

DIFFERENTIATION OF ENDOPLASMIC RETICULUM IN HEPATOCYTES

I. Glucose-6-Phosphatase Distribution *In Situ*

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ABSTRACT

The distribution of glucose-6-phosphatase activity in rat hepatocytes during a period of rapid endoplasmic reticulum differentiation (4 days before birth-1 day after birth) was studied by electron microscope cytochemistry. Techniques were devised to insure adequate morphological preservation, retain glucose-6-phosphatase activity, and control some other possible artifacts. At all stages examined the lead phosphate deposited by the cytochemical reaction is localized to the endoplasmic reticulum and the nuclear envelope. At 4 days before birth, when the enzyme specific activity is only a few per cent of the adult level, the lead deposit is present in only a few hepatocytes. In these cells a light deposit is seen throughout the entire rough-surfaced endoplasmic reticulum. At birth, when the specific activity of glucose-6-phosphatase is approximately equal to that of the adult, nearly all cells show a positive reaction for the enzyme and, again, the deposit is evenly distributed throughout the entire endoplasmic reticulum. By 24 hr postparturition all of the rough endoplasmic reticulum, and in addition the newly formed smooth endoplasmic reticulum, contains heavy lead deposits; enzyme activity at this stage is 250% of the adult level. These findings indicate that glucose-6-phosphatase develops simultaneously within all of the rough endoplasmic reticulum membranes of a given cell, although asynchronously in the hepatocyte population as a whole. In addition, the enzyme appears throughout the entire smooth endoplasmic reticulum as the membranes form during the first 24 hr after birth. The results suggest a lack of differentiation within the endoplasmic reticulum with respect to the distribution of glucose-6-phosphatase at the present level of resolution.

INTRODUCTION

The mechanism of assembly of membrane components into fully differentiated membranes remains an unsolved problem in cell biology. A particularly favorable system for studying this problem is the endoplasmic reticulum (ER)¹ in the

¹Abbreviations used are: ER, endoplasmic reticulum; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; P_i, inorganic phosphate; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl) amino methane.

developing hepatocyte of the rat. As previously reported (1-4), during the time immediately surrounding birth these cells undergo striking morphological and enzymatic changes. Glycogen is built up within the hepatocytes, until at birth a large store has accumulated which is utilized during the first day of life. The amount of rough ER (ER membranes with bound ribosomes), which is limited in hepatocytes 4 days before birth, increases during the last few days of gestation;

at this time the cells still lack smooth ER (ER membranes without ribosomes).

Right after birth there is an extensive proliferation of smooth ER at the edges of the glycogen regions. Dallner et al. (3) have shown that the enzyme activities associated with these rough and smooth ER membranes appear at different times and increase at different rates, so that the enzyme composition of the ER membranes changes during development. Some of the enzyme activities reach adult values several days before birth, some increase slowly after birth, while others show a dramatic increase immediately after birth. The increases in enzyme activity are most likely due to the synthesis of new protein since they can be prevented by treatment of the newborn rat with puromycin or actinomycin.

These changes in the enzymatic properties of the ER were studied exclusively by homogenization and cell fractionation, techniques which cannot provide information on the distribution of the newly formed enzymes within the population of hepatocytes and within the ER of individual cells. Electron microscope cytochemistry on intact liver tissue can provide information on the topography of the differentiation process. With this technique one can ask, for instance, whether the hepatocytes acquire a given enzyme synchronously or at different times, and whether sites of newly formed enzyme activity occur in clusters or are evenly distributed throughout the entire ER of an individual cell. Such information can help distinguish between models of membrane formation which involve simultaneous assembly of all membrane components, and models which involve the independent insertion of certain components (e.g., enzymes) into a pre-existing framework. We have used electron microscope cytochemistry to study the distribution of glucose-6-phosphatase (G6Pase), an ER enzyme whose activity increases dramatically at the time of birth. The work is presented in two parts; this paper contains the cytochemical evidence while its companion (5) reports on cell fractionation studies performed to corroborate the cytochemical results obtained *in situ*.

