electron microscopic study glucagon, injected intraperitoneally into 20-day old rat fetuses, caused within 3-12 hours a depletion of glycogen, an apparent increase in lysosomes and microbodies, vesiculation of RER, and the appearance of SER (Chiu and Phillips, 1974). On the other hand, a quantitative electron microscopic examination of fetal liver, also at 20 days of gestation, revealed no effect of glucagon, thyroxine, or hydrocortisone on several cytological parameters (Greengard et al., 1972). The latter included fraction of liver occupied by parenchymal cells, number of hepatocyte nuclei per unit volume liver, and volume of an average hepatocyte. For glucagon and thyroxine, the measurements were made at those doses and times after injection which gave maximal

enzyme activation (Greengard et al., 1972). These two studies seem to conflict. If glucagon caused depletion of glycogen (Chiu and Phillips, 1974), its injection would also be expected to result in decreased hepatocyte size, as is the case in vivo in hepatocytes one day after birth (Rohr et al., 1971). Clearly, the data are insufficient to provide an explanation for how hormones, if they are involved at all, regulate the large number of morphological changes that occur in perinatal rat liver.

Purpose and rationale of the present study

The primary problem to which this research was directed was the elucidation of factors which regulate in vivo the structural differentiation of rat liver during late gestation and early postnatal life. It was assumed that a certain degree of morphological development is intimately involved with and in fact necessary for the proper functioning of the organ (however, see Discussion for a more detailed evaluation of the correlation between structure and function). It was further concluded from the existing experimental evidence that hormones are likely candidates for this regulatory role. The dilemma then became to select from the large number of known hormones one or two with which to begin to probe the structure of the liver. Once again, the accumulated data provided excellent clues. Thyroid hormones and glucagon were by far the most effective agents in previous attempts at eliciting biochemical or structural changes in fetal rat liver. Furthermore, there is a temporal correlation between major developmental events in the liver and the initial appearance of or changes in circulating levels of these two hormones. The thyroid gland of rats

begins to store iodine at a low level during the last 2-3 days of gestation (Gorbman and Evans, 1943; Feldman et al., 1961). Endogenous peroxidase activity also appears near the time of birth (Strum et al., 1971). Although the alpha cells of the pancreas begin to secrete glucagon early in gestation (Pictet et al., 1975), there is a dramatic increase in circulating levels of this hormone at birth (Girard et al., 1973). Plasma insulin concentration drops at the same time, such that the insulin/glucagon molar ratio drops from 10.5 to 1.0 within one hour after delivery (Girard et al., 1973). Thus there was ample evidence to support a closer examination of the role of thyroid hormones and glucagon in hepatic differentiation.

It was decided to begin by investigating the involvement of thyroid hormones for several reasons. First, the onset of thyroid function precedes the large change in glucagon concentration at birth. Secondly, while both glucagon and thyroxine administration to fetuses initiate changes in activities of several enzymes associated with carbohydrate metabolism, thyroxine additionally affects drug metabolizing enzymes (Greengard and Dewey, 1968; Greengard, 1969). Thus it was thought that the thyroid hormones might be involved in a more general way in preparing the liver for its adult role of participation in a variety of key pathways. Finally there were methods available for examining the effects of thyroid hormones which were thought to be far superior to those by which hormonal influences on hepatic development had been studied previously. The most commonly used technique in earlier in vivo experiments with all hormones, and still probably the best way of evaluating glucagon in vivo, consisted of

injecting the hormone intraperitoneally into the fetus through the maternal uterine wall. This procedure is a difficult one with two important disadvantages. First, it requires anesthetization and laparotomy of the mother, and anesthetics themselves may have profound effects on both enzyme activities and liver ultrastructure. For example, it is well known that certain barbiturates cause a proliferation of SER (Jones and Fawcett, 1966) which is followed closely by increased activity of drug metabolizing enzymes (Ernster and Orrenius, 1965), and ether anesthesia was shown to prevent the stimulation by glucagon of gluconeogenesis as measured by pyruvate incorporation into glycogen (Philippidis and Ballard, 1970). Secondly, the major problem with direct injection of fetuses is that there is a danger of leakage from the injection site and thus uncertainty about the actual dose delivered. Therefore, although this technique was attempted in order to test thyroid hormone effects directly, efforts were concentrated on other approaches.

Thyroid effects on fetal and neonatal liver development can be evaluated by two traditional methods of endocrine research; that is, observing changes resulting from a lack as well as an excess of the hormone. For these experiments hypothyroidism was induced by mixing the thyroid blocking agent propylthiouracil with the mothers' diet. Since this compound both crosses the placenta and is transmitted in the milk to the suckling young, hypothyroid neonates could readily be obtained (Knobil and Josimovich, 1959; Hamburgh et al., 1962). A unique opportunity arose by which the converse situation, that of hyperthyroidism, could be tested. This condition was produced in

fetuses by the intramuscular administration of a recently synthesized analog of thyroxine (Jorgensen et al., 1974b) to pregnant rats. This non-halogenated analog, 3,5-dimethyl-3'-isopropyl-L-thyronine (DIMIT; Fig. 1), was shown to have significant nuclear binding capacity (Koerner et al., 1975) and biological activity, as measured by the ability to induce amphibian metamorphosis (Frieden and Yoshizato, 1974) and to reverse thiouracil-induced goiter. The latter activity was demonstrated first in adult rats, with DIMIT exhibiting 18% of the potency of thyroxine (Jorgensen et al., 1974a), but it was subsequently found that the analog had, on a molar basis, 4.5 times the activity of thyroxine in preventing fetal rat goiter (Comite et al., in press). DIMIT has a lower molecular weight than thyroxine and this feature undoubtedly contributes to its greater ability to cross the placenta. In addition, it is weakly bound to plasma transport proteins and, therefore, more of it is available for transfer to the fetus (Snyder et al., 1976). The reasons for the difference in activity in fetuses versus adults are conjectural, but may be related to the lack in the fetus of systems for metabolizing the analog. DIMIT is more active in the fetus than iodinated analogs (Comite et al., in press), probably because it is not susceptible to inactivation via deiodination to reverse triiodothyronine (Chopra and Crandall, 1975). In short, DIMIT has several characteristics which recommend it as an agent which can be used effectively to study thyroid hormone action in fetuses with a minimum of disturbance to the animals.

Upon completion of the hypo- and hyperthyroidism experiments it was decided to proceed with the investigation of glucagon's role in

hepatic development, even though these studies would have to be done in a more disadvantageous manner, that is, by direct injection into the fetuses. Glucagon was studied alone and in combination with DIMIT, since there is excellent evidence that many physiological events require a simultaneous or sequential action of two or more hormones. For example, synthesis and activation of glycogen synthetase in fetal rat liver explants resulted from the sequential actions of glucocorticoids and insulin (Eisen et al., 1973). In mouse mammary gland explants, insulin, glucocorticoids, and prolactin were required for milk protein synthesis (Mills and Topper, 1970).

In the most thorough previous morphological analysis of hormonal influences on fetal liver development, glucagon effects were evaluated qualitatively (Chiu and Phillips, 1974). This is an acceptable procedure only when an experimental condition results in a very pronounced effect. On the other hand, apparent small changes in quantities of organelles may be artifacts resulting from inadequate sample size or may simply be due to changes in cell size. Conversely, significant but subtle effects may be overlooked entirely when analysis is qualitative. The application of quantitative electron microscopy (morphometrics or stereology) circumvents these problems of interpretation; however, it is an extremely time-consuming technique. Therefore, in the present study a compromise approach was used. Quantitative electron microscopy was employed to evaluate liver ultrastructure whenever some indication of structural changes on a qualitative level was first observed.

In conjunction with the morphological studies, selected liver

functions were assayed in many experiments. Glucose-6-phosphatase was chosen because it is known to be induced by both glucagon and thyroxine (Greengard and Dewey, 1968; Greengard, 1969) and therefore could serve as a check on the technique of direct hormone injection and also on the effectiveness of DIMIT compared with thyroxine. In addition, the enzyme is important in carbohydrate metabolism and thus provided a measure of a major liver function. NADPH-cytochrome c reductase was analyzed for similar reasons. It is known to be prematurely induced in fetal liver by thyroxine (Greengard and Dewey, 1968; Greengard, 1969). However, glucagon had no effect on the enzyme in previous studies, suggesting that the hormonal control of NADPH-cytochrome c reductase is slightly different from that of glucose-6-phosphatase. This difference was also useful as a check on both technique and the biological activity of the thyroid hormone analog. As an enzyme of drug metabolism, NADPH-cytochrome c reductase acted as a measure of a second key liver function. Finally, as a microsomal component, the enzyme could be used to probe the relationship between drug metabolizing enzyme induction and SER proliferation; i.e., to perhaps determine whether these events are obligatorily coupled. The latter viewpoint arose primarily from studies of phenobarbital-treated adult animals, in which hepatic SER and drug metabolizing enzyme activity increased in a coordinated manner (Ernster and Orrenius, 1965; Jones and Fawcett, 1966). The third biochemical measurement, that of cytochrome P-450 concentration, was also used to investigate the relationship between proliferation of SER and drug metabolizing activity. The cytochrome was thought to be particularly useful in this regard since, unlike NADPH-cytochrome c

reductase or glucose-6-phosphatase, it is thought to be a component of the SER alone and not distributed between both the RER and SER (Jones and Mills, 1974).

In summary, the specific objectives of this research were:

1) to investigate the roles of thyroid hormones and glucagon in the structural differentiation of perinatal rat hepatocytes, using quantitative methods, and
2) to determine what, if any, is the correlation between hormonally induced changes in liver structure and perinatal development of important hepatic enzymes.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were obtained from the Holtzman Co. (Madison, WI) on about the 10th day of pregnancy. Sperm positive vaginal smear dates were provided so that the gestational date could be determined accurately. Delivery occurred consistently on the morning of day 22 (Kalter, 1968). The rats were housed individually and given laboratory animal chow (Ralston Purina Co., St. Louis, MO), except when the effects of special diets were being tested, and water, both ad libitum. Animals were maintained on a controlled lighting schedule consisting of 12 hours of light (6 AM-6 PM) and 12 hours of darkness (6 PM-6 AM).

Special diets

For experiments requiring hypothyroid neonates pregnant rats were given a diet of ground laboratory chow containing 6-n-propyl-2--thiouracil (PTU; Eli Lilly & Co., Indianapolis, IN or Sigma Chemical Co., St. Louis, MO), in a final concentration of 0.1%, 0.2%, 0.3%, or 0.5%, by weight. Before mixing in the drug, the ground food was dampened with water to ensure that a uniform preparation of chow coated with PTU was obtained. Control rats received ground chow without PTU. The diets were given ad libitum from the 12th or 15th day of pregnancy until the third postnatal day, at which time the pups were sacrificed by decapitation and livers removed for electron microscopy and/or biochemistry. In some cases, mothers were also sacrificed and blood and/or liver samples collected.

DIMIT injections

Hyperthyroidism was produced in fetal rats by means of 3,5-dimethyl-3'-isopropyl-L-thyronine (DIMIT) injections given intramuscularly to the mothers. In initial experiments, the thyroid hormone analog was dissolved with difficulty in a solution of 50% ethanol in saline (0.9% NaCl), since it was not known how stable the compound would be in acid or alkali. It was later determined that DIMIT is stable and readily soluble in an alkaline solution (E.C. Jorgensen, personal communication) and this was used in subsequent experiments. The DIMIT was first dissolved in a small amount of 0.1 N NaOH prepared in ethanol, and then brought up to volume with alkaline saline. The final solution contained 0.003 N NaOH and 1% ethanol in saline. The solution was adjusted with 0.1 N HCl to a pH of 10.0. There were no differences in experimental results obtained using the two DIMIT solutions.

The following DIMIT dosages and treatment schedules were tested: 1, 2, 5, or 10 μg/100 g maternal body weight (BW)/5 days, and 10 or 20 μg/100 g BW/2 days. The concentrations of all DIMIT solutions were prepared so that a volume of 0.1 ml always contained the correct dose per 100 g BW. Control rats received 0.1 ml of the appropriate vehicle (ethanolic saline or alkaline ethanolic saline) per 100 g BW. Injections were given once daily from the 15th through the 19th days or only on the 18th and 19th days of pregnancy (5 and 2-day schedules, respectively). Solutions were injected into the hind thighs, with right and left sides receiving the injections on alternate days. On the 20th day of gestation, mothers were sacrificed by decapitation.

In some experiments, maternal blood was collected and/or liver samples were taken. Fetuses were removed and sacrificed, and livers were excised for electron microscopy and/or biochemistry.

Direct injection of fetuses

Animals of three fetal ages (15, 17, and 20 days) were used to test the effects of glucagon and thyroxine, injected singly and in combination. Some animals pre-treated for 5 days with DIMIT also received glucagon in these direct injection experiments. Hormone doses and exposure times were those reported by others to have structural or functional effects on fetal rat liver (Greengard and Dewey, 1968; Greengard, 1969; Chiu and Phillips, 1974). The following procedure was used. Pregnant rats were anesthetized with sodium pentabarbital (Nembutal; Abbott Laboratories, North Chicago, IL), 50 mg/kg BW, or sodium methohexital (Brevital; Eli Lilly & Co.), 65 mg/kg BW. They were then laparotomized and the uterus was exposed. Fetuses in one uterine horn were injected intraperitoneally through the maternal uterine wall with hormones: [1] sodium levothyroxine (Letter; Armour Pharmaceutical Co., Phoenix, AZ), 3 μg/fetus in 0.05 ml 0.9% NaCl, pH 10.0; [2] glucagon (Eli Lilly & Co. or Sigma Chemical Co.), 0.05 mg/fetus in 0.05 ml 0.9% NaCl, pH 10.0, or [3] thyroxine plus glucagon, in the above doses and a total volume of 0.05 ml. Fetuses in the other uterine horn served as controls and were each injected with 0.05 ml of vehicle. The uterus was returned to the body cavity and the incision closed with wound clips. Six hours later, mothers were either re-anesthetized or sacrificed by decapitation. Fetuses were removed from the uterus and sacrificed, and fetal livers were removed for

electron microscopy and/or biochemistry.

Serum preparation and thyrotropin assay

Blood from hypothyroid, hyperthyroid, and control mothers was allowed to clot for one hour at room temperature. Clots were rimmed and the blood was centrifuged for 30 min at 1500 rpm in an International Centrifuge (Universal Model UV; Needham, MA). Serum samples were transferred to a second set of tubes and frozen until assayed. Serum thyrotropin (thyroid stimulating hormone, TSH) concentrations were determined by Dr. Orlo Clark of the Surgical Service, Veterans Administration Hospital, San Francisco, CA, using a modification of the radioimmunoassay technique of Odell et al. (1965).

Preparation of tissue for electron microscopy

Fixation, dehydration, embedding. Liver samples from all experiments were processed identically for electron microscopy. After removal from the animal, a piece of liver (always from the left lateral lobe) was immersed in a drop of fixative and cut into one-millimeter cubes with a razor blade. The cubes were transferred to a vial full of fixative and allowed to stand for 2 hours at room temperature. Fixative was prepared according to a modification of the procedure of Karnovsky (1965) and contained the following (final concentrations given): glutaraldehyde (Polysciences, Inc; Warrington, PA), 2.4% and paraformaldehyde, 0.8%, in 0.06 M sodium cacodylate, pH 7.4. Fixative was replaced by 3 changes of 0.1 M sodium cacodylate, pH 7.4, at 30 minute intervals, and the samples were then refrigerated overnight. On the next day tissues were post-fixed for 1 hour on ice in a

final concentration of 2% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.4. Samples were then dehydrated with increasing concentrations of ethanol (50%, 70%, 95%, and 3 changes of 100%), each for 5 minutes. The tissue was gradually brought to room temperature during this procedure. The last change of absolute ethanol was replaced by a solution of propylene oxide and absolute ethanol (1:1, by volume). After 5 minutes the latter solution was followed by 2 changes of propylene oxide, for 5 minutes each. The vials were then filled with a solution (1:1, by volume) of propylene oxide and epoxy resin (Luft, 1961) and allowed to stand capped overnight at room temperature. On the following day the vials were uncapped and left for several hours, to allow the concentration of epoxy to gradually increase as propylene oxide evaporated. After evaporation was complete, the blocks of tissue were finally embedded in polyethylene capsules (BEEM; Ted Pella Co., Tustin, CA) filled with epoxy. The latter was a mixture of two stock solutions, prepared according to instructions provided by the supplier (Ladd Research Industries, Inc.; Burlington, VT). Solution A contained, for every 80 g Epon 812, an amount of dodecenyl succinic anhydride (DDSA), usually 85-90 g, which was based on the epoxide weight per equivalent (WPE). Solution B contained, for every 100 g Epon 812, an amount of nadic methyl anhydride (NMA), usually 70-75 g, similarly based on the WPE. Solutions A and B were usually mixed in a ratio of 3:7, by weight, to which was also added 0.14 ml tri(dimethylaminomethyl) phenol (DMP-30) accelerator for every 10 g of resin. Blocks were placed in a preheated oven at about 70°C without vacuum for 48 hours. This provided blocks

of medium hardness.

Microsomal pellets were prepared for electron microscopic observation in one of two ways. They were either [1] fixed in a pellet and treated exactly according to the above procedure, or [2] suspended in a final concentration of 2% osmium tetroxide in 0.1 M sodium cacodylate, pH 7.4, fixed on ice for 1 hour, pelleted at 98,000 x g in a preparative ultracentrifuge (Beckman Model L2-65; Beckman Instruments, Inc., Palo Alto, CA), dehydrated, and embedded as above.

En bloc staining. For many experiments, an additional step was included during tissue dehydration. After the 5 minutes in 50% ethanol, blocks were stained for 30 minutes at room temperature with uranyl acetate, in a final concentration of 2% (weight/volume) prepared in 70% ethanol. They were then rinsed in 70% ethanol and dehydration was continued as described before. When compared with liver fixed in osmium or glutaraldehyde but not en bloc stained, the cells had well-delineated membranes, but poorly stained glycogen. This was advantageous for quantitative morphological studies, since in darkly stained areas full of glycogen membranes are often difficult to discern (Figs. 2 and 3).

Sectioning, staining, viewing. Thick (0.5 μm) sections of liver were cut with glass knives on an ultramicrotome (Porter-Blum Model MT2; Sorvall, Inc.; Newtown, CT), placed on glass slides, and dried on a hot plate. Heated sections were then stained for 15 seconds with a filtered 1% toluidine blue solution which contained borax in a final concentration of 1%. They were rinsed for 5 seconds in absolute

ethanol and dried. Sections were viewed with a light microscope (Carl Zeiss, Inc.; New York, NY).

Blocks were then trimmed to exclude cells immediately surrounding central veins and portal triads, and thin (~600 Å) sections of the midlobular cells were cut with a diamond knife, collected on copper grids, and stained. Usually, sections were stained first for 15 minutes with a 25% (weight/volume) solution of uranyl acetate in absolute methanol (Stempak and Ward, 1964) and then for 5 minutes in lead citrate (Reynolds, 1963). For the latter stain, 5 ml of 8.8% sodium citrate were mixed with 3.75 ml of 8.9% lead nitrate. Sodium hydroxide (1.0 N, 1-2 ml) was added to clear the solution which was then spun for 15 minutes in a clinical centrifuge. The supernatant was used for staining. Occasionally, grids were stained only with lead citrate for 8 minutes. Sections were viewed and photographed with a Philips 300 electron microscope (Philips Electronic Instruments, Inc., Mount Vernon, NY), using an accelerating voltage of 60 kilovolts.

Morphometric analysis of liver ultrastructure

Sampling. For quantitative electron microscope studies, a standard sampling procedure was rigorously followed. After placement in the electron beam, the first grid square encountered that [a] was completely covered by tissue and [b] contained in one corner a field of view more than half occupied by parenchymal tissue was photographed at Tap 3, a magnification equal to about 4500X. If the diagonally opposite corner of the grid square also contained a predominance of hepatocytes, it was photographed as well. The above two criteria were then applied to an adjacent grid square. This procedure was repeated

until 5 such low magnification photographs had been taken. Five high magnification electron micrographs (Tap 9, about 15,000X) were then taken. The criteria were the same except that only fields of view more than half occupied by hepatocyte cytoplasm were photographed. The exact magnifications obtained at Tap 3 and Tap 9 were determined at the beginning of each morphometric study using a calibration grid containing 28,800 lines per inch.