MATERIALS AND METHODS

Animals

Male rats (150–300 g each) of the Sprague-Dawley strain fed *ad libitum* on Purina Lab Chow provided the adult liver tissue. The rats were not starved

before sacrifice in order to parallel the conditions found in fetal and newborn rats, in which the glycogen content of the liver could not be reduced by starvation. Pregnant female rats, in most cases from Holtzmann Co., Madison, Wis., were received between the 14th and 17th days of pregnancy and were maintained on the same diet. At least four females mated on the same day were obtained at a time. Some of them were sacrificed on the desired date of pregnancy; two were kept as controls and allowed to deliver normally. The latter almost always gave birth on the 22nd day after mating, generally within a few hours of one another. Newborn animals were allowed to nurse normally, although animals used immediately after birth (1–3 hr) had not yet begun to nurse.

Reagents

Glucose-6-phosphate (G6P), barium and dipotassium salts, was obtained from Sigma Chemical Co., St. Louis, Mo. For routine enzyme assays the dipotassium salt was used without further purification. To obtain highly purified G6P for the cytochemical incubation mixture (less than 1% inorganic phosphate), the purer but poorly soluble barium salt was converted to the more soluble dipotassium salt, either by treatment with sulfuric acid or by passage over a Dowex-50 ion exchange column (Dow Chemical Co., Midland, Mich.). The free acid thus obtained was neutralized with KOH and the salt was stored at -20°C . Glutaraldehyde was obtained from Fischer Co., Fairlawn, N. J., as a 50% aqueous solution. Sodium cacodylate was from Amend Drug & Chemical Co., New York.

Perfusion Fixation

The livers of adult rats under ether anesthesia were perfused via the portal vein, first with 50 ml of buffer (0.1 M sodium cacodylate, pH 6.6, containing 6 mM G6P), then with 100 ml of fixative (2% glutaraldehyde in 0.1 M cacodylate buffer, pH 6.6, containing 6 mM G6P), and finally with another 50 ml of buffer. After perfusion with fixative that lasted from 3 to 5 min, the liver, which became firm and light pink, was excised, cut into pieces, and maintained at 4°C in cold buffer. In experiments in which both unfixed and fixed tissues were to be obtained from the same liver, the right lobe of the liver was ligated and removed after the initial perfusion with buffer. Since no fixative leaked through the cut, a closed perfusion system was maintained.

For work with fetuses, female rats at the desired stage of pregnancy were anesthetized with ether; the fetuses were removed and decapitated and their livers were slowly and carefully perfused via the hepatic portal vein, each with 5 ml of the same fixa-

tive used for the adult, and then with 5 ml of the buffer. The initial perfusion with buffer was omitted because of the fragility of the fetal liver, but after fixation the organ was solid enough to permit the final buffer wash. Generally the right lobes of the liver became light pink and appeared well fixed.

For work with newborn animals, the livers were perfused with 10 ml of fixative and then with 10 ml of buffer. In each experiment, littermates were used for all time points after birth. The age of the animals was estimated by taking the midpoint of delivery as time zero.

Cytochemistry and Electron Microscopy

Small cubes of fixed liver were chopped with an automatic tissue chopper (6) into thin (50–100 μ) slices which were kept at 0°C for approximately 1 hr before being incubated for 30 or 60 min at 24–25°C in a shaking water bath. The histochemical medium, modified from that of Wachstein and Meisel (7), contained 1 mM G6P (dipotassium salt), 2 mM lead nitrate, and 0.05 M cacodylate buffer, pH 6.6. A concentration of lead twice that of the substrate was used to ensure the presence of sufficient lead ions to trap all of the released phosphate. In the absence of tissue, no precipitate formed in the medium during a 60 min incubation.

The tissue slices were transferred directly from the incubation medium into 2% OsO₄ in 0.1 M cacodylate buffer, pH 6.6, to stop the reaction and to postfix the tissue. Following postfixation for 60 min at 4°C, and several washes with cacodylate and Veronal-acetate buffer (0.029 M sodium Veronal, 0.029 M sodium acetate, 0.02 N HCl, pH approximately 6.8), the specimens were stained for 60 min at room temperature with 0.5% uranyl acetate prepared in the same buffer (8). Samples were dehydrated through graded alcohols and propylene oxide, and were embedded in Epon (9). An entire slice was embedded and oriented with its long dimension perpendicular to the plane of sectioning.

After polymerization, the blocks were trimmed so that each section would include the complete width of a tissue slice. In this way, two opposite edges which were exposed to the medium during the histochemical incubation could be examined, and the distance of penetration of the substrate and trapping agent into the slice could be observed.