Five low and five high power electron micrographs were taken, as described above for each block of liver. This number of micrographs was necessary in order to achieve a high level of confidence in sampling (see Schmucker et al., 1974, for formula). Three blocks of tissue were randomly selected and examined for each fetus or neonate. The total number of animals included in a morphometric experiment varied but usually 3 fetuses or neonates from each of 3 mothers were used. In short, a typical comparison of two groups of animals involved 540 electron micrographs. In a few instances, only 3 low and 3 high magnification photographs were taken per block; therefore, in these experiments a comparison of two conditions required a total of 324 electron micrographs.

Counting and calculations. All negatives of electron micrographs were printed with an enlargement of 3X. The final magnifications of the low and high power views were, therefore, about 14,000X and 45,000X, respectively. Low power micrographs were overlaid with a clear piece of film on which was printed a coherent double lattice test system of course and fine lines (Weibel, 1973; Fig. 4). Intersections of these lines provided a system of 30 course and 480 fine points with which

only the intralobular liver tissue was evaluated. Thus, portal triads and hepatic and central veins, which in the adult rat occupy about 4% of the total liver volume (Weibel et al., 1969), were excluded. Using the course points, "hits" on extrahepatocyte space (sinusoids, Disse spaces, and bile canaliculi), hepatocytes, and hepatocyte nuclei, cytoplasm, mitochondria, and lipid droplets were counted. Fine points were used to count "hits" on hepatocyte microbodies and lysosomes. From these data, volume densities (volumes per unit volume intralobular liver tissue; relative volumes; per cent) of all the above components were calculated according to the method of Weibel (1973), and expressed as cm^3/cm^3. High magnification micrographs were overlaid with a coherent multipurpose test system (Weibel, 1973) consisting of 50 lines, each 2 cm long, arranged in a regular triangular lattice (Fig. 5). The ends of the lines served as points for recording "hits" on hepatocyte cytoplasm. Intersections of the test lines with RER, SER, and Golgi membranes were counted and used to calculate the surface densities (surface areas per unit volume liver cell cytoplasm) of these organelles, according to the formula: $S_V = 4 \cdot I_i / P_T . z$, where S_V is the surface density of a given membrane system, I_i is the number of intersections of the membranes with test lines, P_T is the number of test points, and z is test line length corrected to account for the magnification factor (Weibel, 1973). The surface densities were then converted to values per unit volume intralobular liver tissue, using the volume density of hepatocyte cytoplasm determined on the low magnification electron micrographs. Data were expressed as m^2/cm^3.

Specific volumes of hepatocytes and hepatocyte nuclei, cytoplasm,

mitochondria, lysosomes, microbodies, and lipid droplets, and specific surface areas of RER, SER, and Golgi membranes were also calculated, according to the method of Loud (1968). One light micrograph of thick sectioned liver from each fetus or neonate was taken at an initial magnification of 200X with a Zeiss Universal photomicroscope (Carl Zeiss, Inc.), enlarged to 1000X, and overlaid with a test system of lines, the intersections of which provided 100 points. These points were used to count "hits" on hepatocytes and hepatocyte nuclei. An ocular micrometer was employed to measure the diameter of all hepatocyte nuclei within each test area. From these data, the number of hepatocyte nuclei per unit volume liver (hepatocyte nuclear numerical density) was calculated, and the specific volume of an average mononuclear hepatocyte was estimated. An independent check on hepatocyte volume density was also obtained during the course of the calculations. Once the specific hepatocyte volume was obtained, the specific volumes and surface areas of the various cellular components could be calculated using the previously determined volume and surface densities. All specific volumes were expressed in μm^3 and specific surface areas in μm^2.

Statistics. An attempt was made to maintain a confidence level of at least 95% for all determinations of volume densities. As calculated according to the method described by Schmucker et al. (1974), this level was achieved for all parameters except lipid droplets (about 85% confidence) and microbodies (90-95% confidence). It was judged not to be worthwhile to count more of these relatively rare elements just to reach a higher confidence level, since changes in

lipid droplets or microbodies were not observed in any of the experiments. All data were also subjected to a probability analysis (student's t-test), and were expressed ± the standard error.

Preparation of microsomes

Livers were excised and kept on ice in a small volume of 0.05 M potassium phosphate buffer containing 0.25 M sucrose (final pH, 7.4) until they were all collected. Livers of all fetuses or neonates from a single mother were pooled if they had been treated alike (i.e., where half the litter represented control animals and half were experimental, two pools were obtained). Each pooled sample was then blotted on filter paper, weighed, and homogenized by hand in about 10 times its volume of phosphate-buffered sucrose, on ice, using a glass homogenizing vessel and teflon pestle. The homogenates were transferred to high-speed polycarbonate centrifuge bottles (Beckman Instruments, Inc.) and spun at 4°C and 18,000 x g for 10 minutes in a preparative ultracentrifuge (Beckman Model L2-65), using a Type 40 fixed angle rotor. Supernatants were transferred to a second set of bottles and centrifuged for 60 minutes at 4°C and 98,000 x g. Supernatants from the second spin were discarded and microsomal pellets were resuspended in buffered sucrose, aliquoted, and frozen. An attempt was made to suspend the microsomes in a final concentration of approximately 10 mg microsomal protein per ml final suspension. The quality of the microsomal preparation obtained by the above procedure is shown in Fig. 6.

In several experiments, a microsomal sample of adult rat liver was also prepared and assayed for comparative purposes. A piece of the

liver similar in weight to an average pooled fetal or neonatal sample was homogenized, fractionated, suspended, and aliquoted exactly as described above.

Biochemical assays

Protein determination. The concentration of microsomal protein was measured by the technique of Lowry et al. (1951), using bovine serum albumin (25-200 μg) to obtain a standard curve. Duplicate samples within the above range, standards, and reagent blanks were brought to 1.0 ml with distilled water. To each tube was added 5.0 ml of a reagent prepared 30 minutes in advance and containing, in a ratio of 1:1:100 by volume: 2% sodium potassium tartrate, 1% cupric sulfate, and 2% sodium carbonate prepared in 0.1 N NaOH. The tubes were mixed and 10 minutes later, 0.5 ml of 1 N phenol reagent (Folin-Ciocalteau; Fisher Scientific Co., Pittsburgh, PA) was added with mixing. After 30 more minutes, the absorbance at 540 nm was read on a spectrophotometer (Gilford Model Stasar II; Gilford Instrument Laboratories, Inc., Oberlin, OH). Protein concentration was expressed in mg microsomal protein per ml original suspension.

Cytochrome P-450. The technique of Estabrook et al. (1972) was used to determine cytochrome P-450 concentration. One to three mg microsomal protein were brought to a final volume of 2.5 ml with 0.05 M potassium phosphate buffer, pH 7.4. A few crystals of sodium dithionite were added to the diluted sample and mixed in thoroughly. The microsomal suspension was then divided between two semi-micro cuvettes and a baseline scan of absorbance from 420 to 500 nm at 25°C

was recorded on a dual beam spectrophotometer (Aminco-Chance; American Instruments Co., St. Louis, MO). One cuvette was then gassed with carbon monoxide for one minute and the absorbance over the above range was recorded again. The absorbance at 490 nm was subtracted from that at 450 nm to obtain the absorbance change. Cytochrome P-450 concentration was calculated using an extinction coefficient of 91 $mM^{-1}cm^{-1}$ and was expressed in nmoles per mg microsomal protein or nmoles per g liver.

NADPH-cytochrome c reductase. This enzyme was assayed at 25°C according to the method of Masters et al. (1967). A recording spectrophotometer (Gilford Model 240; Gilford Instrument Laboratories, Inc.) was first calibrated at 550 nm using as a reference solution: 0.7 ml 0.05 M potassium phosphate buffer containing 10^{-4} M ethylenediamine tetraacetic acid, pH 7.7; 0.4 ml 10^{-4} M cytochrome c, and 0.05 ml 10^{-2} M potassium cyanide. A baseline absorbance for the samples was then recorded. Sample cuvettes contained: 0.5 ml buffer; 0.4 ml cytochrome c; 0.05 ml potassium cyanide, and 0.1 ml microsomal suspension (containing approximately 1 mg protein). After a stable baseline reading was obtained, 0.1 ml 10^{-3} M reduced nicotinamide adenine dinucleotide phosphate (NADPH) was added to the sample cuvette and stirred in rapidly. The reaction was recorded for 1-3 minutes. Absorbance change per minute was determined from the straight line portion of the graph, and the rate of reduction of cytochrome c was calculated using an extinction coefficient of 21 $mM^{-1}cm^{-1}$. NADPH--cytochrome c reductase activity was expressed as nmoles cytochrome c reduced per minute per mg microsomal protein or per g liver.

Glucose-6-phosphatase. The enzymatic procedure of Zakim and Vessey (1973) was used to assay the phosphotransferase activity of this multifunctional enzyme. To a set of centrifuge tubes was added: 0.1 ml 1.0 M sodium acetate, pH 5.5; 0.4 ml 0.2 M sodium pyrophosphate, pH 5.5; 0.4 ml 1.0 M glucose, and 0.1 ml distilled water. This was brought to 37°C in a shaking water bath at medium speed. An aliquot of microsomes approximately equal to 1 mg protein (usually 0.1 ml) was then added and the reaction, in which glucose-6-phosphate was formed, was allowed to proceed for 5 minutes. This step was timed precisely. The reaction was stopped by placing the tubes into a boiling water bath for 3 minutes. Tubes were centrifuged for 10 minutes at 1000 rpm in an International centrifuge (Universal Model UV) and the supernatants were transferred to a second set of tubes. The amount of glucose-6-phosphate formed in the above reaction was measured enzymatically. First, a recording spectrophotometer (Gilford Model 240) was calibrated at 340 nm using as a reference solution: 0.1 ml 1.0 M Tris-HCl buffer, pH 8.0; 0.025 ml nicotinamide adenine dinucleotide phosphate (NADP), 12.5 mg/ml; 0.002 ml glucose-6-phosphate dehydrogenase (Zwischenferment; Sigma Type VII from baker's yeast; Sigma Chemical Co.), and 0.873 ml distilled water. A baseline absorbance for the samples was then recorded. Sample cuvettes contained: 0.1 ml Tris--HCl buffer; 0.002 ml glucose-6-phosphate dehydrogenase; 0.773 ml distilled water, and 0.1 ml supernatant from the first reaction. After a stable baseline reading was obtained, 0.025 ml NADP was added and stirred in quickly. The reduction of NADP was recorded to completion, i.e., until a plateau was reached. The initial absorbance was subtracted from that reached at the plateau to obtain the absorbance

change. An extinction coefficient of 6.22 X 10^3 $mM^{-1}cm^{-1}$ was used to calculate glucose-6-phosphatase activity, which was expressed as pmoles glucose-6-phosphate formed per minute per mg microsomal protein or per g liver. Standard solutions of glucose-6-phosphate were also run in the second reaction as a check on the method.

Statistics. All enzyme activities and cytochrome P-450 concentrations were subjected to a probability analysis (student's t-test) and were expressed ± the standard error.

RESULTS

Qualitative observations of normal developing rat liver

Light (Figs. 7-10) and electron (Figs. 11-15) microscopic examination of rat liver from the 15th gestational to the 3rd postnatal day was undertaken in order to determine if there were any substantial differences in the hepatic morphology of developing Sprague-Dawley, Holtzman strain rats when compared with the accepted descriptions outlined earlier in the Introduction. The latter represent the combined observations of several investigators and deal with a variety of rat strains.

The proportion of the liver occupied by hematopoietic and other non-parenchymal cells appeared to exceed 50% until about the 17th day of gestation (Fig. 7), dropped sharply between the 17th and 20th days (Figs. 7 and 8), and decreased more slowly thereafter. On the 3rd day after birth (Fig. 10), non-parenchymal cells were still present but they appeared to occupy less than 20% of the liver.

Hepatocyte size underwent an overall increase over the time period studied. The largest increase occurred between the 17th and 20th days of gestation and was associated with glycogen accumulation (Figs. 7 and 8, 12 and 13). Small patches of glycogen were occasionally observed in hepatocytes at 17 days, but usually were not present until one or two days later, and then storage proceeded at an extremely rapid rate. At 21 days, fetal hepatocytes reached both maximum size and glycogen content (Figs. 9 and 14). Between the 21st gestational day and the 3rd postnatal day most of the glycogen was mobilized from the hepatocytes (Figs. 10 and 15). However, cell-to-cell variability

was great, with some cells entirely depleted of glycogen while others still contained large masses of this material. The overall major loss of glycogen was accompanied by decreased cell size; nevertheless, hepatocytes at 3 days after birth still appeared larger than they had been before glycogen storage began.

On the 15th day of gestation (Fig. 11), flattened stacks of RER and large masses of particulate material, representing free ribosomes, were observed in the hepatocyte cytoplasm. The RER was more abundant on the 17th and 20th fetal days and appeared not to change significantly after that. By the 20th day of gestation (Fig. 13), the large areas of free ribosomes had disappeared, although small numbers of these organelles could be found at any time.

Only very rarely was any SER observed in hepatocytes before birth. On the 3rd postnatal day, however, substantial amounts of this membrane system were found, particularly in glycogen areas and adjacent to patches of RER (Fig. 15). Frequent connections between RER and SER were observed.

Large, well-developed Golgi systems were present at all five time points examined. In fetal animals the Golgi apparatus was nearly always located close to the nucleus (Figs. 12 and 13). At 3 days after birth, a perinuclear position was still very common, but the Golgi was also found with increasing frequency near borders with other hepatocytes and particularly near the bile canaliculus (not shown in Fig. 15, but see Fig. 18).

Mitochondria were present in moderate numbers throughout the late fetal period. On the third day after birth, however, mitochondria seemed more numerous, even with the decreased hepatocyte size taken

into consideration (compare Figs. 11-14 with Fig. 15). There were no obvious changes in mitochondrial size at any time point examined.

By at least the 17th fetal day both microbodies and dense bodies, presumably lysosomes, were present (Fig. 12), although the former were more numerous. The numbers of both organelles gradually increased thereafter. However, lysosomes must have increased more rapidly, since the numbers of microbodies and lysosomes appeared about equal by the 3rd day after birth.

Lipid droplets could be observed at all time periods studied, but were rather infrequent before birth. After birth there was a variable accumulation of lipid in the hepatocytes. Many cells contained none of this material on the 3rd postnatal day (Fig. 15), and others contained large quantities (Fig. 10). Cells with and without lipid droplets were often found immediately adjacent to one another (Fig. 10).

Quantitative comparison of 20-day fetal and 3-day postnatal rat liver

Morphometric data obtained from control animals used in experiments to be described later are shown in Tables 2 and 3. Rat liver structure at 20 days of gestation was compared with that at 3 days after birth. The volume density and specific volume data (Table 2) for the most part agreed with impressions derived from qualitative observation at these two time points. For example, a highly significant increase in volume density as well as specific volume of hepatocyte mitochondria, lipid droplets, lysosomes, and microbodies was confirmed with the quantitative technique. On the other hand, there was slight disagreement regarding the proportion of liver occupied by

Table 2. Comparison of volume densities and specific volumes of liver components from 20-day fetal and 3-day postnatal Sprague-Dawley, Holtzman strain rats.

Parameter	Volume density (cm^3/cm^3)[a]		P	Specific volume (μm^3)		P
	20-day fetal (7)[b]	3-day postnatal (9)		20-day fetal (7)	3-day postnatal (9)	
Extrahepatocyte space	0.15 ± 0.02[c]	0.16 ± 0.02	NS	-	-	-
Hepatocyte	0.85 ± 0.02	0.84 ± 0.02	NS	2985 ± 136[d]	3583 ± 280	NS
nuclei	0.11 ± <0.01	0.07 ± 0.01	<.01	395 ± 25	316 ± 44	NS
cytoplasm	0.74 ± 0.02	0.76 ± 0.02	NS	2590 ± 114	3267 ± 264	NS
mitochondria	0.07 ± 0.01	0.15 ± 0.01	<.001	253 ± 19	639 ± 45	<.001
lipid droplets	0.006 ± 0.003	0.052 ± 0.013	<.01	20 ± 11	219 ± 54	<.01
lysosomes	0.003 ± <0.001	0.006 ± <0.001	<.005	10 ± 1	24 ± 3	<.005
microbodies	0.002 ± <0.001	0.006 ± <0.001	<.001	5 ± <1	27 ± 4	<.001

[a]Volume per unit volume intralobular liver tissue; per cent.

[b]Number of animals in each group is in parentheses.

[c]Values are means ± SE.

[d]Volume of average mononuclear hepatocyte. All other specific volumes are based on this calculated average.

Table 3. Comparison of surface densities and specific surface areas of hepatocyte membrane systems from 20-day fetal and 3-day postnatal Sprague-Dawley, Holtzman strain rats.

Parameter	Surface density (m^2/cm^3)[a]		P	Specific surface area (μm^2)[b]		P
	20-day fetal (7)[c]	3-day postnatal (9)		20-day fetal (7)	3-day postnatal (9)	
Rough endoplasmic reticulum	3.38 ± 0.08[d]	2.80 ± 0.19	<.025	11866 ± 546	12058 ± 1288	NS
Smooth endoplasmic reticulum	1.43 ± 0.13	3.05 ± 0.17	<.001	4969 ± 385	13247 ± 1457	<.001
Golgi	0.49 ± 0.13	0.32 ± 0.07	NS	1650 ± 384	1317 ± 283	NS

[a]Surface area of component per unit volume intralobular liver tissue.

[b]Surface area of component per average mononuclear hepatocyte.

[c]Number of animals in each group is in parentheses.

[d]Values are means ± SE.

hepatocytes. Whereas the initial observations had suggested a difference between 20-day fetal and 3-day postnatal animals in this parameter, the morphometric method showed that about 85% of the liver was occupied by parenchymal cells at either age. A decrease in volume density of hepatocyte nuclei at 3 days was not explained by statistically significant changes in specific volumes of nuclei or hepatocytes or in nuclear numerical densities, which were 289 X 10^6 at 20 days of gestation versus 256 X 10^6 at 3 days after birth. However, there was a trend toward larger cells and smaller nuclei at 3 days. This combined with a high degree of variability may account for the observed effect on nuclear numerical density.

Data on quantities of intracellular membranes are presented in Table 3. The major effect of age was a large increase in both surface density and specific surface area of SER, which confirmed the preliminary observations. A slight decrease in surface density but not specific surface area of RER may be explained by the tendency toward larger hepatocytes at 3 days. There was no difference in quantity of Golgi membranes at the 2 days examined.

Direct injection of fetuses with hormones

Fetuses in utero at ages 15, 17, and 20 days were injected intraperitoneally with 50 µg glucagon or vehicle, and the livers were fixed for electron microscopy 6 hours later. Qualitative examination of hepatic morphology revealed no differences between controls and experimentals. Similar studies in which the effects of 3 µg thyroxine or 3 µg thyroxine plus 50 µg glucagon were tested in fetuses at age 17 and 20 days also produced no discernible changes in liver ultra-

structure. No biochemistry was done with livers from fetuses used in these experiments. Instead, it was decided to turn to other methods of investigating hormone, and particularly thyroid hormone, effects on developing liver.

Quantitative effects of hypothyroidism on neonatal liver ultrastructure

One of the most striking and important events in perinatal hepatic development is the appearance and proliferation of SER. Experiments with a thyroid-blocking agent were designed to test a hypothesis that thyroid hormones act as a stimulus for this earliest SER formation. Accordingly, rats were given 0.1% PTU in the diet from the 15th day of pregnancy until the third postnatal day, at which time neonates were sacrificed for electron microscopic examination of their livers. Since significant SER is normally present by 3 days after birth, it was thought likely that if there was an inhibition of this organelle's development due to thyroid insufficiency it could be easily detected by day 3. Preliminary comparison of several electron micrographs from control and experimental animals in fact suggested such a PTU-induced reduction in SER, and a morphometric study was therefore undertaken. The results of this and other PTU experiments are shown in Tables 4-8. The quantitative analysis revealed no significant effects of 0.1% PTU on surface density or specific surface area of SER or any other membrane system (Tables 6 and 7). Neither was there any effect on the volume densities or specific volumes of the liver components listed in Tables 4 and 5, except for a small increase in mitochondrial specific volume (Table 5). This result is not supported by any other significant changes. Therefore, it is probably a

Table 4. Effect of PTU on volume densities of 3-day postnatal liver components.[a]

Parameter	Volume density (cm^3/cm^3)[b]											
	Control (9)[c]		0.1% PTU (6)		P[d]	0.2% PTU (6)		P	0.5% PTU (6)		P	
Extrahepatocyte space	0.16 ± 0.02[e]		0.11 ± 0.02		NS	0.18 ± 0.04		NS	0.15 ± 0.03		NS	
Hepatocyte	0.84 ± 0.02		0.89 ± 0.02		NS	0.82 ± 0.04		NS	0.86 ± 0.03		NS	
nuclei	0.07 ± 0.01		0.07 ± 0.02		NS	0.08 ± 0.01		NS	0.07 ± 0.01		NS	
cytoplasm	0.76 ± 0.02		0.81 ± 0.02		NS	0.74 ± 0.04		NS	0.78 ± 0.03		NS	
mitochondria	0.15 ± 0.01		0.17 ± 0.01		NS	0.15 ± 0.01		NS	0.17 ± 0.01		NS	
lipid droplets	0.052 ± 0.013		0.056 ± 0.021		NS	0.052 ± 0.018		NS	0.025 ± 0.009		NS	
lysosomes	0.006 ± <0.001		0.005 ± 0.001		NS	0.007 ± <0.001		NS	0.007 ± <0.001		NS	
microbodies	0.006 ± <0.001		0.005 ± 0.001		NS	0.006 ± 0.001		NS	0.007 ± 0.001		NS	

[a]PTU was administered in the diet from the 15th day of pregnancy (0.1% PTU) or from the 12th day of pregnancy (0.2% and 0.5% PTU) until the 3rd day after delivery, at which time neonates were sacrificed.