Sections were cut on a Porter-Blum Sorvall MT2 automatic microtome using a du Pont (E. I. du Pont de Nemours & Company, Wilmington, Del.) diamond knife. Gold, silver, or gray sections were collected on bar grids (E. F. Fullam, Inc., Schenectady, N. Y.) covered with 2% Formvar (Belden Mfg. Co., Chicago, Ill.) and carbon films. The sections were stained with alcoholic uranyl acetate (10) for 1 min and basic lead citrate (8, 11)

for 3–5 min, and were examined in a Siemens Elmiskop electron microscope operated at 80 kv with a double condenser, a 400 μ condenser aperture, and a 50 μ objective aperture.

Light Microscopy

Incubated slices of perfusion-fixed tissue were washed with 0.05 M cacodylate buffer immediately after incubation, and were then rinsed briefly in dilute ammonium sulfide. Following further rinses with buffer and water they were dehydrated and embedded as described above. 0.5 μ sections were cut from polymerized blocks, mounted on slides, and examined and photographed in a Zeiss Ultraphot microscope fitted with oil-immersion phase-contrast lenses.

Biochemistry

Adult male rats were anesthetized with ether, newborn rats were decapitated, and fetal rats were decapitated after being removed from anesthetized mothers. When liver tissue was to be used solely for biochemical experiments, livers were removed without previous perfusion. Homogenates, 10, 20, or 25% w/v, were prepared at 4°C in 0.25 or 0.88 M sucrose using a motor-driven Potter-Elvehjem homogenizer (12) fitted with a Teflon pestle.

Homogenization did not disrupt fixed tissue extensively and evenly enough to permit reliable G6Pase and protein determinations. Therefore, when G6Pase activity was assayed in fixed tissue, homogenates prepared from both fixed and unfixed liver were sonicated using a Branson Sonic Power Co., (Danbury, Conn.) "Sonifer" set at 4–6 amp. The homogenates were kept in an ice bath during the sonication and 10-sec intervals of sonication were separated by 15 sec of cooling. The sonication was monitored by phase-contrast microscopy. Total sonication time varied (Table I), since in each experiment it was continued until only a few unbroken cells remained in the homogenate from the fixed tissue.

The G6P assay, modified from those of Swanson (13) and de Duve et al. (14), was performed in duplicate for 5–60 min at 37°C. The 1.0 ml assay medium contained 30 μ moles of cacodylate buffer, pH 6.6, and 30 μ moles of G6P (dipotassium salt). Under the reaction conditions used, the release of phosphate was directly proportional to enzyme concentration and was independent of substrate concentration; the rate of phosphate release was constant for 30 min. The reaction was stopped by the addition of 1.0 ml 10% trichloroacetic acid (TCA). The precipitated protein was sedimented at low speed and the supernatant was assayed for phosphate released during the reaction using the method of either

Fiske and SubbaRow (15) or Ames and Dubin (16). Assay medium to which a TCA-denatured tissue sample was added at time zero served as a blank. The pellets were resuspended in 1.0 ml of 1 N NaOH for protein determination by the method of Lowry et al. (17) using bovine serum albumin (BSA) as standard. To determine protein concentration in homogenates of fixed tissue, the TCA precipitate was heated in 1 N NaOH in capped tubes for 30 min at 90–100°C to solubilize the protein. Similarly treated BSA was used as standard. Routinely, specific activity was determined as micromoles of inorganic phosphate released per milligram protein per unit time.

RESULTS

The Development of Glucose-6-Phosphatase Activity

Fig. 1 illustrates the development of G6Pase activity in whole homogenates prepared from animals ranging in age from 4 days before birth to 72 hr after birth; similar results were obtained with isolated rough microsomes. The enzyme specific activity increases slowly before birth, reaching a value approximating that of the adult at the time of birth. Then, during the next 24 hr

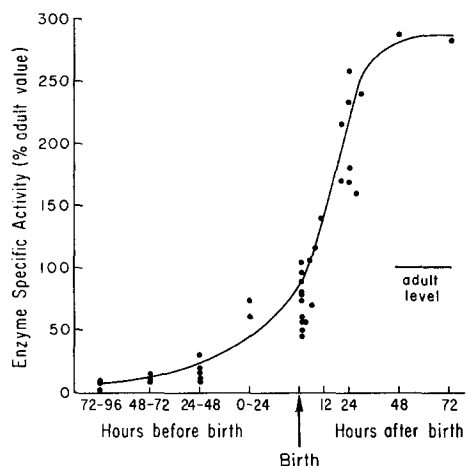


FIGURE 1 G6Pase activity in liver homogenates during development. The adult value (1.86 μ moles P_i /mg protein per 20 min, 37°C) is the average of six experiments each carried out on a single adult liver. Each point on the curve represents a homogenate prepared from the combined livers of three to five animals from the same litter. Duplicate points at the same stage represent different litters. In a single experiment, littersmates were used for all time points after birth. All determinations were done in duplicate.

the specific activity increases rapidly, overshooting the adult value by 2–3-fold. Similar curves have been reported by Burch et al. (1), Dallner et al. (3), and Greengard (4), although the time and degree of overshoot are varied. The increase in activity after birth is most likely due to new enzyme synthesis since it can be prevented by puromycin or actinomycin treatment (3). Dallner et al. (3) have shown that the activity drops off several days after birth and slowly decreases to the adult level. Greengard and Dewey (18) have postulated that the slow accumulation of G6Pase activity during the last few days of gestation reflects the increased metabolic activity of the fetus caused by the thyroid, which becomes functional at this time. In fact, they found that injection of thyroxine (18) or glucagon (19) can induce precocious development of G6Pase activity in the late rat fetus. The dramatic increase right after birth is probably a response to postnatal hypoglycemia (19, 20); the increased enzyme activity facilitates the conversion of stored glycogen into free glucose.

Parameters of the Cytochemical Reaction

FIXATION: One of the most difficult steps in cytochemical work is the selection of a method of tissue fixation which meets two criteria: reliable morphological preservation and retention of enzyme activity. Glutaraldehyde, a fixative widely employed in cytochemical work, has been reported to inhibit G6Pase activity when used for extended periods of time (21). However, we have found that a short perfusion fixation is sufficient to permit good preservation of the liver tissue without inactivating the enzyme. Table I shows that at all ages the amount of activity recovered after a short perfusion fixation was 70–85% of that present before fixation. This high recovery of activity implies that the cytochemical reaction product is produced by the bulk of the enzyme present in the unfixed tissue. Neither purification of glutaraldehyde by passage through Norit (22) (American Norit Co., Jacksonville, Fla.), nor lowering the fixative concentration to 1% decreased enzyme inactivation; moreover, the lower concentration of fixative resulted in poor fixation. Although inclusion of G6P during fixation did not protect the activity appreciably, it was routinely included during fixation as an added precaution since the enzyme is relatively unstable (14, 23, 24). This exposure of the tissue to substrate does not contribute any

TABLE I

Glucose-6-Phosphatase Activity after Fixation by Perfusion

Each experiment represents a different perfused liver. In the experiments on adult liver, specific activity after fixation is compared to that found in the same liver before fixation. In the newborn, the comparison is made between fixed and unfixed tissue from litter mates.

Homogenates from both fixed and unfixed tissue were subjected to equal sonic treatment which was continued until the number of unbroken cells in the homogenate of fixed tissue was approximately equal to that in the unsonicated homogenate of fresh tissue. All specific activity figures refer to sonicated homogenates and are averages of two determinations.

Age	Fixative	Sonication	Fixed		Control	Activity remaining
			sec	$\mu\text{moles } P_i/\text{mg protein per 20 min}$		
Adult	2% glutaraldehyde unpurified	35	1.18	1.69		70
"	2% glutaraldehyde purified*	35	1.26	1.66		76
"	2% glutaraldehyde unpurified + 6 mM G6P	70	1.26	1.60		79
"	1% glutaraldehyde purified*	30	1.30	1.66		78
Newborn (2 hr)	2% glutaraldehyde unpurified + 6 mM G6P	60	0.86	1.04		83

* By filtration through Norit.

TABLE II
*Lack of Effect of 2 mM $Pb(NO_3)_2$ on
Glucose-6-Phosphatase Reaction*

A. Effect on enzyme activity*	
Incubation conditions	$\mu\text{moles } P_i/\text{mg protein}$
no Pb^{++}	1.18
+2 mM $Pb(NO_3)_2$	1.18
B. Effect on substrate†	
Minutes of incubation	$\mu\text{moles } P_i/\text{ml}$
0	0.038
30	0.025
60	0.033

All values are averages of two determinations.
* 10 mg (wet weight) adult liver homogenate were incubated in 1 mM G6P, 0.05 M cacodylate buffer, pH 6.6, at 25°C for 30 min in the presence or absence of 2 mM lead nitrate.