[b]Volume per unit volume intralobular liver tissue; per cent.

[c]Number of animals in each group is in parentheses.

[d]P values are given for each PTU group compared with controls.

[e]Values are means ± SE.

Table 5. Effect of PTU on specific volumes of 3-day postnatal liver components.

Parameter	Specific volume (μm^3)						
	Control (9)[a]	0.1% PTU (6)	P[b]	0.2% PTU (6)	P	0.5% PTU (6)	P
Hepatocyte	3583 ± 280[cd]	4520 ± 464	NS	3688 ± 171	NS	3310 ± 18	NS
nuclei	316 ± 44	381 ± 100	NS	374 ± 64	NS	284 ± 28	NS
cytoplasm	3267 ± 264	4138 ± 404	NS	3313 ± 133	NS	3024 ± 41	NS
mitochondria	639 ± 45	875 ± 88	<.025	688 ± 47	NS	655 ± 18	NS
lipid droplets	219 ± 54	326 ± 123	NS	232 ± 77	NS	99 ± 35	NS
lysosomes	24 ± 3	25 ± 5	NS	31 ± 2	NS	25 ± 2	NS
microbodies	27 ± 4	24 ± 4	NS	25 ± 3	NS	27 ± 2	NS

[a]Number of animals in each group is in parentheses.

[b]P values are given for each PTU group compared with controls.

[c]Values are means ± SE.

[d]Volume of an average mononuclear hepatocyte. All other specific volumes are based on this calculated average.

Table 6. Effect of PTU on surface densities of 3-day postnatal hepatocyte membrane systems.

Parameter	Surface density (m^2/cm^3)[a]						
	Control (9)[b]	0.1% PTU (6)	P[c]	0.2% PTU (6)	P	0.5% PTU (6)	P
Rough endoplasmic reticulum	2.80 ± 0.19[d]	2.94 ± 0.33	NS	2.76 ± 0.26	NS	2.26 ± 0.17	NS
Smooth endoplasmic reticulum	3.05 ± 0.17	3.60 ± 0.20	NS	2.79 ± 0.16	NS	2.78 ± 0.21	NS
Golgi	0.32 ± 0.07	0.38 ± 0.12	NS	0.30 ± 0.05	NS	0.19 ± 0.09	NS

[a]Surface area of component per unit volume intralobular liver tissue.

[b]Number of animals in each group is in parentheses.

[c]P values are given for each PTU group compared with controls.

[d]Values are means ± SE.

Table 7. Effect of PTU on specific surface areas of 3-day postnatal hepatocyte membrane systems.

Parameter	Specific surface area (μm^2)[a]					
	Control (9)[b]	0.1% PTU (6)	P[c]	0.2% PTU (6)	P	0.5% PTU (6)	P
Rough endoplasmic reticulum	12058 ± 1288[d]	15509 ± 2711	NS	12400 ± 1069	NS	8710 ± 531	NS
Smooth endoplasmic reticulum	13247 ± 1457	18342 ± 2069	NS	12593 ± 786	NS	10718 ± 686	NS
Golgi	1317 ± 283	1835 ± 469	NS	1365 ± 204	NS	752 ± 369	NS

[a]Surface area of component per average mononuclear hepatocyte.

[b]Number of animals in each group is in parentheses.

[c]P values are given for each PTU group compared with controls.

[d]Values are means ± SE.

Table 8. Effect of PTU on nuclear numerical densities.

Treatment	nuclei/cm^3 liver tissue ($X\ 10^6$)	P
Control (9)[a]	256 ± 25[b]	-
0.1% PTU (6)	219 ± 25	NS[c]
0.2% PTU (6)	218 ± 9	NS
0.5% PTU (6)	260 ± 8	NS

[a]Number of animals in each group is in parentheses.

[b]Values are means ± SE.

[c]P values are given for each PTU group compared with controls.

consequence of the high variability in cell size (Table 5) and nuclear numerical density (Table 8), which suggest a tendency toward fewer, larger hepatocytes.

It was next decided to test two higher doses of PTU and to start the treatment on the 12th day of pregnancy, since the possibility existed that the 0.1% PTU dose had not sufficiently inhibited thyroid hormone production. The results of these experiments are also shown in Tables 4-8. Control data were pooled with those from the 0.1% PTU study, since there was no significant difference between them. The higher PTU doses had no effect on any of the ultrastructural parameters measured morphometrically. Figures 16-18 show that hepatic morphology at the various PTU doses was well-preserved and appeared quite normal when compared with the 3-day postnatal control (Fig. 15).

Effects of hyperthyroidism on fetal liver structure and function

Experiments with the thyroid hormone analog DIMIT were carried out in order to determine if an earlier than normal exposure to thyroid hormones could accelerate hepatic development, i.e., the converse of the PTU experiments. DIMIT was administered to pregnant rats by intramuscular injection and 20-day fetal livers were examined for changes in fine structure and certain microsomal functions. Since this compound had never been analyzed with regard to effects on liver, a dose response study was first performed in which two microsomal enzymes, NADPH-cytochrome c reductase and glucose-6-phosphatase, were assayed. The results are shown in Table 9. A significant elevation of glucose-6-phosphatase activity was observed only when 10 µg DIMIT were administered per 100 grams maternal BW for the two days prior to

Table 9. Effect of various DIMIT doses and treatment schedules on microsomal NADPH-cytochrome c reductase and glucose-6--phosphatase activities in 20-day rat fetuses.

Treatment	n	NADPH-cytochrome c reductase				Glucose-6-phosphatase			
		Activity[a]/mg microsomal protein	P[b]	Activity/g liver	P	Activity[c]/mg microsomal protein	P	Activity/g liver	P
Vehicle[d]	3[e]	2.4 ± 0.6[f]	-	18.0 ± 7.1	-	49.7 ± 14.2	-	375.3 ± 152.8	-
20 µg DIMIT/100 g BW/2 days[g]	3	5.3 ± 1.1	NS	46.1 ± 13.8	NS	69.8 ± 7.8	NS	600.0 ± 118.8	NS
10 µg DIMIT/100 g BW/2 days	3	6.2 ± 0.4	<.01	52.2 ± 4.0	<.01	95.1 ± 1.7	<.025	806.6 ± 47.8	<.05
10 µg DIMIT/100 g BW/5 days[h]	3	9.2 ± 0.8	<.005	73.5 ± 2.2	<.001	61.1 ± 8.5	NS	503.6 ± 103.4	NS
5 µg DIMIT/100 g BW/5 days	3	7.3 ± 0.7	<.005	53.2 ± 5.8	<.02	84.5 ± 12.2	NS	614.2 ± 97.7	NS
2 µg DIMIT/100 g BW/5 days	3	7.2 ± 0.6	<.005	60.9 ± 12.4	<.05	88.2 ± 16.0	NS	758.7 ± 226.4	NS

[a]Activity expressed as nmoles cytochrome c reduced/minute.
[b]P values are given for each treatment group compared with controls.
[c]Activity expressed as pmoles glucose-6-phosphate produced/minute.
[d]Absolute ethanol/0.9% NaCl (1:1).
[e]Fetal livers from each of 3 litters were pooled.
[f]Values are means ± SE.
[g]Treatments once daily on the 18th and 19th days of pregnancy.
[h]Treatments once daily on the 15th-19th days of pregnancy.

sacrifice. The NADPH-cytochrome c reductase demonstrated a more typical pattern of dose response, with 10 μg DIMIT per 100 g BW for 5 days clearly the most effective dose. Therefore, this latter treatment schedule was selected for subsequent studies.

The 10 μg/5 days DIMIT dose was next tested for its effectiveness in causing hyperthyroidism, as demonstrated by suppression of maternal serum TSH concentrations. Five control females had a mean TSH level (± SE) of 568 ± 159 ng per ml on the 20th day of pregnancy. Three DIMIT-treated females all had TSH levels below 250 ng/ml, which was the lower limit of sensitivity of the radioimmunoassay. The normal range was 366-1158 ng/ml.

The results of further studies of DIMIT effects on hepatic microsomes from 20-day fetal rats, using larger numbers of samples, are presented in Table 10. A highly significant effect on NADPH--cytochrome c reductase activity was confirmed (3.8-fold increase), and this time an effect, though smaller, on glucose-6-phosphatase activity was also observed (1.5-fold increase). Adult activities determined simultaneously were, for the reductase, 31.3 ± 2.9 nmoles/min/mg protein and, for the phosphatase, 146.5 ± 10.5 pmoles/min/mg protein. The heme protein cytochrome P-450 was not detectable in either control or DIMIT-treated fetuses. This was not attributable to a technical problem since adult rat liver microsomes prepared at the same time as the fetal samples were used as internal controls and had acceptable concentrations of cytochrome P-450 (0.52 ± 0.05 nmoles/mg microsomal protein; n = 8).

The effects of DIMIT on 20-day fetal liver morphology are shown in light and electron micrographs (Figs. 19-22). Hepatic structure

Table 10. Effect of DIMIT on 20-day fetal microsomal NADPH--cytochrome c reductase, glucose-6-phosphatase, and cytochrome P-450.

Microsomal function	Activity[a]/mg microsomal protein			Activity/g liver		
	Control (8)[b]	DIMIT (8)	P	Control (8)	DIMIT (8)	P
NADPH-cytochrome c reductase (nmoles cytochrome c reduced/min)	2.5 ± 0.2[c]	9.6 ± 0.7	<.001	19.6 ± 2.5	80.0 ± 6.8	<.001
Glucose-6-phosphatase (pmoles glucose-6--phosphate produced/min)	50.7 ± 5.5	76.7 ± 8.9	<.05	400.4 ± 59.3	647.5 ± 88.4	<.05
Cytochrome P-450 (nmoles)	not detectable			not detectable		

[a]Activity refers to units listed for each microsomal function, e.g., nmoles/min, pmoles/min, or nmoles.

[b]Number of litters assayed. Livers from each litter were pooled for microsome preparation.

[c]Values are means ± SE.

was generally well preserved in both control and experimental animals. Glycogen was not observed in most DIMIT-treated hepatocytes (Figs. 20 and 22), and by qualitative examination these cells appeared to be much smaller than control hepatocytes.

A decrease in the average volume of fetal hepatic parenchymal cells after DIMIT administration was confirmed by morphometric analysis (Table 11). However, this large (46%) reduction in specific hepatocyte volume was much greater than that measured for hepatocyte volume density (15%). Such a discrepancy suggested either a very large simultaneous decrease in liver weight and/or an increase in the number of hepatocytes per unit volume of liver. In order to resolve this problem, both liver weights and hepatocyte nuclear numerical densities were determined. The mean weight of 8 DIMIT-treated fetal livers (± SE) was 193 ± 4 mg, as compared with 215 ± 10 mg for 6 controls. This 10% decrease in liver weight of experimental animals was significant ($P < .05$), but insufficient to account for the marked difference between the volume density and the specific volume of hepatocytes from DIMIT-treated fetuses. On the other hand, the hepatocyte nuclear numerical density in the experimental group was 451 X 10^6 per cm^3 of intralobular liver tissue, which represented a 56% increase over the control value of 289 X 10^6. Neither light nor electron micrographs revealed many binucleate hepatocytes in either control or treated animals. Thus, the data clearly suggest that DIMIT promoted cell division.

The DIMIT-induced decrease in average hepatocyte size was accompanied by comparable reductions in the specific volumes of both

Table 11. Effect of DIMIT on volume densities and specific volumes of 20-day fetal liver components.

Parameter	Volume density (cm^3/cm^3)[a]			Specific volume (μm^3)		
	Control (7)[b]	DIMIT (8)	P	Control (7)	DIMIT (8)	P
Extrahepatocyte space	0.15 ± 0.02[c]	0.28 ± 0.02	<.001	-	-	
Hepatocyte	0.85 ± 0.02	0.72 ± 0.02	<.001	2985 ± 136[d]	1614 ± 76	<.001
nuclei	0.11 ± <0.01	0.10 ± 0.01	NS	395 ± 25	216 ± 28	<.001
cytoplasm	0.74 ± 0.02	0.62 ± 0.01	<.001	2590 ± 114	1398 ± 68	<.001
mitochondria	0.07 ± 0.01	0.11 ± <0.01	<.001	253 ± 19	252 ± 11	NS
lipid droplets	0.006 ± 0.003	0.002 ± 0.001	NS	20 ± 11	6 ± 2	NS
lysosomes	0.003 ± <0.001	0.004 ± <0.001	NS	10 ± 1	8 ± 1	NS
microbodies	0.002 ± <0.001	0.002 ± <0.001	NS	5 ± <1	5 ± 1	NS

[a]Volume per unit volume intralobular liver tissue; per cent.

[b]Number of animals in each group is in parentheses.

[c]Values are means ± SE.

[d]Volume of average mononuclear hepatocyte. All other specific volumes are based on this calculated average.

cytoplasmic and nuclear compartments (Table 11). The volume density of hepatocyte cytoplasm was also decreased, but less dramatically, while volume density of nuclei was unaffected. The latter result may also reflect an increased mitotic index, with nuclei smaller but more numerous during a period of active cell division. Nuclear/cytoplasmic ratios remained quite constant (0.15-0.16) in both control and experimental animals.

Mitochondrial volume density was significantly elevated by DIMIT treatment, although the volume of mitochondria per average cell remained unchanged. There was no apparent difference in mitochondrial size. Thus, mitochondrial division must have occurred at a rate similar to that of hepatocellular division, with the result that the normal number of mitochondria per cell was maintained in the DIMIT--treated hepatocytes.

No significant changes in relative or specific volumes of lipid droplets, lysosomes, or microbodies were found after administration of the thyroid hormone analog.

Measurements of RER, SER, and Golgi membranes, expressed both as surface densities and specific surface areas, are shown in Table 12. The surface densities of these components were unaffected by DIMIT. However, the specific surface areas of all three membrane systems were greatly decreased. This probably reflects the large increase in number of hepatocytes that occurred after hormone treatment. The data suggest that significant membrane synthesis did not occur or, possibly, that the rate of membrane synthesis was equal to the rate of membrane degradation.

Table 12. Effect of DIMIT on surface densities and specific surface areas of 20-day fetal hepatocyte membrane systems.

Parameter	Surface density (m^2/cm^3)[a]			Specific surface area (μm^2)[b]		
	Control (7)[c]	DIMIT (8)	P	Control (7)	DIMIT (8)	P
Rough endoplasmic reticulum	3.38 ± 0.08[d]	3.17 ± 0.12	NS	11866 ± 546	7151 ± 515	<.001
Smooth endoplasmic reticulum	1.43 ± 0.13	1.28 ± 0.12	NS	4969 ± 385	2881 ± 307	<.001
Golgi	0.49 ± 0.13	0.33 ± 0.08	NS	1650 ± 384	708 ± 155	<.05

[a]Surface area of component per unit volume intralobular liver tissue.

[b]Surface area of component per average mononuclear hepatocyte.

[c]Number of animals in each group is in parentheses.

[d]Values are means ± SE.

Effects of 0.3% PTU on neonatal liver structure and function

After determining that an early exposure to thyroid-like activity could indeed affect liver development both structurally and functionally, the hypothyroid condition in neonates was once again examined, but from a different point of view. The DIMIT experiments had clearly shown that microsomal enzyme activities could be stimulated without an increase in endoplasmic reticulum. Therefore, it was possible that PTU treatment could also affect enzyme activities even though the earlier morphometric studies with this compound had demonstrated no effect on intracellular membrane quantities.

A dose of 0.3% PTU in the diet was chosen at this time because newly available data showed that this dose was highly effective in producing hypothyroidism in fetal rats (Comite et al., in press). The PTU was administered to rats from the 12th day of pregnancy onward as before. On the third day after delivery, two mothers were sacrificed and their serum TSH concentrations were determined to be 3871 ± 583 ng/ml, as compared with 412 ± 62 ng/ml for two controls ($P < .01$).

The effect of PTU on microsomal functions is presented in Table 13. There was a significant reduction in glucose-6-phosphatase activity. NADPH-cytochrome c reductase and cytochrome P-450 were unchanged.

In order to confirm that there was nothing unusual about the 0.3% PTU dose in terms of its effect on hepatic ultrastructure, a morphometric analysis was done (Tables 14 and 15). Like the other PTU doses, this level produced no measurable change in any of the parameters examined.

Table 13. Effect of PTU on 3-day postnatal hepatic microsomal NADPH-cytochrome c reductase, glucose-6-phosphatase, and cytochrome P-450.

Microsomal function	Activity[a]/mg microsomal protein			Activity/g liver		
	Control (4)[b]	PTU (4)	P	Control (4)	PTU (4)	P
NADPH-cytochrome c reductase (nmoles cytochrome c reduced/min)	7.4 ± 0.7[c]	6.6 ± 0.9	NS	61.8 ± 6.4	55.7 ± 6.1	NS
Glucose-6-phosphatase (pmoles glucose-6-phosphate produced/min)	151.2 ± 3.9	120.3 ± 3.8	<.001	1261.0 ± 48.6	1027.8 ± 55.1	<.02
Cytochrome P-450 (nmoles)	0.18 ± 0.02	0.19 ± 0.02	NS	1.51 ± 0.17	1.58 ± 0.10	NS

[a]Activity refers to units listed for each microsomal function, e.g., nmoles/min, pmoles/min, or nmoles.

[b]Number of litters assayed. Livers from each litter were pooled for microsome preparation.

[c]Values are means ± SE.

Table 14. Effect of 0.3% PTU on volume densities and specific volumes of 3-day postnatal liver components.

Parameter	Volume density (cm^3/cm^3)[a]			Specific volume (μm^3)		
	Control (4)[b]	PTU (4)	P	Control (4)	PTU (4)	P
Extrahepatocyte space	0.13 ± 0.01[c]	0.16 ± 0.02	NS	-	-	
Hepatocyte	0.87 ± 0.01	0.84 ± 0.02	NS	3498 ± 58[d]	3481 ± 189	NS
nuclei	0.09 ± 0.01	0.09 ± 0.01	NS	378 ± 40	353 ± 34	NS
cytoplasm	0.78 ± 0.01	0.75 ± 0.01	NS	3120 ± 40	3128 ± 62	NS
mitochondria	0.15 ± <0.01	0.16 ± 0.01	NS	594 ± 16	673 ± 45	NS
lipid droplets	0.074 ± 0.032	0.042 ± 0.013	NS	300 ± 130	179 ± 55	NS
lysosomes	0.005 ± 0.001	0.005 ± 0.001	NS	19 ± 3	22 ± 3	NS
microbodies	0.005 ± 0.001	0.006 ± 0.001	NS	21 ± 3	27 ± 2	NS

[a]Volume per unit volume intralobular liver tissue; per cent.

[b]Number of animals in each group is in parentheses.

[c]Values are means ± SE.

[d]Volume of average mononuclear hepatocyte. All other specific volumes are based on this calculated average.

Table 15. Effect of 0.3% PTU on surface densities and specific surface areas of 3-day postnatal hepatocyte membrane systems.

Parameter	Surface density (m^2/cm^3)[a]			Specific surface area (μm^2)[b]		
	Control (4)[c]	PTU (4)	P	Control (4)	PTU (4)	P
Rough endoplasmic reticulum	3.37 ± 0.08[d]	3.66 ± 0.15	NS	13551 ± 374	15218 ± 885	NS
Smooth endoplasmic reticulum	2.87 ± 0.25	3.45 ± 0.30	NS	11545 ± 964	14317 ± 1267	NS
Golgi	0.23 ± 0.06	0.28 ± 0.04	NS	926 ± 246	1166 ± 164	NS

[a]Surface area of component per unit volume intralobular liver tissue.