† 1 mM G6P was incubated in 0.05 M cacodylate buffer, pH 6.6, at 25°C with 2 mM lead nitrate. Samples at the beginning of the incubation, and after 30 and 60 min were immediately assayed for inorganic phosphate.

background precipitate detectable biochemically or cytochemically.

To assay G6Pase activity in fixed tissue, sonication of the homogenate was necessary. Such sonication of homogenates from unfixed tissue caused a loss of enzyme activity which varied between 0

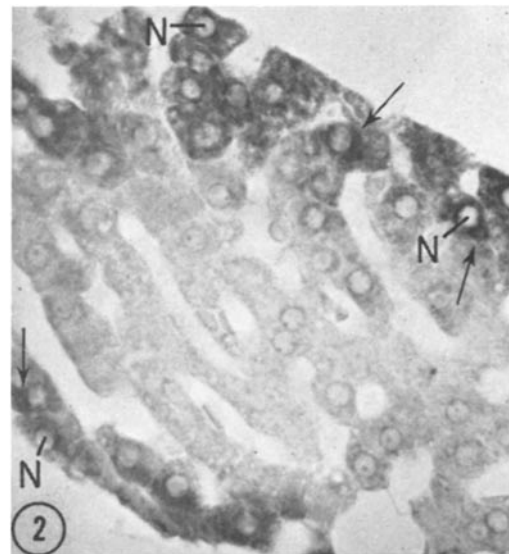


FIGURE 2 Light micrograph of tissue slice ($\sim 90 \mu$ across) from adult liver incubated for 30 min for G6Pase activity. The section, $\sim 0.5 \mu$ thick, is unstained so the contrast is due solely to the reaction product. Note the localization of the deposit in the cytoplasm of the hepatocyte (arrows) and the lack of deposit in the nuclei (N). $\times 680$.

and 23%; it was assumed that a similar loss occurred during sonication of homogenates from fixed tissue. The difference between the specific activities of sonicated-unfixed and sonicated-fixed

tissue is thus assumed to be due to inactivation of the enzyme by fixation.

EFFECT OF LEAD IONS: Since lead ions have been shown to inhibit some phosphatases (25-27), and since 2 mM lead nitrate is routinely used in the G6Pase incubation medium, the effect of this lead concentration on G6Pase activity was assayed under the conditions of the cytochemical reaction. After 30 min of incubation, samples were removed and were treated with 5% TCA. Pre-

liminary experiments showed that 5% TCA completely solubilizes precipitated lead phosphate and releases it from the tissue; the inorganic phosphate determined thus includes the phosphate initially present as lead phosphate. The upper part of Table II illustrates that 2 mM lead nitrate had no effect on G6Pase activity. This concentration of lead nitrate is the highest that can be used in the incubation mixture without precipitation occurring. Previous investigators (28), using higher