[b]Surface area of component per average mononuclear hepatocyte.

[c]Number of animals in each group is in parentheses.

[d]Values are means ± SE.

Effect of combined DIMIT and glucagon treatment on 20-day fetuses

Rats were treated as before with 10 µg DIMIT per 100 g BW or vehicle from the 15th through the 19th day of pregnancy. On the 20th day of gestation the fetuses were given 50 µg glucagon or saline by the direct intraperitoneal injection technique used in the glucagon experiments described previously. Six hours later the fetuses were sacrificed for liver microsome preparation and biochemical assay (Table 16). DIMIT treatment produced the expected elevation in NADPH--cytochrome c reductase activity, expressed either per mg microsomal protein ($P < .001$) or per g liver ($P < .01$). DIMIT-treated microsomes also had greater glucose-6-phosphatase activity per mg microsomal protein ($P < .05$) but this time the increase was not significant when expressed per g liver. Administration of glucagon did not produce any change in activity of either enzyme. None of the 4 treatment groups had detectable concentrations of cytochrome P-450.

Table 16. Effect of DIMIT + glucagon on 20-day fetal microsomal NADPH-cytochrome c reductase, glucose-6-phosphatase, and cytochrome P-450.

Treatment	n	NADPH-cytochrome c reductase		Glucose-6-phosphatase	
		Activity[a]/mg microsomal protein	Activity/g liver	Activity[b]/mg microsomal protein	Activity/g liver
+ DIMIT + glucagon	3[c]	10.7 ± 1.1[d]	39.4 ± 9.3	68.5 ± 20.3	229.5 ± 33.1
+ DIMIT - glucagon	3	12.4 ± 0.8	50.1 ± 12.1	72.7 ± 6.9	295.6 ± 79.6
- DIMIT + glucagon	3	2.7 ± 0.4	13.4 ± 4.1	29.9 ± 6.5	133.7 ± 22.0
- DIMIT - glucagon	3	3.1 ± 1.1	12.5 ± 3.4	50.6 ± 16.4	214.1 ± 53.7

[a]Activity expressed as nmoles cytochrome c reduced per minute.

[b]Activity expressed as pmoles glucose-6-phosphate produced per minute.

[c]Fetal livers from each of 3 litters were pooled.

[d]Values are means ± SE.

DISCUSSION

Correlation of cellular structure with function: general comments and relevance of the present study

That every change in function must be reflected in an alteration in structure (and vice-versa) is a basic tenet of cell biology. In the light of experiments such as those presented here, it is appropriate to critically review and evaluate this concept.

The correlation between structure and function may be thought of in terms of two levels of control. On a coarse level, there is absolute correspondence between structure and function. The genetic material is duplicated in the nucleus, oxidative phosphorylation takes place in mitochondria, proteins are assembled on ribosomes, and so forth. Each function can be assigned to a cellular structure or compartment. It is on a finer level of control that the association can be questioned. When the activity of a particular function is increased or decreased, is there a change in the corresponding structure? Conversely, if the structure changes, is there a functional change?

The answer to these two questions is, clearly, "yes" in many cases. For example, after tenotomy of a skeletal muscle synergist, the remaining muscle will undergo a compensatory hypertrophy. In response to the increased work load on this muscle, the mitochondrial volume density will also increase (Seiden, 1976). In rat kidney, thyroid hormone administration results in a stimulation of $Na^+ + K^+$--activated adenosine triphosphatase activity. This is due to a proportional increase in the number of sodium pump sites (Edelman, 1976).

In the jejunum of patients with tropical sprue, the loss of certain absorptive capabilities is accompanied by shortening of intestinal epithelial microvilli and other irregularities in these organelles (Mathan et al., 1975). In livers of phenobarbital-treated animals, SER is hypertrophied and drug metabolizing enzyme activities are increased (Ernster and Orrenius, 1965; Jones and Fawcett, 1966).

On the other hand, the answer to the questions posed above is, apparently, "no" in certain circumstances. For example, oxandrolone administration to rats results in an increase in the quantity of SER (Schmucker and Jones, 1975) that is not paralleled by changes in activities of drug metabolizing enzymes measured so far (Anthony, 1976). In the present study, NADPH-cytochrome c reductase and glucose--6-phosphatase activities were elevated and no change in surface density of ER was observed.

The examples which do not support a structure-function correlation need to be examined more closely. Hypertrophy of SER in oxandrolone--treated animals may well be reflected in a functional change not tested. Alterations in drug metabolizing enzyme activities may be accompanied by changes in membrane structure that are more subtle than simple increases or decreases in quantity of membrane. The study of Jothy et al. (1974) provides an excellent example. They applied spin labeling techniques and found a molecular change in rat liver RER structure after triiodothyronine administration.

Although the present study does not reveal a functional change for every structural alteration, or vice-versa, one cannot conclude that the correspondence does not exist. Even a simple chemical reaction involves molecular interactions which in the strictest sense

are structural changes. Thus, structure and function cannot truly be separated, and our ability to observe the appropriate correlative events is the limiting factor.

Development of liver structure: comparison of Holtzman with other strains of rats

In general, both the qualitative and quantitative aspects of developing Sprague-Dawley, Holtzman rat liver morphology agreed quite well with those presented by previous investigators for other rat strains. However, in several aspects, particularly with regard to glycogen storage, the Holtzman rats appeared to develop slightly ahead of some other strains. For example, Holtzman rat liver sometimes contained a small amount of glycogen as early as the 17th day of gestation and it invariably had large masses of this material by the 20th gestational day. Livers of Carworth CFN strain rats were similar in that they contained rather large glycogen deposits by about the 19th day of gestation (Dallner et al., 1966a), while Wistar rats possessed only very small quantities at the same stage of development (Rohr et al., 1971). In addition, Holtzman strain rats may have mobilized glycogen less rapidly after birth. These differences in turn affected certain other parameters such as cellular composition of the liver and hepatocyte size. Hepatocytes of Holtzman rats had a mean volume of 2985 ± 136 μm^3 at 20 days of gestation compared with 1392 μm^3 for Wistar rats at 19 days (Rohr et al., 1971), a large difference which was probably due to the earlier onset of glycogen storage in the former strain. At 20 days of fetal development Holtzman rat liver was already composed of 85% parenchymal cells by volume, whereas NEDH strain liver was composed of only 68%

parenchymal cells at 20 days (Greengard et al., 1972) and, even at birth on the 22nd day, Wistar rat liver still contained only 76% hepatocytes by volume.

Twenty-day old fetal Holtzman rat liver contained about 7% hepatocyte mitochondria by volume. This compares well with 5% measured for Wistar rats at 19 days of gestation (Rohr et al., 1971) but is much less than the 13% reported for 20-day fetal rats of the Kx strain (Herzfeld et al., 1973). However, the latter was expressed in terms of a cm^3 of liver cell cytoplasm rather than of intralobular liver tissue. If one assumes a volume density for cytoplasm of about 0.60, which is a reasonable estimate given a hepatocyte volume density of 0.70 (Greengard et al., 1972) and a nuclear volume density that is relatively constant (0.08-0.10), then a cm^3 of intralobular tissue from a 20-day Kx fetus contained about 8% mitochondrial volume. This is very similar to the figure obtained for other strains.

A few lipid droplets, lysosomes, and microbodies were present during the last few days of gestation not only in Holtzman rats but in the other strains as well. However, in previous studies these rare cell components were not quantitated and, therefore, comparison of data is impossible. Measurements from other strains for comparison with those for fetal Holtzman Golgi surface density are likewise not available.

Surface densities of ER were determined in only one previous analysis of perinatal liver development, and were found to be 4.3 and 0.8 m^2 per cm^3 liver cell cytoplasm, respectively, for RER and SER of 20-day fetuses (Herzfeld et al., 1973). Converting these values to a base of intralobular liver tissue as before, one arrives at values of

2.6 m^2 RER and 0.5 m^2 SER per cm^3. Holtzman rats had 3.4 m^2 RER and 1.4 m^2 SER per cm^3 liver tissue. These are not very great differences when one takes into account sample variability and inconsistencies in methodology which may be present between laboratories. For example, membranes which are mostly ribosome-studded may also have large ribosome-free areas, especially at dilated ends of the flattened stacks. Some investigators might arbitrarily count these areas as entirely belonging to the RER, whereas in the present study they were included with the SER. In addition, there were many small smooth membrane vesicles (excluding coated vesicles) that were included here with the SER even though they did not have the typical tubular configuration. Herzfeld et al. (1973) did not explain how their measurements were made.

When compared with published data for adult rats of the same strain (Anthony et al., in press; Schmucker, 1976; Schmucker et al., 1974), 20-day fetal Holtzman rat livers already contained the adult percentage of parenchymal cells. They contained, per cm^3 of intralobular liver tissue, approximately 35% of the mitochondrial volume, 75% of the lysosomal volume, and 20% of the peroxisomal volume of adult Holtzman rat liver. Golgi surface density had already reached the adult level, while RER surface density had at least achieved and perhaps exceeded the adult value. (Published adult RER surface densities were highly variable.) Finally, 20-day fetal liver contained about 30% of the adult quantity of SER when expressed in terms of surface density. (Before comparison with fetal data, all adult surface densities were converted from a base of ground substance to liver

tissue, using a volume density of 0.6 for ground substance; Schmucker, 1976.)

A similar presentation of data for other strains can only be incomplete, and available values require conversion to a common denominator, i.e., a base of intralobular liver tissue, before comparison with Holtzman rats is possible. By the 20th fetal day both NEDH and Kx rats were about 80% mature in terms of parenchymal cell contribution to liver volume (Greengard et al., 1972; Herzfeld et al., 1973). Given an adult hepatocyte volume density of 88% (Herzfeld et al., 1973) and an estimated nuclear volume density of 0.1, a cytoplasmic volume per cm^3 liver tissue of 0.8 was used in the subsequent mathematical conversions. Twenty-day fetal Kx rats were then calculated to have 50% of the adult mitochondrial volume density, 100% of the adult RER surface density, and 30% of the adult SER surface density (Herzfeld et al., 1973).

Although by three days after birth Holtzman rats had lost much of their glycogen, many hepatocytes still contained considerable quantities of this material. In Wistar rats, on the other hand, the typical neonatal mobilization of glycogen was nearly complete within 24 hours after birth (Rohr et al., 1971). Even so, hepatocytes were not entirely depleted of glycogen, and thus a precise comparison between strains would require a tissue glycogen determination. A hepatocyte size of 3583 ± 280 μm^3 in Holtzman rats compared with 2713 μm^3 for Wistar rats (Rohr et al., 1971) would, however, support a more rapid glycogen loss from the latter strain. Measurement of NEDH rat hepatocytes at 2 days after birth (2600 μm^3) suggests they

may be more like Wistar than Holtzman rats in terms of glycogen mobilization (Greengard et al., 1972).

Hepatocyte volume density at 3 days after birth was 0.84 in Holtzman rats and 0.81 in Wistar rats (Rohr et al., 1971). It was 0.83 in NEDH rats on postnatal day 2 (Greengard et al., 1972) and assumed to be the same in Kx rats by Herzfeld et al. (1973).

Volume density of mitochondria was 0.15 in Holtzman rats and 0.22 in Wistar rats (Rohr et al., 1971) at 3 days of age. It can be estimated at 0.17 in Kx rats at 2 days after birth if one assumes a hepatocyte cytoplasmic volume per cm^3 liver tissue of 0.75 (Herzfeld et al., 1973). This is reasonable if one accepts the hepatocyte volume density data for Kx rats presented above.

There are no data from other strains to compare with those for postnatal Holtzman lipid droplet volume density or Golgi surface density. Holtzman rats had volume densities of 0.006 each for hepatic lysosomes and microbodies, while in 3-day old Wistar rat livers, lysosomes occupied 0.004 cm^3 and microbodies occupied 0.012 cm^3 of each cm^3 of intralobular liver tissue.

Surface densities of RER and SER from 3-day old Holtzman rats were 2.8 m^2/cm^3 and 3.0 m^2/cm^3, respectively. At 2 days after birth, Kx rats had 3.5 m^2 RER per cm^3 liver tissue, after mathematical conversion as described above (Herzfeld et al., 1973). Since RER surface density does not change significantly after birth, the 2-day and the 3-day values can probably be compared and, considering in-strain animal variability and methodology differences discussed before, the data are quite similar. SER was still very sparse in Kx rats at 2

days after birth (about 0.45 m^2/cm^3 liver tissue; Herzfeld et al., 1973), but the adult quantity had been achieved by the next time point examined, which was 12 days after birth. No surface density measurements were made at 3 days in this or any strain before the present study, so absolute comparisons are impossible. However, the SER volume density data of Rohr et al. (1971) confirm an increase in this parameter from the time of delivery until at least 8 days after birth and, especially, between the first and third postnatal days. That the greatest developmental formation of SER occurs in early postnatal life is certain for all rat strains so far examined either qualitatively or quantitatively. However, not enough time points have been examined quantitatively for any strain to precisely determine the pattern of this development, let alone to allow for accurate day-by--day comparisons between strains. What data are available do support Rohr et al.'s (1971) contention that neonatal glycogen mobilization precedes SER proliferation and thus that there is no direct involvement of SER in glycogenolysis at this time. That SER does play a role in hepatic glycogen breakdown was suggested previously by several investigators (Peters et al., 1962; Jones and Fawcett, 1966).

When compared with adult Holtzman rat livers (Anthony et al., in press; Schmucker, 1976; Schmucker et al., 1974), 3-day neonatal Holtzman livers had the same hepatocyte volume density. They also were characterized by 75%, 150%, and 50% of the adult volume densities of mitochondria, lysosomes, and microbodies, respectively. Rohr et al. (1971) also noted a very high postnatal lysosomal volume density in Wistar rats. In their studies, however, it was observed on the first rather than the 3rd day after birth, and was followed by a decline to

less than the adult level. An adult Golgi surface density was found in 3-day Holtzman neonates, as it had been in 20-day fetuses. The same was true of RER. Finally, the 3 day old rat livers contained about 70% of the adult quantity of SER, expressed in terms of surface density.

A few comparative (% adult) measurements are available for Wistar rats (Rohr et al., 1971; Weibel et al., 1969). At 3 days after birth "percent adult" volume densities were 98% for parenchymal cells, 100% for mitochondria, 60% for lysosomes, and 100% for microbodies. By two days after birth, Kx and NEDH rats were about 95% mature in terms of hepatocyte volume density (Greengard et al., 1972; Herzfeld et al., 1973). Kx rats at this stage of development also had achieved an adult mitochondrial volume density and RER surface density. As indicated earlier, SER surface area per cm^3 liver tissue increased rapidly between the 2nd (30% of adult) and the 12th (100% of adult) postnatal days (Herzfeld et al., 1973).

A summary of the above comparisons between late fetal, early neonatal, and adult livers is presented in Table 17. It includes only Holtzman and Kx rats, however, since data in all 3 age categories are available for just these 2 strains.

Direct intraperitoneal injection of 20-day old fetuses with glucagon, thyroxine, or a combination of both hormones

A dose of 3 µg thyroxine was chosen for these experiments because the same amount was successfully used in earlier studies to produce premature increases in NADPH-cytochrome c reductase and glucose-6-phosphatase activities of late fetal rats (Greengard and Dewey, 1968).

Table 17. Quantities of hepatocyte components in Holtzman and Kx strain rats presented as percentages of adult values for the same strain.

Parameter	Holtzman[a]		Kx[b]	
	20-day fetal	3-day postnatal	20-day fetal	3-day postnatal[c]
Volume density[d]				
hepatocytes	100	100	80	95
mitochondria	35	75	50	100
lysosomes	75	150	-	-
microbodies	20	50	-	-
Surface density[e]				
RER	100	100	100	100
SER	30	70	30	>30[f]
Golgi	100	100	-	-

[a]Adult values were obtained from Anthony et al. (in press), Schmucker (1976), and Schmucker et al. (1974). Fetal and neonatal measurements were made during the course of the present study.
[b]Data for Kx strain were obtained from Herzfeld et al. (1973).
[c]Measurements in the Kx strain were actually made on the 2nd day after birth. Therefore, values given for 3 days are minimum percentages.
[d]Volume densities were all expressed as cm^3 of component per cm^3 intralobular liver tissue before further comparison.
[e]Surface densities were all expressed as m^2 of membrane per cm^3 intralobular liver tissue before further comparison.
[f]A value of 30% was determined for the 2nd postnatal day. An increase occurs soon after but the precise day was not determined (Herzfeld et al., 1973).

The dose of 50 μg glucagon significantly stimulated glucose-6--phosphatase in the same experiments (Greengard and Dewey, 1968). This quantity of glucagon was also reported to induce several normal but premature structural changes in 20-day fetal hepatocytes, including an increase in the number of lysosomes and microbodies and in the quantity of SER (Chiu and Phillips, 1974). The morphological effects of glucagon were visible by 6 hours after injection (Chiu and Phillips, 1974) and the biochemical effects of both hormones were significant within 5 hours, although thyroxine was a more effective inducer at 24 than at 5 hours after injection (Greengard and Dewey, 1968). In view of these results, a time point of 6 hours after injection was chosen in the present morphological studies. No effects of either hormone alone or the hormone combination were observed by routine electron microscopic examination. That thyroxine did not cause changes in liver structure is not surprising. Greengard et al. (1972) were unable to demonstrate a structural hepatic effect of a much higher dose of thyroxine (30 μg) even after 24 hours. Theirs was a morphometric analysis, albeit of only a few parameters, but should have revealed subtle changes that would be undetected by qualitative examination. Furthermore, there is no reason to assume that structural effects of thyroid hormone would necessarily be produced as rapidly as biochemical changes.

A lack of a morphological effect of glucagon in the present study is more disturbing, in view of Chiu and Phillips' (1974) results with the same experimental design. There are at least three possible explanations for the discrepancy in results. First, it may be that

there was a problem of technique and that the full dose of glucagon was not delivered to the fetuses in the present experiments. This must be considered as a strong possibility for both the glucagon and thyroxine experiments. As discussed earlier, the technique has many inherent difficulties of which leakage from the injection site is a major one. Secondly, the experiments were qualitative rather than quantitative, and small but significant changes in hepatic ultrastructure may have been missed. Finally, the study of Chiu and Phillips (1974) was not quantitative either and, as a result, many of the reported morphological effects of glucagon may have been interpreted incorrectly. For example, cell size was not determined and a decrease in this parameter could have resulted in an apparent, rather than a real, increase in lysosomes and microbodies. It is in fact likely that cell size was reduced, since the glucagon treatment resulted in glycogen depletion. RER was vesiculated in glucagon-treated fetuses. If the amount of RER was also decreased--for example, by degranulation--an increased quantity of SER would have been observed. The normal postnatal proliferation of SER, in contrast, is not at the expense of RER (Rohr et al., 1971).

There is one morphometric study of hepatic effects of glucagon injected into 20-day fetal rats (Greengard et al., 1972). Only a few parameters were measured (hepatocyte volume density and specific volume, nuclear numerical density); however, no significant changes were observed. It must be concluded that there is still no definitive evidence that a single injection of thyroxine or glucagon injected into a fetus in late gestation can stimulate structural differentiation of

hepatocytes along a premature but otherwise normal path.

Morphological effects of 0.1%, 0.2%, and 0.5% PTU on neonatal rat liver

Morphometric studies with the above three doses of PTU revealed no changes at all in hepatocyte ultrastructure of 3-day old rats. The only previous work which is available for comparison is a quantitative analysis of liver from young adult rats 21 days after thyroidectomy (Riede et al., 1975). Removal of the thyroid gland resulted in decreased hepatocyte size and also a reduction in specific volumes (per hepatocyte) of most organelles including nuclei, mitochondria, microbodies, lysosomes, and RER. There was, however, an increase in surface density of SER. It is difficult to interpret these data in view of the fact that no information on volume densities, nuclear numerical densities, or liver weights was provided and no electron micrographs were shown. Thus it is impossible to determine whether or not the reported decreases were real effects of thyroidectomy or simply due to tissue shrinkage. The observed increase in SER could have been due to degranulation of RER. Unfortunately, not enough information was given to determine whether the surface area of total endoplasmic reticulum changed as a result of thyroidectomy. The lack of an effect of PTU in the present experiments is therefore neither supported nor seriously challenged by Riede et al.'s (1975) study. Given just the morphometric data, one is forced to conclude either that thyroid hormones are not necessary for the perinatal morphological changes that are under consideration here, or that low circulating levels of thyroid hormones survived the PTU treatment and were sufficient to stimulate hepatocyte differentiation. It was not

possible to choose between these alternatives until the results of later experiments with the thyroid hormone analog, DIMIT, and with 0.3% PTU were obtained.

Effects of DIMIT on hepatocyte structure and function

Since the naturally occurring thyroid hormones are not readily transferred across the placenta (Chopra and Crandall, 1975; Dubois et al., 1977), their injection into pregnant rats is clearly not an effective means of producing hyperthyroid fetuses. On the other hand, it has been shown that the thyroxine analog, DIMIT, readily crosses the placenta and is highly effective in a fetal rat antigoiter assay (Comite et al., in press). Possible reasons for the enhanced placental transfer of this compound were presented earlier and included its small molecular weight relative to triiodothyronine and thyroxine and weakness of binding to plasma transport proteins.

When given to pregnant rats in the present study, DIMIT caused several changes in fetal hepatic structure and function. They were all changes that normally occur within the first three days after birth. For example, the glycogen content of hepatocytes usually increases during the last 3 days of fetal life and then drops sharply within 24 hours after birth (Rohr et al., 1971). DIMIT-treated 20-day fetal livers were, however, largely devoid of glycogen. Massive doses of thyroxine and diets containing desiccated thyroid had the same effect in adult rats (Battarbee, 1974; Callas and Cannon, 1975), at least partly as a result of activation of glycogen phosphorylase (Takahashi and Suzuki, 1975).

Concomitant with the pattern of glycogen accumulation and

depletion observed during late fetal and early neonatal stages there occurs an increase and a subsequent decrease in average hepatocyte volume. A 47% reduction in liver cell volume between birth and the 3rd postnatal day was reported in one morphometric study of developing rat liver (Rohr et al., 1971). DIMIT treatment resulted in a 46% decrease in the specific volume of 20-day fetal hepatocytes, from 2985 μm^3 to 1614 μm^3. Greengard et al. (1972) were unable to demonstrate an effect of thyroxine on hepatocyte size in fetuses of a comparable age. However, these investigators gave only a single dose of thyroxine, and this may be insufficient to elicit certain morphological changes.

According to the quantitative studies of Rohr et al. (1971), fetal rat hepatocytes divide once during the first 3 days of postnatal life. In the present study, DIMIT induced a marked increase in numerical density of fetal hepatocyte nuclei. These data also conflict with those of Greengard et al. (1972), who found no significant change in nuclear numerical density when thyroxine was injected into fetal rats.

In normal developing rat liver, the volume density of mitochondria more than doubles during the first day after birth, while the number of mitochondria per hepatocyte remains nearly constant (Rohr et al., 1971). Thus, mitochondrial division also occurs during this period of active cell division. In 20-day old fetal rats, DIMIT promoted a similar response, i.e., the volume density of mitochondria was significantly increased. Although the number of mitochondria per hepatocyte was not determined, the specific volume of this organelle was unchanged

by DIMIT. Since qualitative examination of hepatocyte fine structure did not reveal any obvious changes in the volume of individual mitochondria in the DIMIT-treated fetuses, the data suggest that DIMIT stimulated mitochondrial division. In adult rats, hyperthyroidism was variously reported to result in an increase (Paget and Thorp, 1963) or no change (Callas and Cannon, 1975) in the number of mitochondria in liver cells.

The microsomal enzymes, NADPH-cytochrome c reductase and glucose--6-phosphatase, become detectable during late gestation in rats (Dallner et al., 1966b). According to several studies with various rat strains (Greengard, 1969; Greengard and Dewey, 1968; Lang, 1965), 20-day old fetuses have about 10% of the adult hepatic NADPH-cytochrome c reductase activity and 25% of the adult glucose-6-phosphatase activity. By 2 days after birth, NADPH-cytochrome c reductase has reached approximately 65% of the adult level of activity. Glucose-6--phosphatase, on the other hand, surpasses the adult activity by about 12 hours after birth, reaches 200% of the adult level by the 2nd day, and then gradually returns to adult activity.

Developing Holtzman strain rats had a very similar overall pattern of enzyme differentiation, although adult activity of NADPH-cytochrome c reductase was reached more slowly. At 20 days of gestation it was 8% of that measured for adults. By 3 days after birth it had increased to 24% of adult activity. Twenty-day fetuses had 29% of the adult glucose-6-phosphatase activity. This also exceeded 100% soon after birth and then declined. It was measured at 103% of the adult activity on the 3rd postnatal day.

A dose of 10 μg DIMIT per 100 g maternal body weight significantly increased NADPH-cytochrome c reductase in 20-day fetal Holtzman liver to 29% of the adult activity (3.8-fold increase, $P < .001$), a level usually not attained until several days after birth. In fact, doses of DIMIT as low as 2 μg/100 g body weight were nearly as effective (3-fold increase, $P < .005$) as the 10 μg dose in increasing the activity of this enzyme. Glucose-6-phosphatase was also significantly stimulated by DIMIT, although the response was inconsistent and did not follow a typical dose response curve. There was no effect at the lowest doses of DIMIT tested. These data are in good agreement with those of most other investigators who have studied thyroxine effects on the above two microsomal enzymes in both fetuses (Greengard, 1969; Greengard and Dewey, 1968) and adults (Battarbee, 1974; Tata et al., 1963). However, the variability of an effect on glucose-6-phosphatase is also supported. Boxer et al. (1974) found no effect of thyroxine on this enzyme in fetal liver organ cultures.

Both NADPH-cytochrome c reductase and glucose-6-phosphatase are present initially on the RER. After birth, as SER begins to proliferate, these enzymes become distributed approximately equally between the two membrane systems. This evidence has been used to support the concept that the SER is derived, in part, from the RER (Dallner et al., 1966b). Additional evidence is provided by morphometric measurements, which show that RER undergoes a quantitative increase in late fetal life, probably between days 19 and 20. Thus an increase in RER immediately precedes the postnatal increase in SER (Rohr et al., 1971). In the present study, DIMIT induction of NADPH-cytochrome c reductase and glucose-6-phosphatase apparently occurred entirely within the RER

compartment and independently of net membrane synthesis. At 20 days of gestation control hepatocytes had an adult complement of RER and very little SER. Neither RER nor SER proliferation was induced by DIMIT. Thus the DIMIT experiments are in agreement with previous studies demonstrating that the enzymes are found first on the RER, but do not shed any light on the origin of SER.

Cytochrome P-450 is thought to reside on the SER (Jones and Mills, 1974). Strong evidence for this is provided by the observation that during normal development the cytochrome increases in concentration in parallel with the appearance of SER (Dallner et al., 1966b). In the present study cytochrome P-450 was undetectable in 20-day control or DIMIT-treated fetuses. This result plus the lack of an effect of the analog on SER support the proposed location of cytochrome P-450 on SER.

To date, investigation of thyroid hormone effects on hepatic ER have been confined to adult animals and the data are somewhat variable. For example, Douglas (1964) did not observe any alterations in either the SER or RER following the administration of thyroxine. In other studies, a slight dilatation of one or the other of these membrane systems was reported (Callas and Cannon, 1975; Shamoto, 1968). Callas and Cannon (1975) reported a slight increase in SER, although morphometry was not employed to verify this observation. In no case have striking changes in quantity or morphology of ER been found after thyroid hormone treatment. On the other hand, molecular changes in RER structure, which may help to elucidate the mechanism of enzyme activation were found using spin labeling techniques (Jothy et al., 1974). These investigators found that triiodothyronine administration

to thyroidectomized rats resulted in a change in the protein, but not the lipid, region of the RER. They speculated that either de novo protein synthesis or a conformational change in the membrane could account for their observations.

In the earlier description of changes that occur in normal developing rat liver, quantitative evidence for a lack of SER involvement in glycogen mobilization was cited. Further support is provided by the DIMIT experiments. In analog-treated livers, glycogen was essentially absent and no membrane development was observed. Since available evidence suggests that glycogen deposition is under the control of glucocorticoids and insulin (Eisen et al., 1973), it is unlikely that DIMIT interfered with this step. Rather, it is probable that glycogen degradation was stimulated.

The overall effect of DIMIT in the present experiments, then, was to initiate prematurely several morphological and functional changes in the liver which normally occur during the early postnatal period. Conspicuous by their absence were changes in the quantity of SER and cytochrome P-450, which during normal development occur concomitantly with alterations in those parameters that were affected by the hormone analog. The studies therefore support the theory that initiation of fetal thyroid hormone synthesis is the natural stimulus for many, but not all, of the hepatic changes typically observed around the time of birth. The DIMIT results also suggest that in the PTU experiments presented earlier enough thyroid hormone still remained in the fetal circulation to initiate all the structural liver changes regulated by the thyroid gland at this time of develop-

ment. The conclusions reached thus far were further refined upon consideration of the 0.3% PTU experiments.

Effects of 0.3% PTU on structure and function of neonatal liver

The morphometric analysis of livers from 3-day old rats after 0.3% PTU treatment agreed completely with results of the earlier PTU experiments, i.e., there was no measurable effect of the drug on hepatic structure. Treatment with 0.3% PTU did, however, produce one significant biochemical effect. It reduced the microsomal glucose-6--phosphatase activity by 20% ($P < .001$). Paradoxically, there was no effect on NADPH-cytochrome c reductase. The latter enzyme was significantly stimulated by even the lowest doses of DIMIT tested in the previous experiments, while glucose-6-phosphatase did not respond at all to the lower doses. Why should the responses of these two enzymes be exactly reversed in the hypothyroid as compared with the hyperthyroid state, and what is the significance of this observation for the developing animal? If thyroid hormones act as a trigger for the initiation of certain developmental changes in the rat liver one might expect that even low concentrations of hormone would be effective. The PTU experiments and the DIMIT dose response results support this proposal and suggest that thyroid hormones may normally regulate (directly or indirectly) the following perinatal hepatic changes: glycogen depletion with reduction in hepatocyte size, hepatocyte division, mitochondrial division, and increased NADPH--cytochrome c reductase activity. The PTU and DIMIT experiments further suggest that thyroid hormones affect but are not the major or sole regulators of glucose-6-phosphatase development, since this enzyme

requires a much higher concentration of hormone to be stimulated. The inhibition of glucose-6-phosphatase activity by 0.3% PTU is, instead, indicative of a permissive effect of thyroid hormone, with some other factor exerting the primary control. There is precedence for this type of thyroid hormone involvement, e.g., in the regulation of hepatic β-hydroxy-β-methylglutaryl coenzyme A reductase (Rodwell et al., 1976) and malic enzyme (Goodridge and Adelman, 1976). Finally, the lack of any demonstrable effects of either DIMIT or PTU on SER and cytochrome P-450 indicates that the postnatal changes in these hepatic components have nothing directly to do with thyroid hormone levels, but are regulated by another mechanism.

Effects of DIMIT plus glucagon on microsomal enzymes of fetal liver

The collective results of all the previous experiments suggested that the full complement of hepatic changes that typically occur during the last few days of gestation and the first few days after birth are initiated by the combined or sequential actions of fetal thyroid hormones and some other factor(s). Several other examples of cellular regulation of specific functions by hormone combinations are well--established. For example, glycogen synthesis in fetal rat liver in vitro depends on the development of glycogen synthetase, which is stimulated by glucocorticoids and subsequently activated by insulin (Eisen et al., 1973). In mouse mammary gland explants, insulin, hydrocortisone, and prolactin are required in sequence for the production of milk protein (Mills and Topper, 1970). In the case of perinatal hepatic enzyme differentiation, glucagon has been suggested as the second stimulus which acts together or in sequence with thyroid

hormone (Greengard and Dewey, 1968). Experiments in which fetuses were pre-treated with DIMIT and then injected with glucagon were designed to test the hypothesis. They confirmed the previously observed DIMIT effects but revealed nothing of a definitive nature about glucagon's role in liver development. The absence of any effect of glucagon on glucose-6-phosphatase again suggested a technical problem with direct injection of fetuses, since the same dose of hormone given to rats of a comparable age was previously shown to significantly increase the activity of this enzyme (Greengard, 1969; Greengard and Dewey, 1968). In view of the lack of confirmation of this well-documented glucagon effect, it seemed unlikely that proliferation of SER had been induced and, therefore, a morphometric analysis of hepatic ultrastructure was not undertaken. It was concluded that, in the future, better methods for testing the effects of glucagon on hepatic structure and function would have to be found.

FUTURE STUDIES

A continuation of research into the regulation of hepatocyte differentiation will focus primarily on determining the identity of the other factor(s) that cooperate with thyroid hormone in bringing about the full spectrum of perinatal changes. For a variety of reasons already presented, glucagon is a likely candidate for this role, but its involvement could not be demonstrated using the methodology attempted so far. The technique of monolayer cell culture will be the next approach. Although it is not a physiological condition and results must be interpreted cautiously, the method offers an opportunity to observe hormone effects directly and without interference from endocrine or other systems. Preparatory studies defining optimum culture conditions for fetal hepatocytes have already begun. When these are complete, triiodothyronine and DIMIT will first be tested to see if the in vivo thyroid effects can be confirmed in vitro and thus to determine if they were direct or indirect effects. Examples of the latter type of regulation include: [1] a general increase in basal metabolic rate, and [2] interaction with another mediator whose activity was either stimulated or inhibited. Experiments with glucagon, thyroid hormones plus glucagon, and perhaps other hormones will then be conducted.

CONCLUSIONS

1. Sprague-Dawley, Holtzman strain rats are qualitatively similar in their hepatic development to those strains of rats which have previously been examined. There are, however, a few quantitative differences.
2. The thyroid hormone analog, DIMIT, can produce in fetal rats changes that mimic certain aspects of early neonatal development. These include glycogen depletion, hepatocyte division, mitochondrial division, and NADPH-cytochrome c reductase induction. The results suggest that thyroid hormones may be the physiological regulators of these events.
3. Both glycogen depletion and increased microsomal enzyme activity can be achieved independently of smooth endoplasmic reticulum development.
4. A combination of thyroid hormones with some other factor(s) is required to produce the full complement of structural and functional changes that occur perinatally in the rat. This second factor, or the combination, regulates glucose-6-phosphatase and cytochrome P-450 development, and the proliferation of smooth endoplasmic reticulum.
5. Glucagon is a likely candidate for the role of second stimulus, but its involvement could not be demonstrated using the techniques applied.
6. Monolayer cell culture will be used in the future investigation of hormone effects on hepatocyte differentiation.

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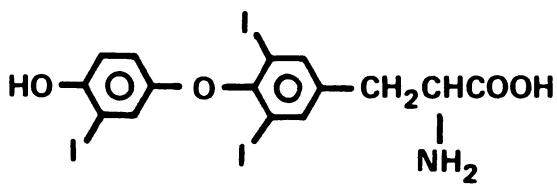
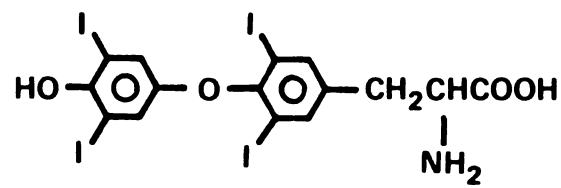
FIGURES

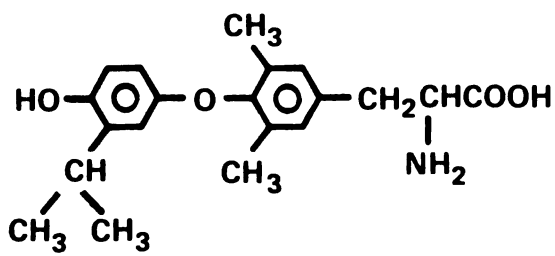
Figure 1. Structures of the two naturally occurring thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4), and the synthetic analog, 3,5-dimethyl-3'--isopropyl-L-thyronine (DIMIT).



L-TRIIODOTHYRONINE (T_3)



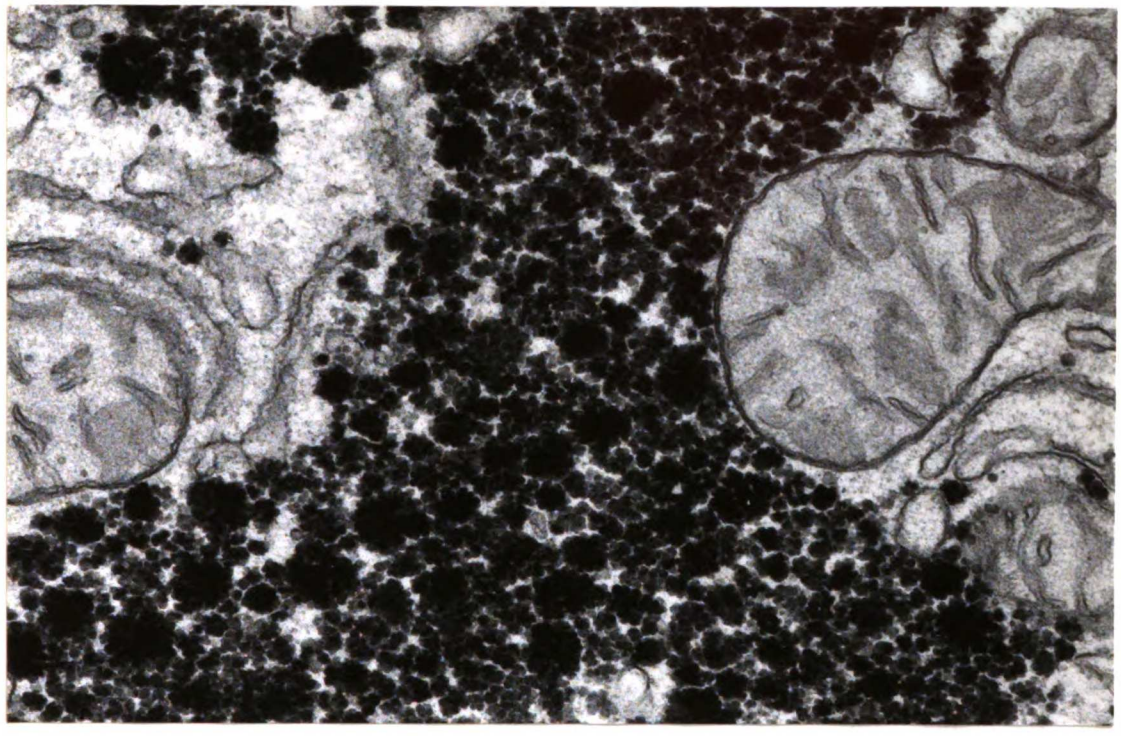
L-THYROXINE (T_4)



L-3,5-DIMETHYL-3'-ISOPROPYLTHYRONINE (DIMIT)

Figure 2. Glycogen of liver fixed in osmium. Tissue was not *en bloc* stained; however, sections were double stained with uranyl acetate and lead citrate (∿45,000X).

Figure 3. Glycogen of liver fixed in glutaraldehyde--paraformaldehyde and *en bloc* stained with uranyl acetate. Sections were double stained as in Figure 2 (∿45,000X).



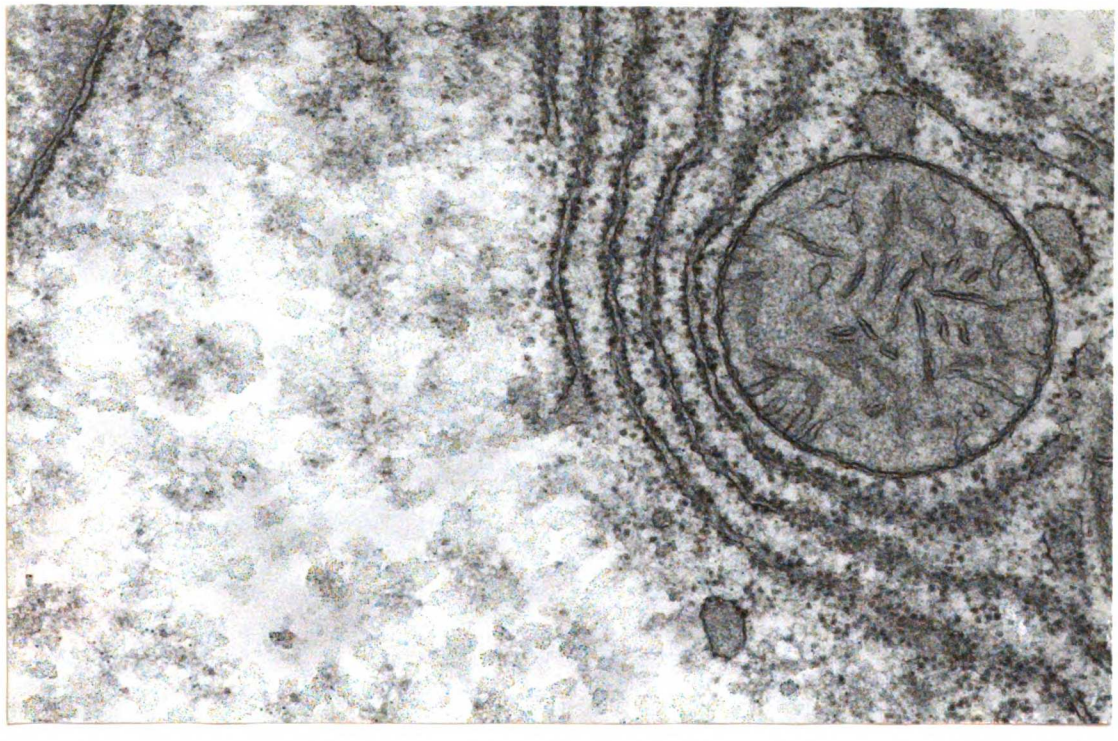


Figure 4. Low magnification electron micrograph overlaid with coherent double lattice test system used for determining volume densities (~14,000X).

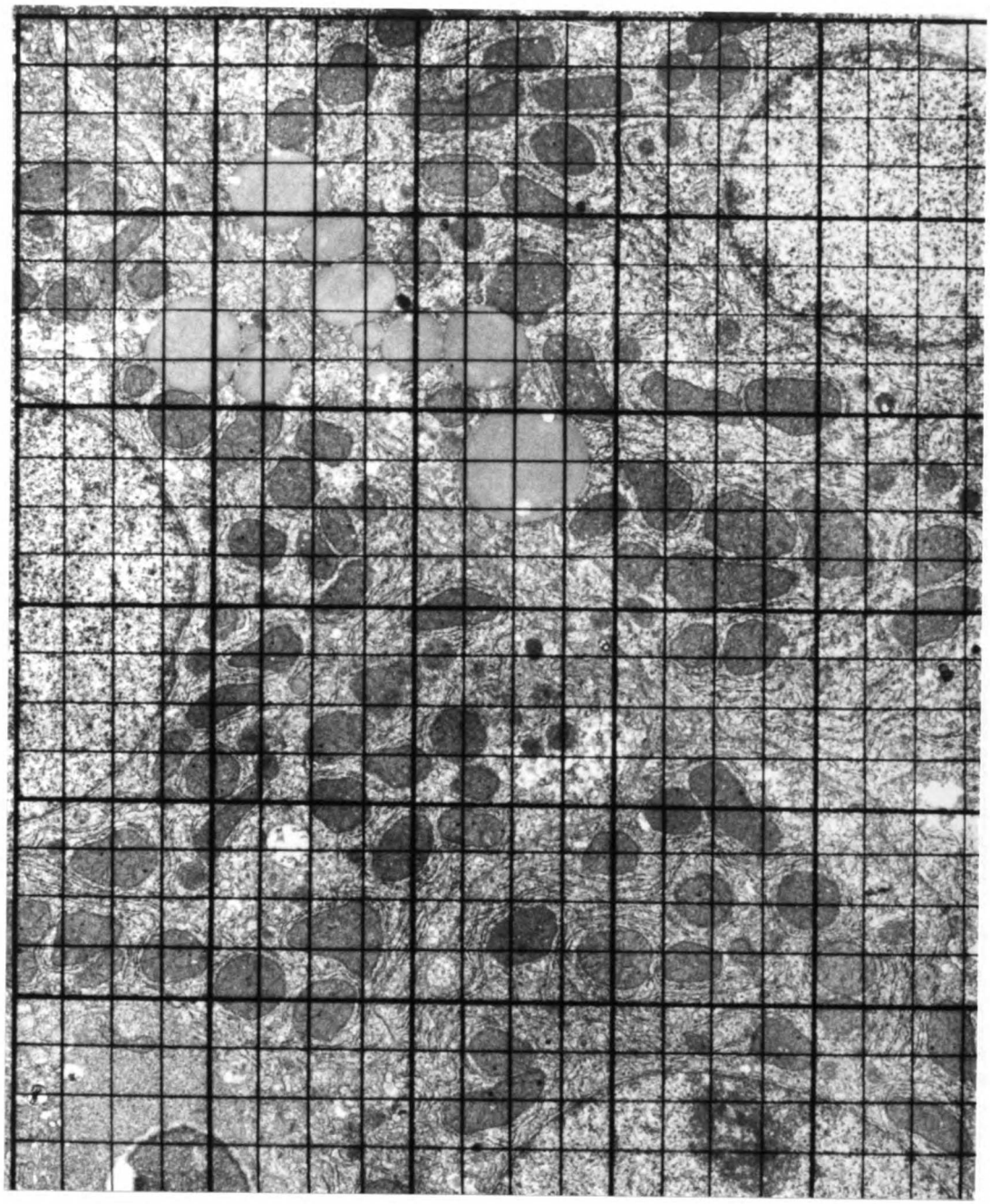


Figure 5. High magnification electron micrograph overlaid with coherent multipurpose test system used for determining surface densities (∿45,000X).

Figure 6. Three-day old neonatal rat liver microsome preparation fixed in cacodylate-buffered osmium. Sections were double stained with uranyl acetate and lead citrate. The microsomes are free of nuclei and mitochondria; however, a considerable amount of glycogen is present and gives the background of the micrograph a granular appearance (∿14,000X).

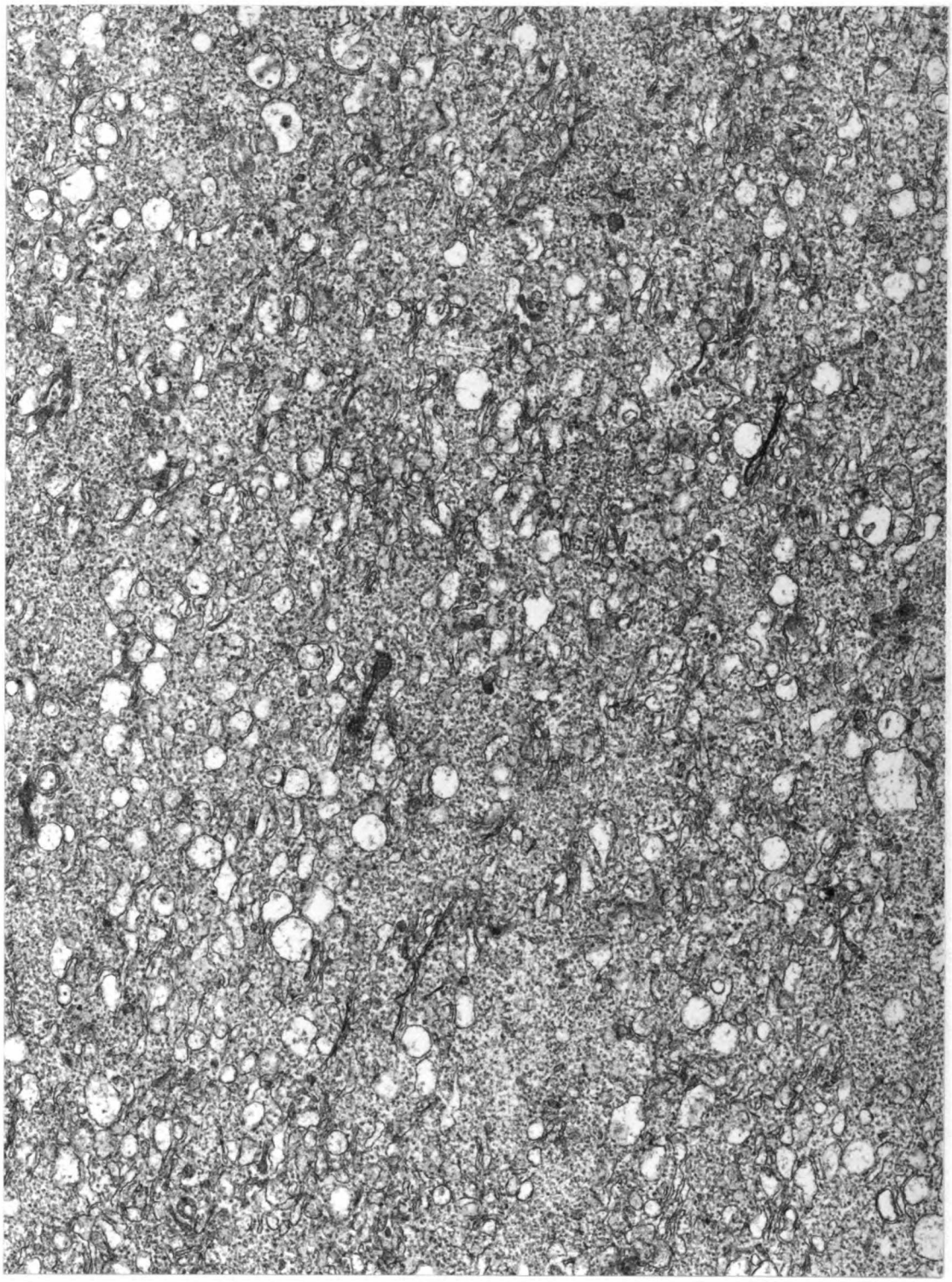
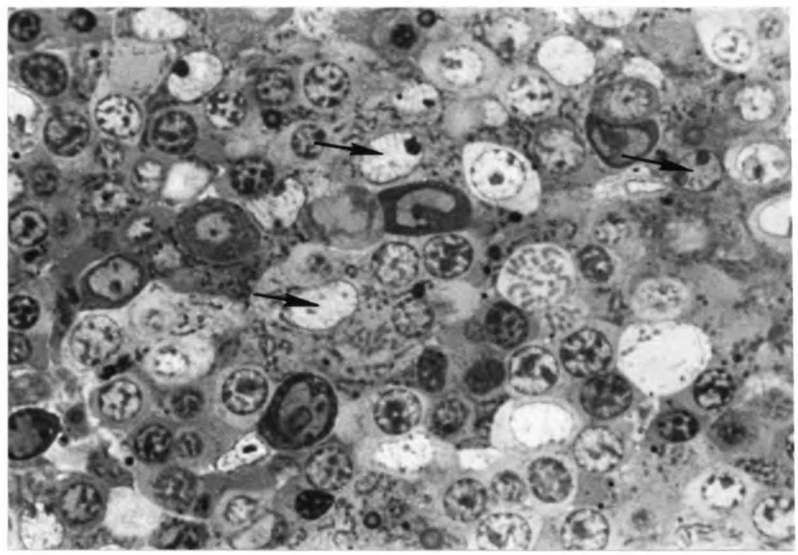


Figure 7. Light micrograph of fetal liver on the 17th day of gestation. Note the large number of hematopoietic cells still present at this stage (arrows indicate nuclei of some hepatocytes; 1000X).

Figure 8. Fetal liver on the 20th day of gestation. Hepatocytes are larger and more numerous than on the 17th day. They contain large masses of glycogen (arrows) which appear white or pale gray due to the *en bloc* uranyl acetate staining (1000X).



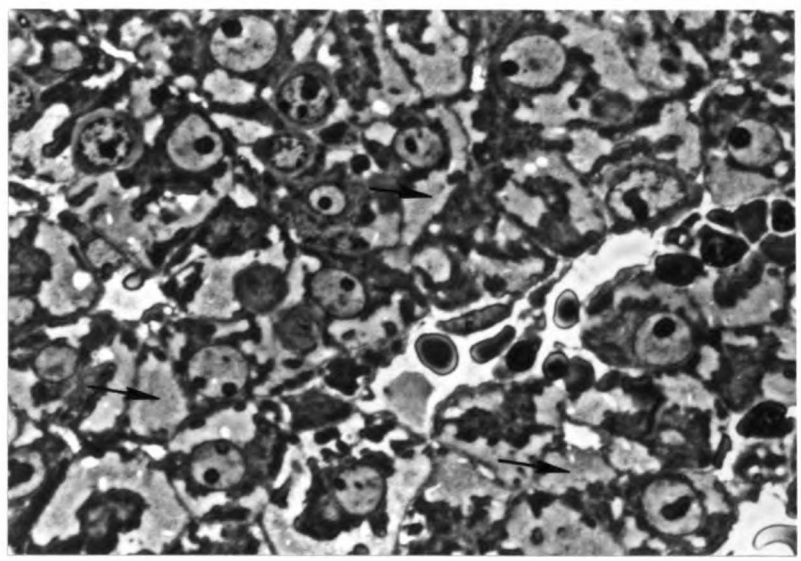
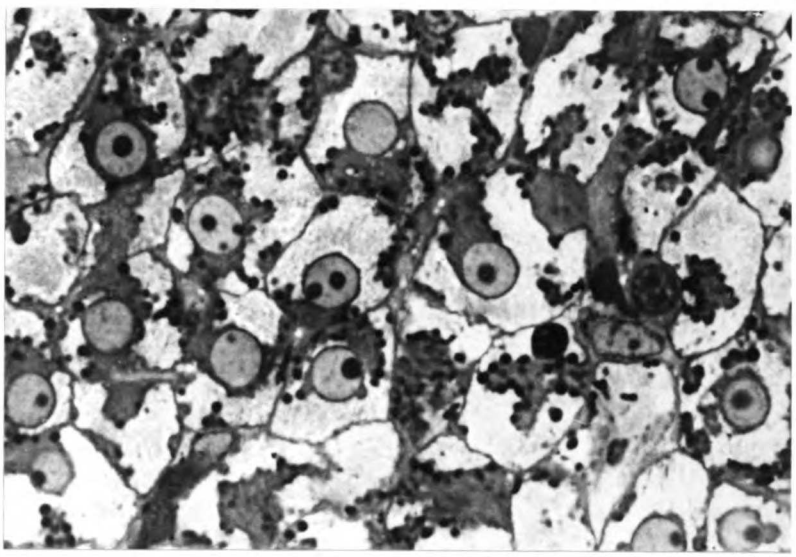


Figure 9. Fetal liver on the 21st day of gestation. Hepatocytes have nearly reached maximum size and glycogen content (1000X).

Figure 10. Liver from 3 day old neonate. Most of the glycogen has been mobilized and hepatocytes are reduced in size. Many hepatocytes contain large numbers of darkly stained lipid droplets (arrows; 1000X).



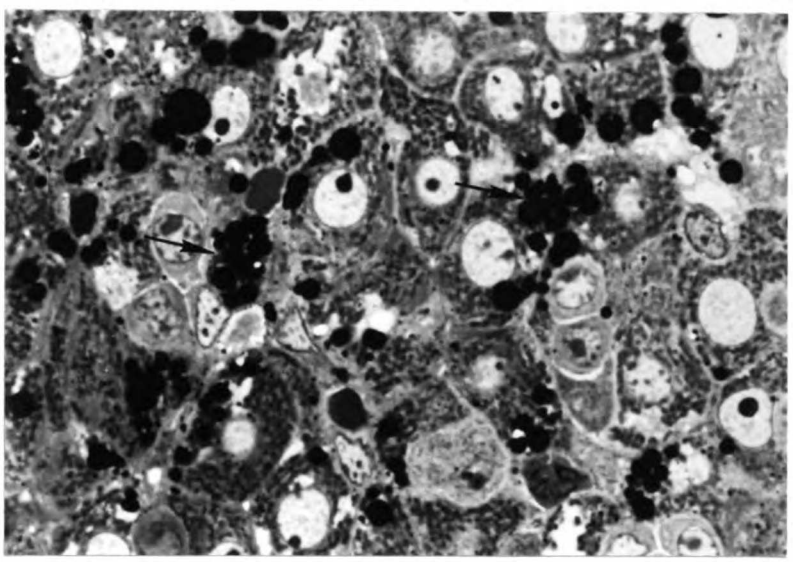


Figure 11. Electron micrograph of fetal liver on the 15th day of gestation. H, hematopoietic cell; N, nucleus; M, mitochondrion; L, lipid droplet; RER, rough endoplasmic reticulum (∿14,000X).

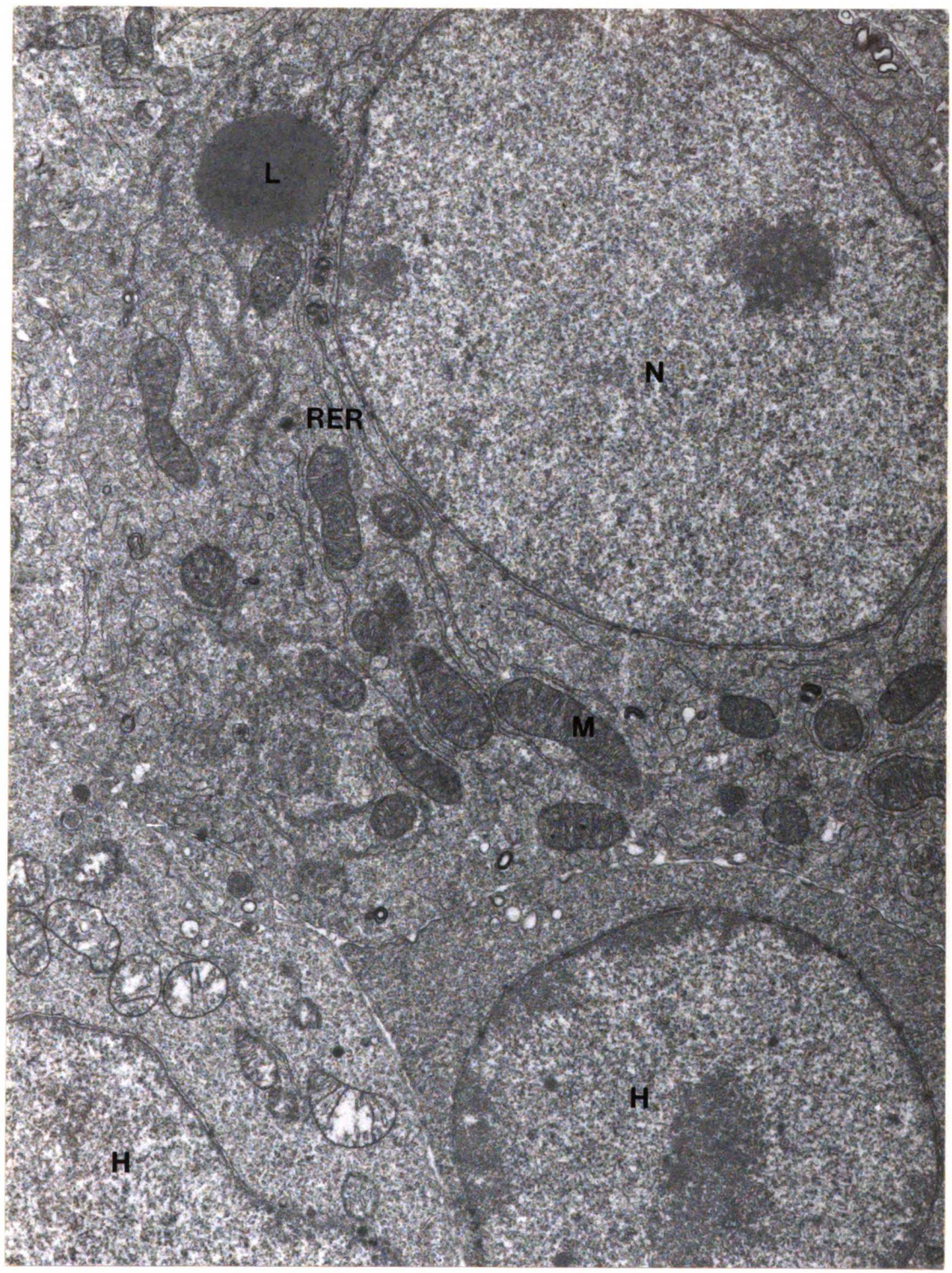

L
N
RER
M
H
H

Figure 12. Fetal liver on the 17th day of gestation. H, hematopoietic cell; N, nucleus; M, mitochondrion; RER, rough endoplasmic reticulum; G, Golgi (∿14,000X).

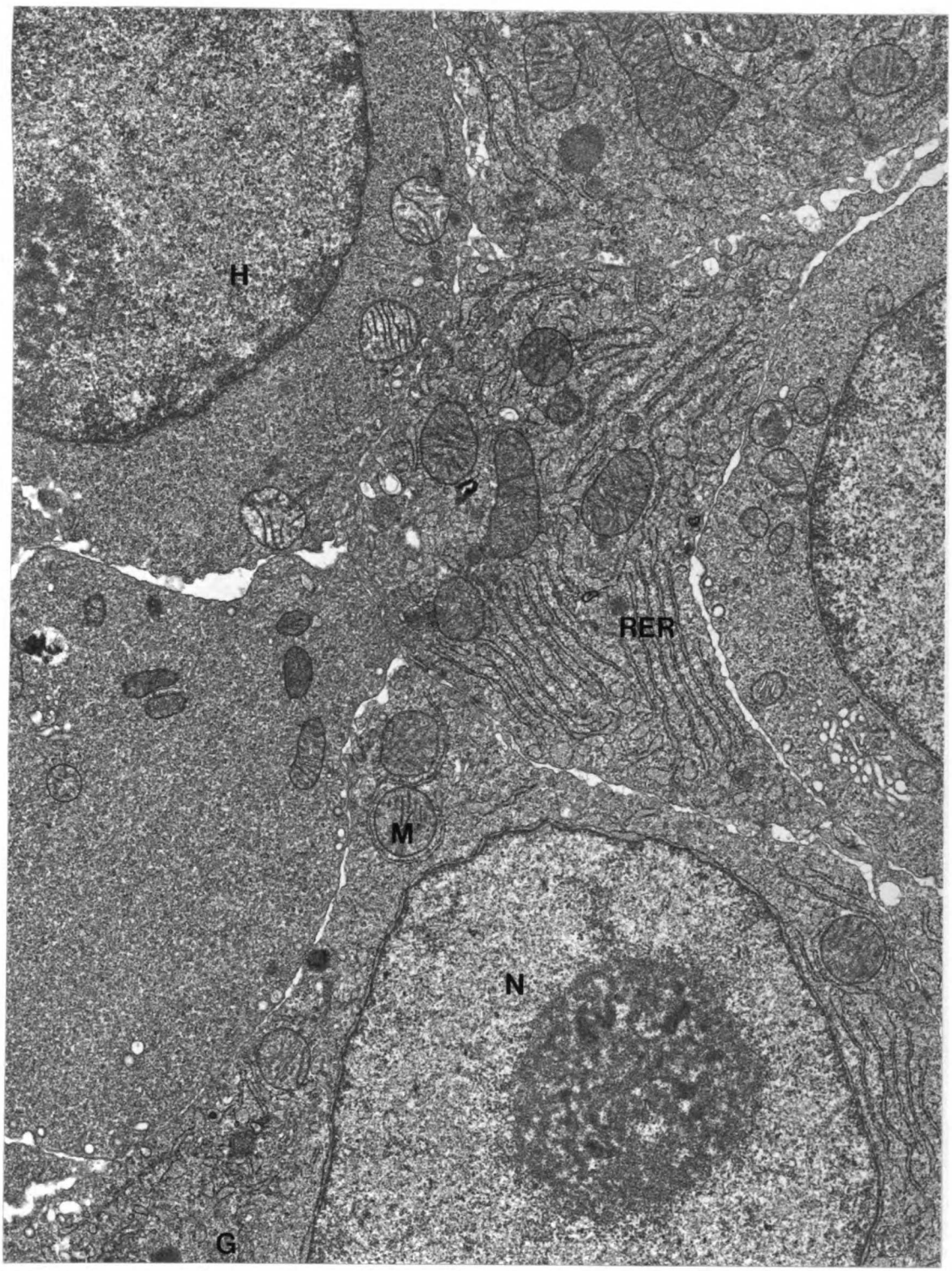

H
RER
M
N
G

Figure 13. Fetal liver on the 20th day of gestation. N, nucleus; M, mitochondrion; RER, rough endoplasmic reticulum; G, Golgi; mi, microbody; gly, glycogen (∿14,000X).

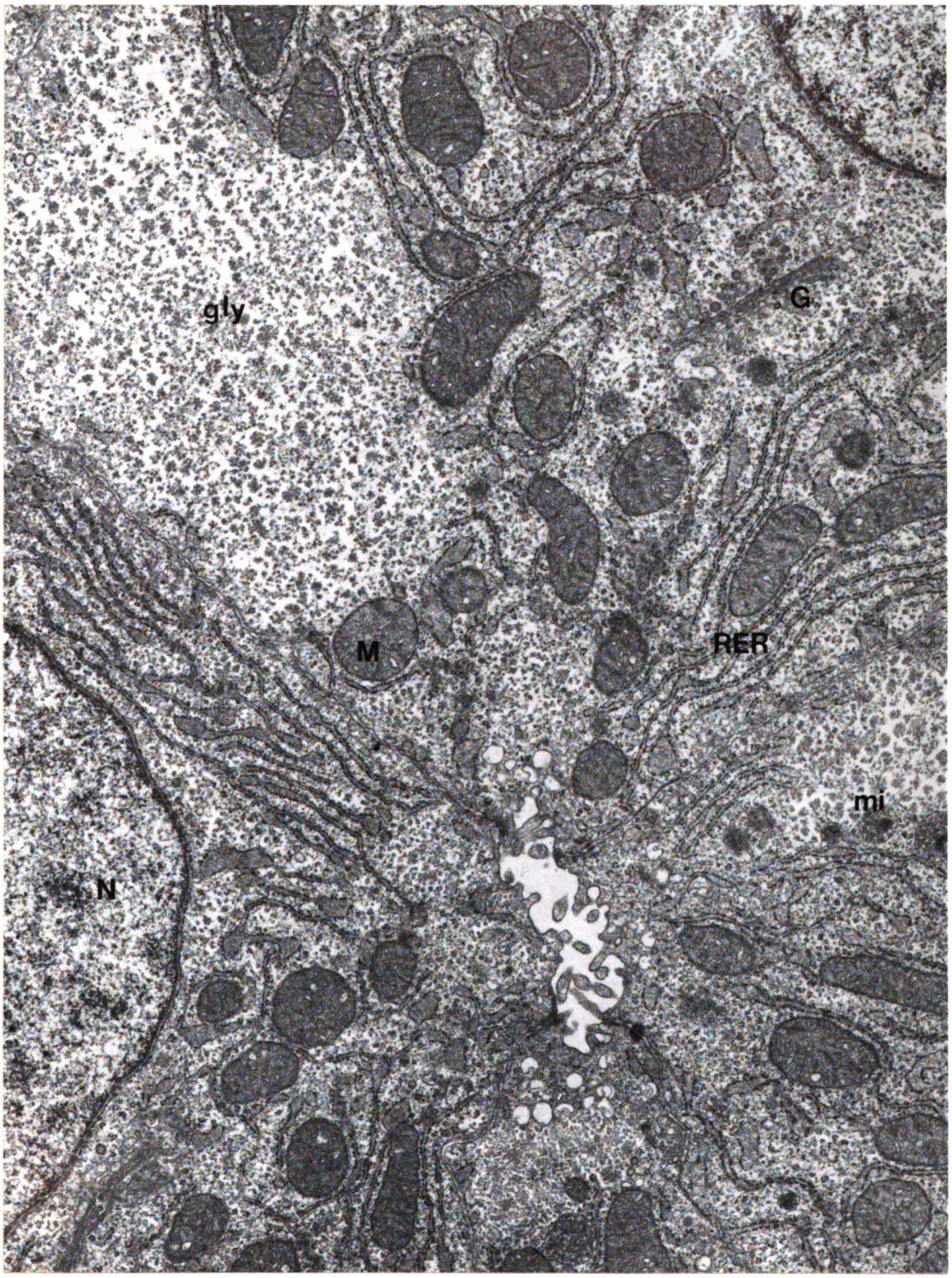

gly
G
M
RER
mi
N

Figure 14. Fetal liver on the 21st day of gestation. N, nucleus; M, mitochondrion; RER, rough endoplasmic reticulum; gly, glycogen (∿14,000X).

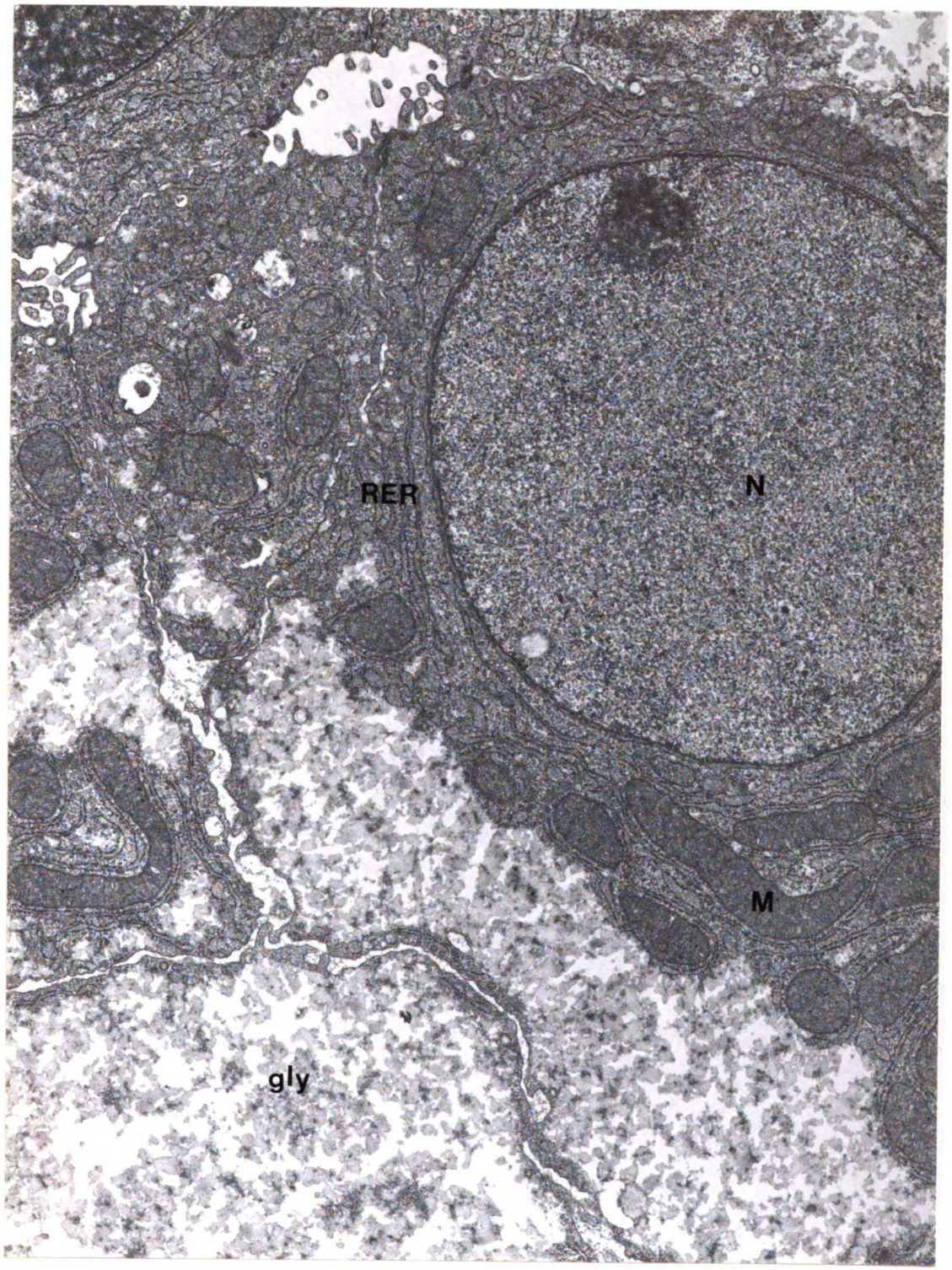

RER
N
M
gly

Figure 15. Liver from 3 day old neonate. N, nucleus; M, mitochondrion; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; gly, glycogen (∿14,000X).

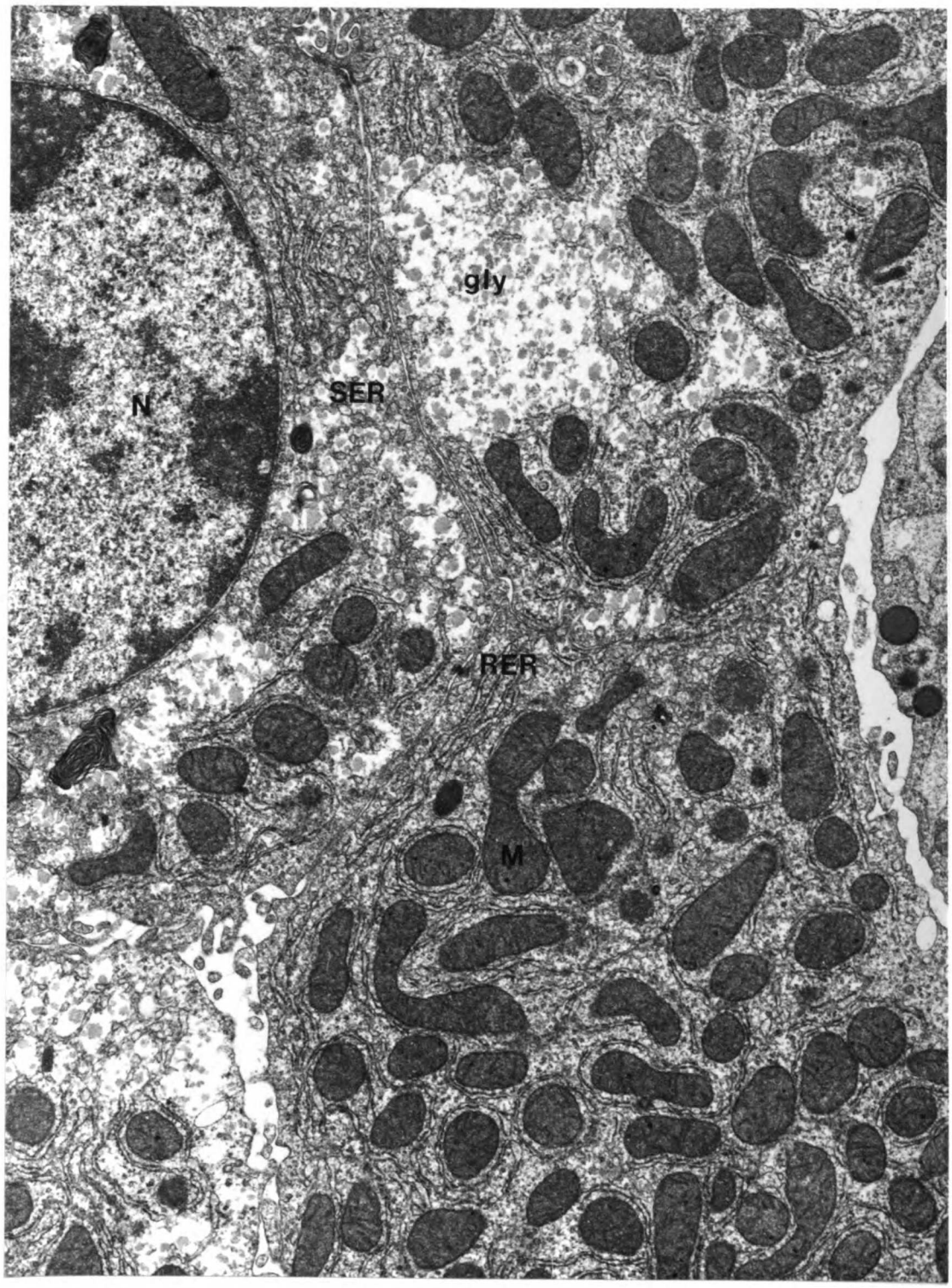

gly
N
SER
RER
M

Figure 16. Liver from 3 day old neonate treated with 0.1% propylthiouracil (PTU). N, nucleus; M, mitochondrion; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; mi, microbody; gly, glycogen (∿14,000X).

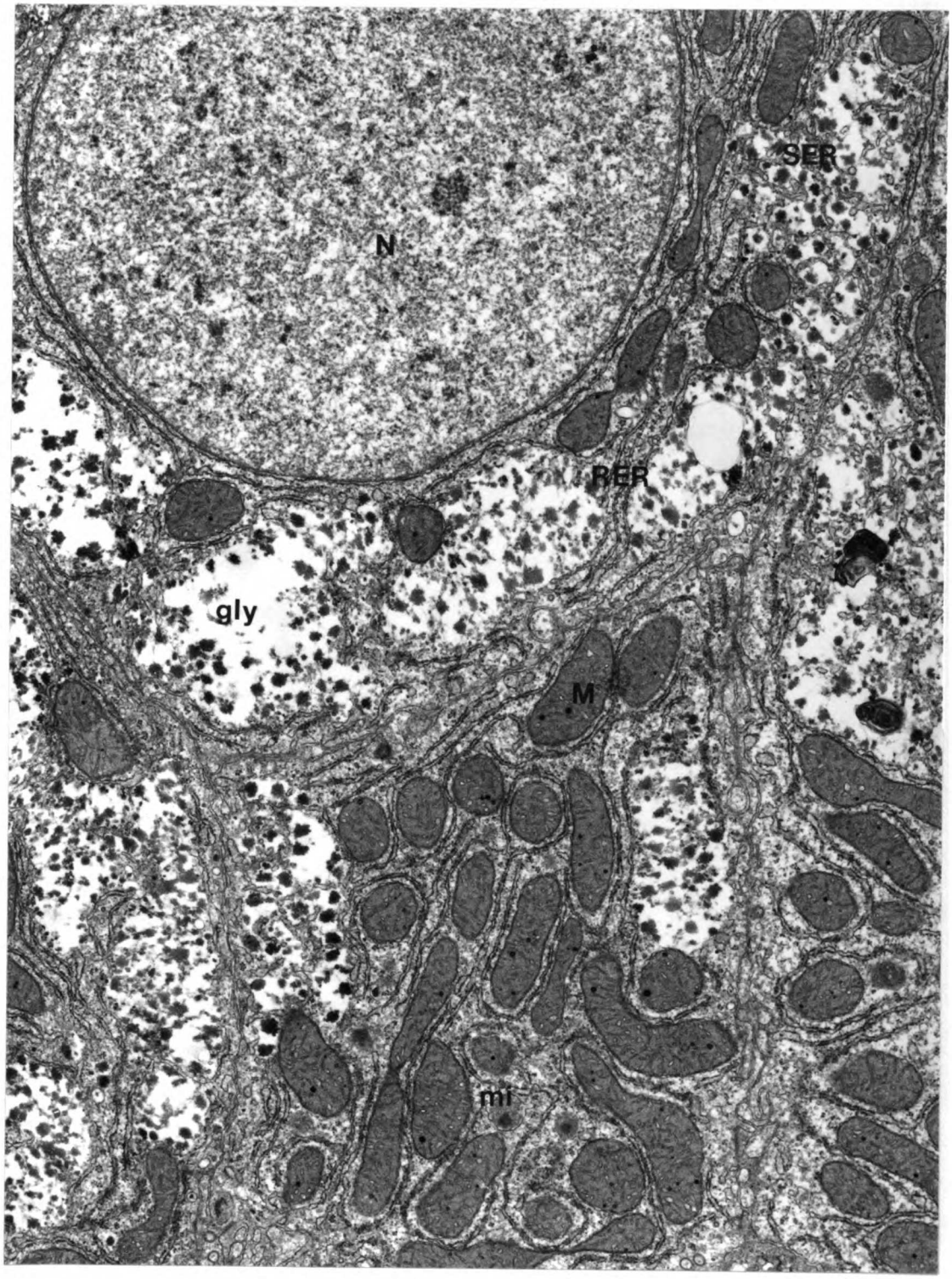

N
SER
RER
gly
M
mi

Figure 17. Liver from 3 day old neonate treated with 0.2% PTU. N, nucleus; M, mitochondrion; RER, rough endoplasmic reticulum; L, lipid droplet; mi, microbody; gly, glycogen (∿14,000X).

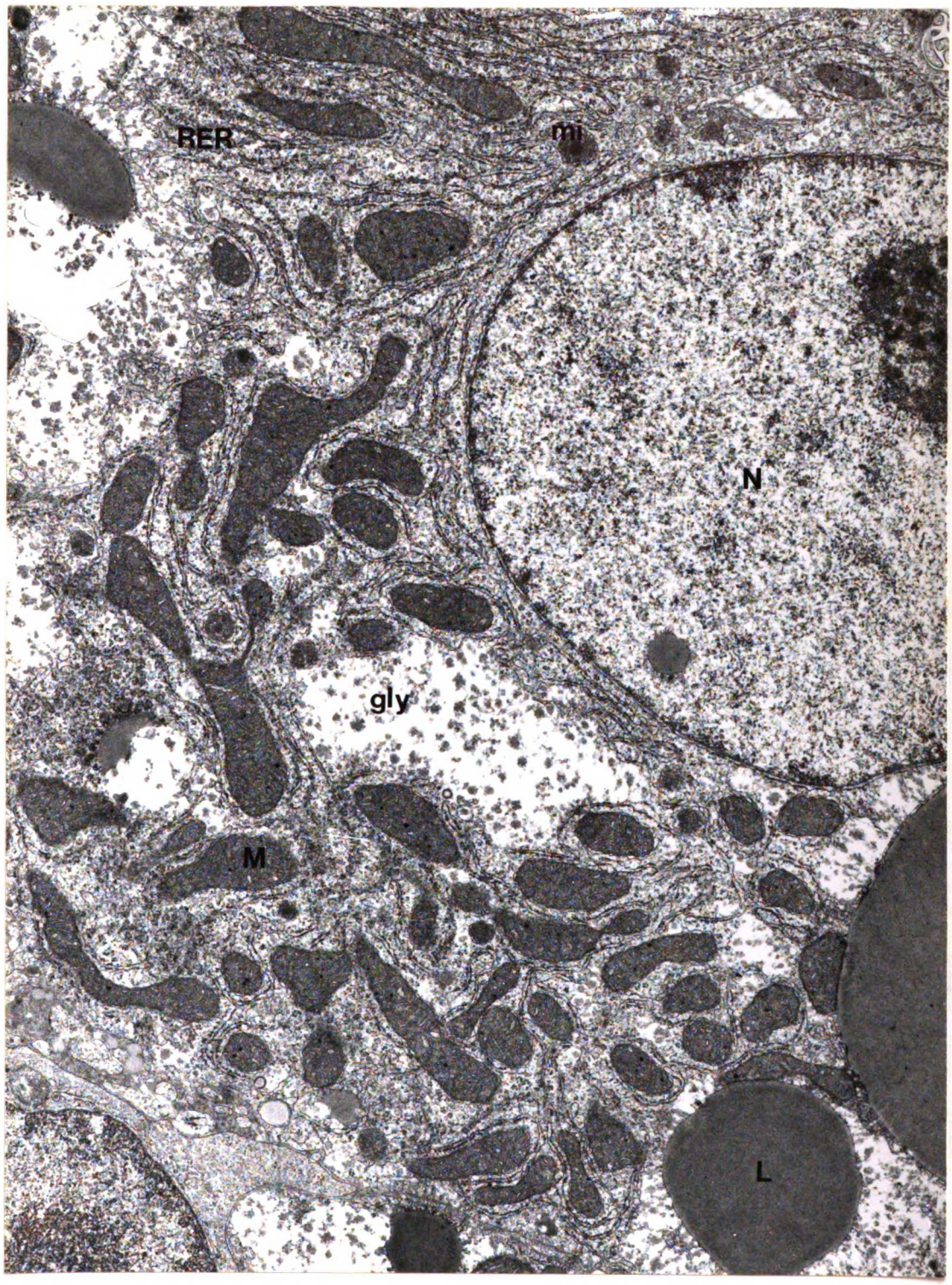

RER
mi
N
gly
M
L

Figure 18. Liver from 3 day old neonate treated with 0.5% PTU. N, nucleus; M, mitochondrion; RER, rough endoplasmic reticulum; SER, smooth endoplasmic reticulum; G, Golgi; L, lipid droplet; bc, bile canaliculus (∿14,000X).

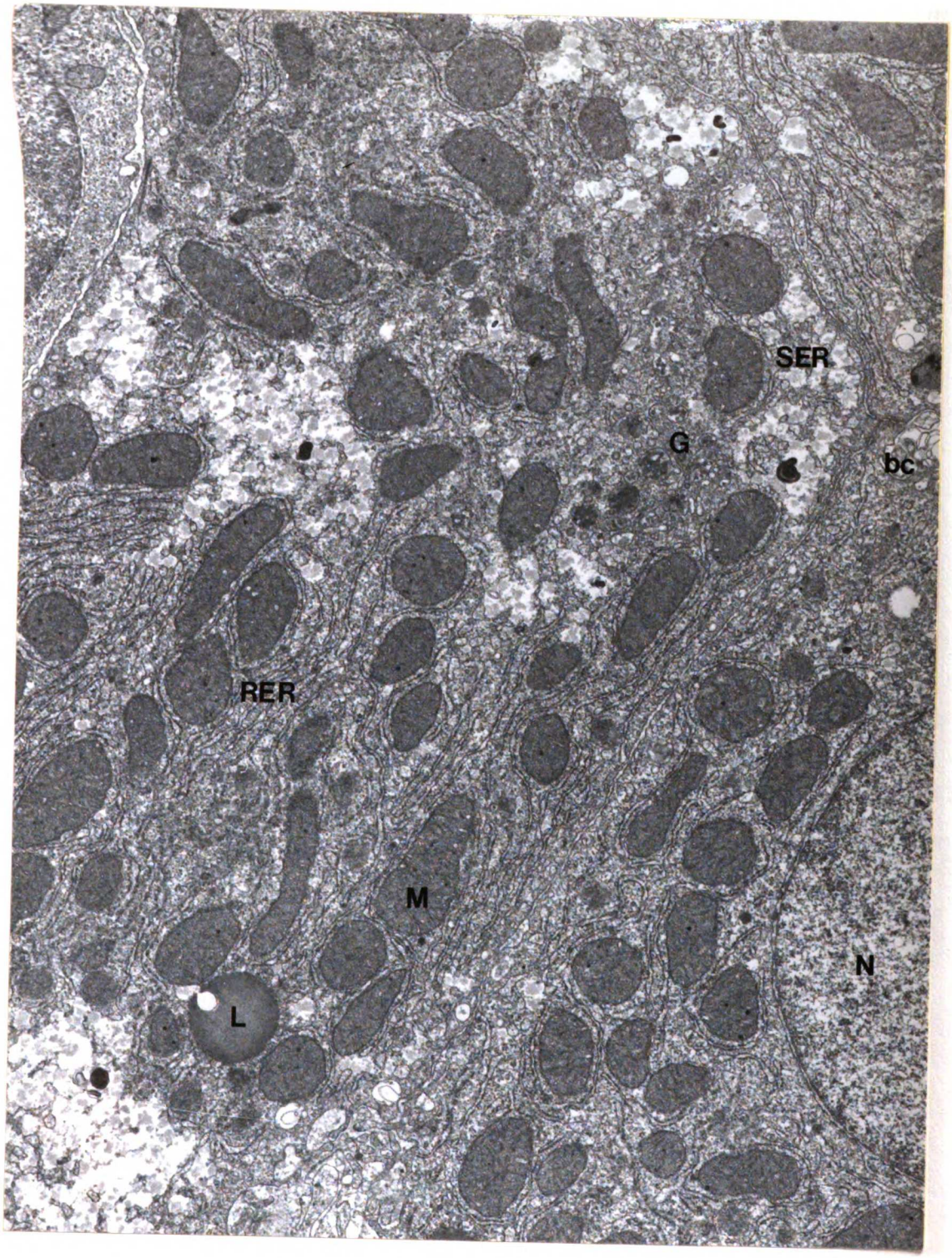
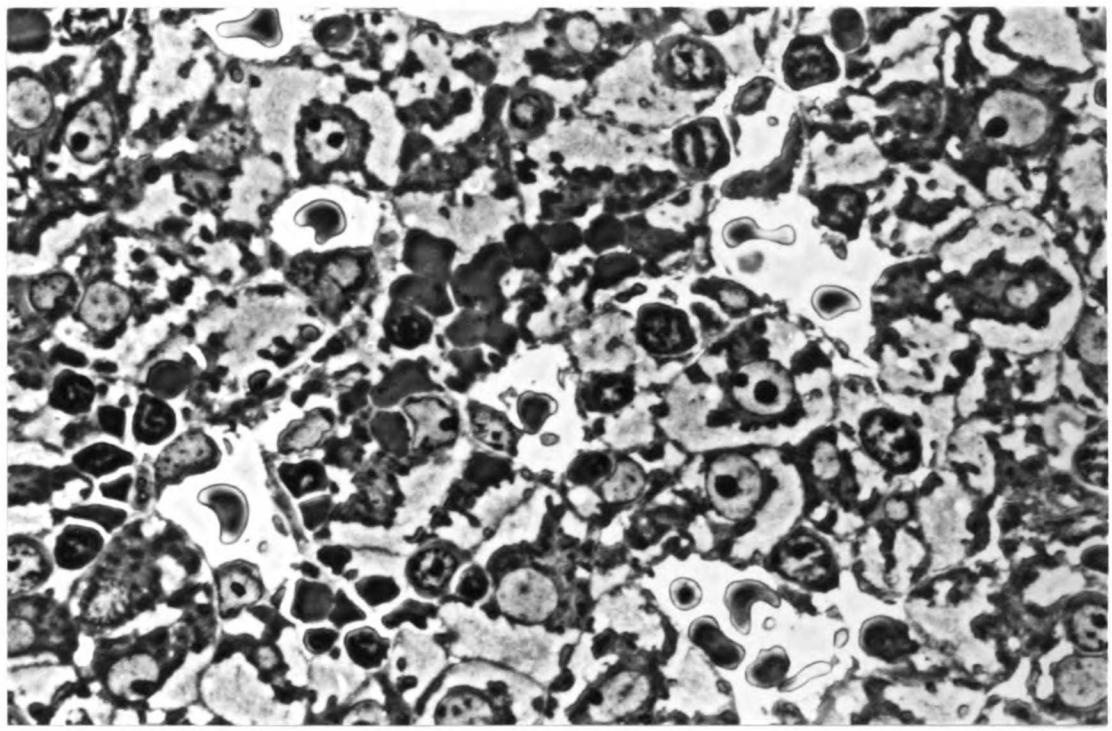

SER
G
bc
RER
M
N
L

Figure 19. Light micrograph of control liver on the 20th day of gestation. Glycogen is prominent in the large hepatocytes (1000X).

Figure 20. Liver from 20-day old fetus that was treated for 5 days with 10 µg DIMIT per 100 g maternal body weight. Very little glycogen is present and hepatocytes are much smaller than in control animals (1000X).



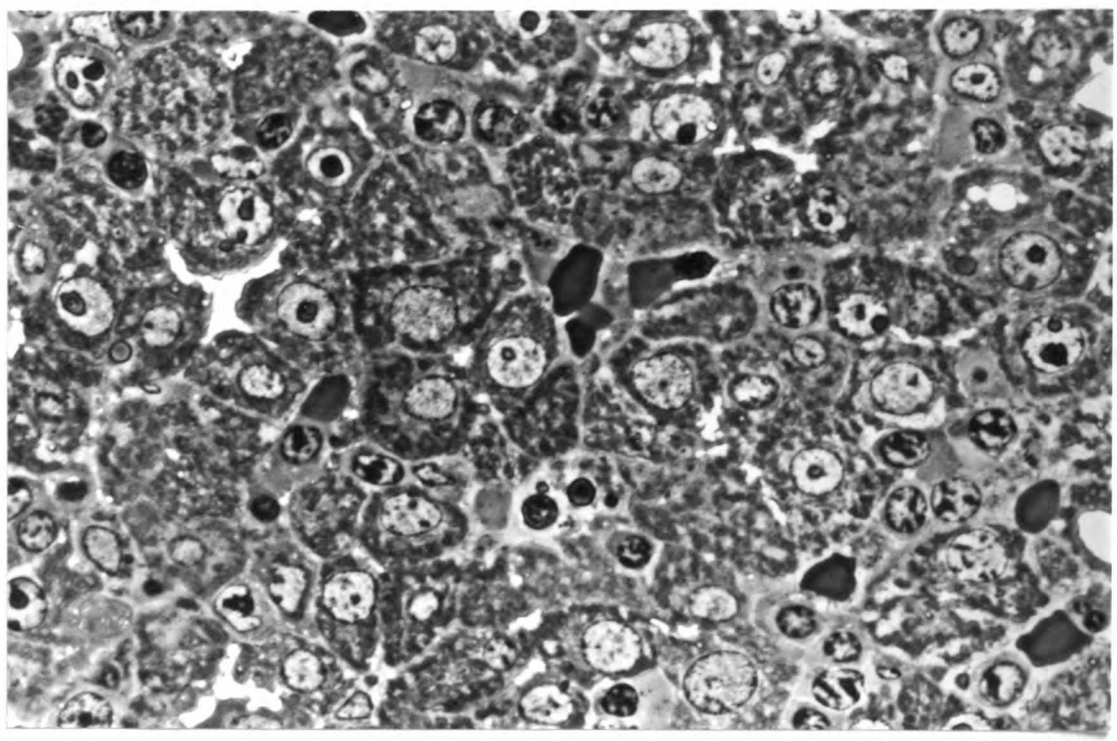


Figure 21. Electron micrograph of hepatocytes from control fetus on the 20th day of gestation. N, nucleus; M, mitochondrion; RER, rough endoplasmic reticulum; G, Golgi; gly, glycogen (∿14,000X).

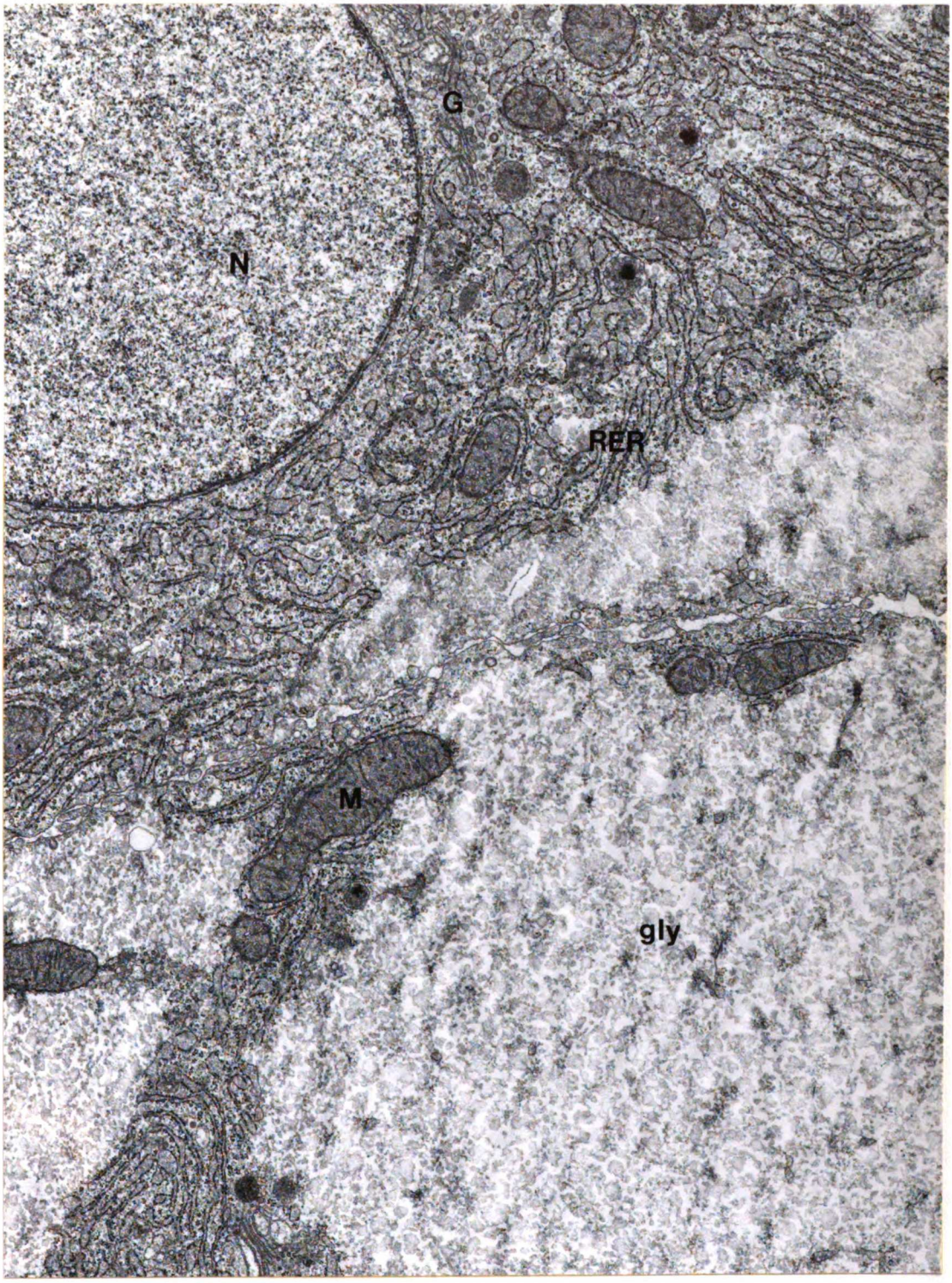
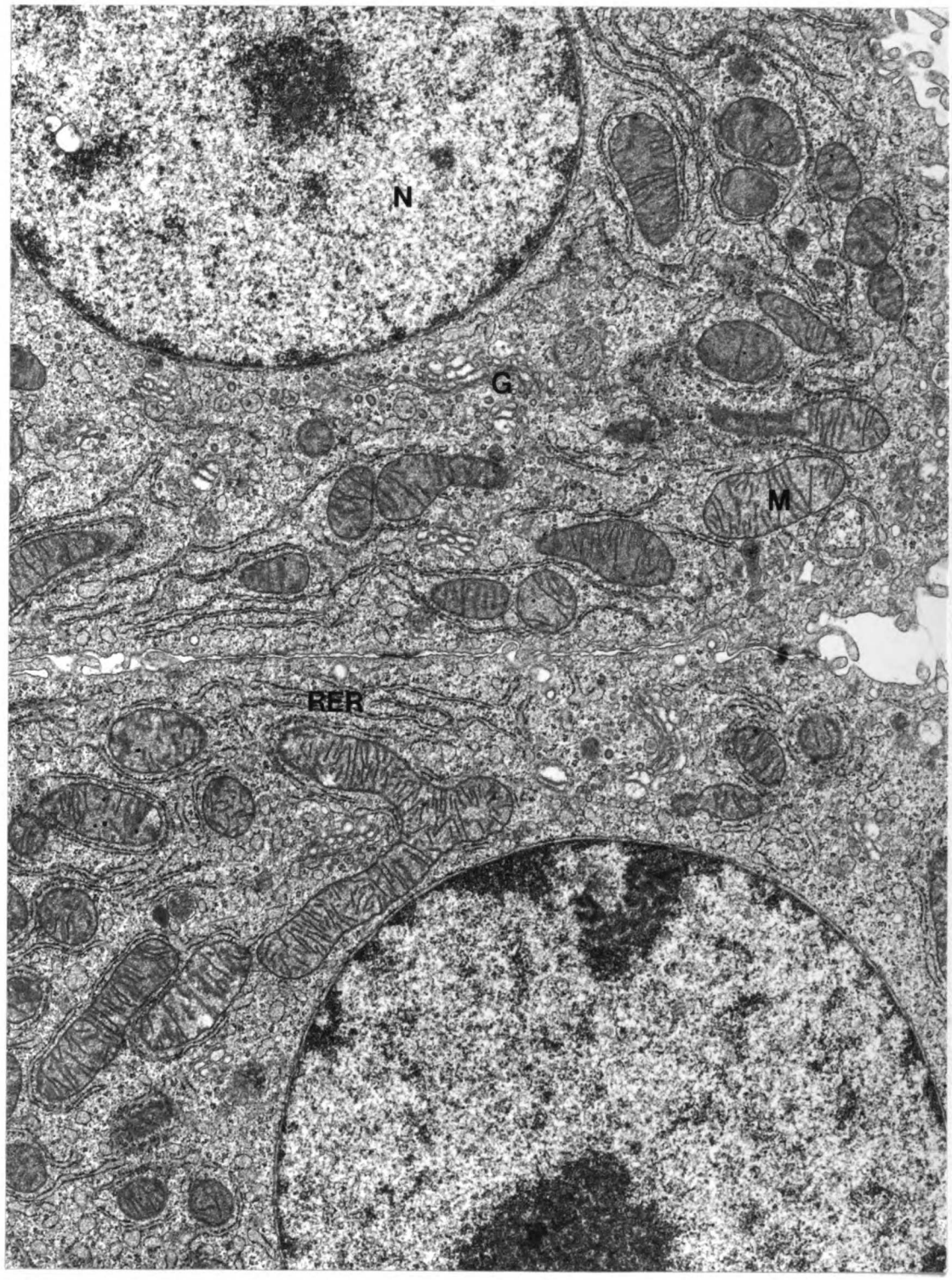

G
N
RER
M
gly

Figure 22. Hepatocytes from DIMIT-treated 20-day old fetus. N, nucleus; M, mitochondrion; RER, rough endoplasmic reticulum; G, Golgi (∿14,000X).


N
G
M
RER