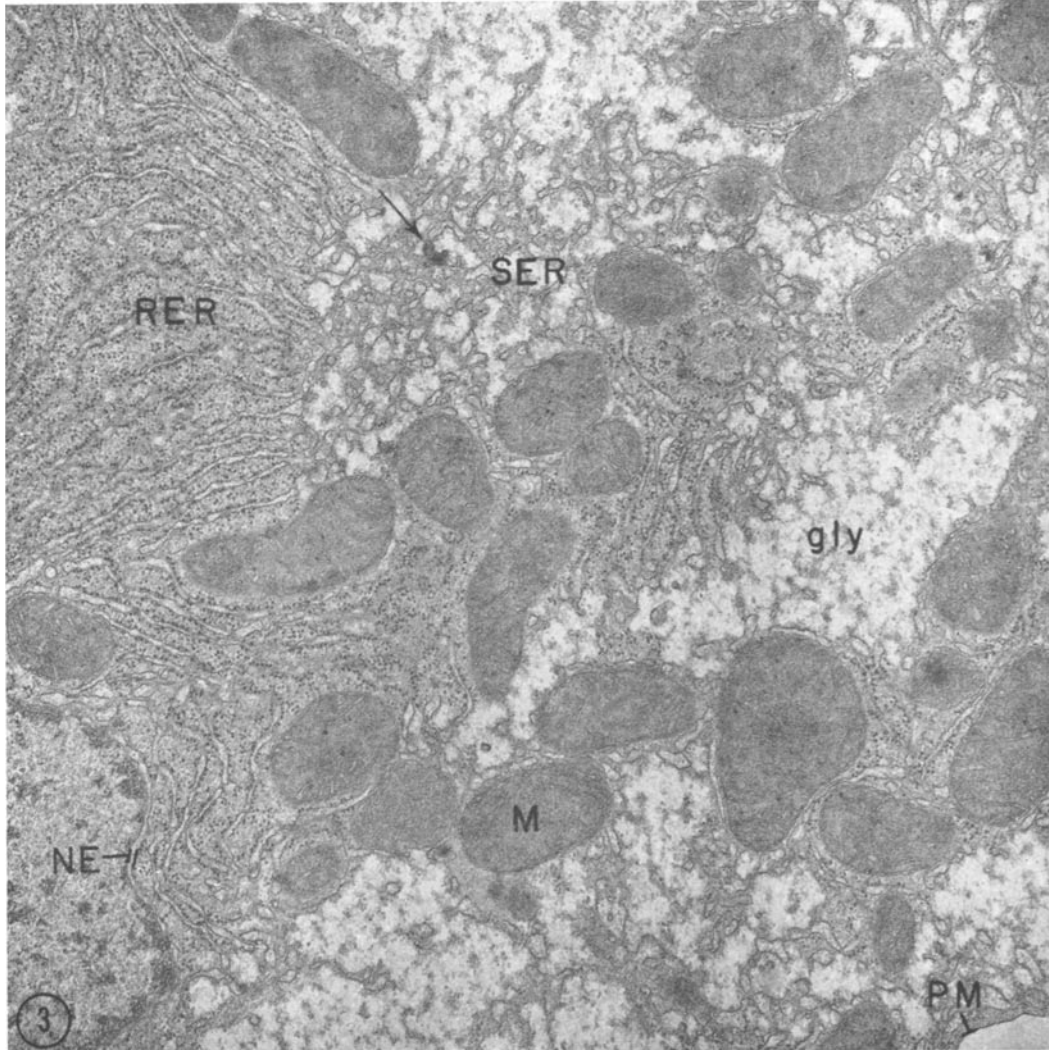


FIGURE 3 Electron micrograph of a hepatocyte from an adult liver fixed by perfusion. The specimen was not incubated for the G6Pase reaction. Note the difference between the parallel arrangement of the rough ER cisternae (*RER*) and the tightly meshed network of smooth ER tubules (*SER*). *NE*, nuclear envelope; *M*, mitochondria; *PM*, plasma membrane; *gly*, glycogen region. The arrow points to a myelin figure produced during fixation. $\times 18,500$.

concentrations, mentioned the formation of such a precipitate.

Another possible source of artifact is the reported ability of lead ions to hydrolyze various phosphate esters (29-32). Since under some conditions this nonspecifically released inorganic phosphate is precipitated preferentially in certain organelles (30), interpretation of the results becomes ambiguous in the presence of such a nonenzymatic hydrolysis. To determine whether lead-catalyzed, nonenzymatic hydrolysis was occurring in our system, G6P and lead were incubated under the same conditions as those used for the cytochemical reaction, but without tissue. Table II (bottom) shows that in the presence of lead only, there is no significant increase in the amount of inorganic phosphate in the medium. The initial level of free phosphate can be accounted for by the contamination of the reagents.

PENETRATION OF SUBSTRATE AND TRAPPING AGENT: During the 30 or 60 min incubation, lead and substrate do not completely penetrate the 50-100- μ m-thick tissue slices. To obtain reproducible results it is necessary to determine the depth of penetration of these reagents. By embedding the entire incubated slice, and trimming and sectioning it so as to include the two opposite edges exposed to the reaction medium, it is possible to examine the entire width of the slice. Fig. 2 shows a light micrograph from adult liver tissue which was processed for light microscopy and then embedded in the manner just outlined. The cytochemical product is clearly seen at the edges of the slice where the deposit is heavy and is limited to the cytoplasm; the intensity of the reaction product is relatively even over one to three rows of cells and then decreases rapidly towards the interior of the slice. The deposit in the outer regions, which was specific and reproducible, increased in intensity in a manner roughly paralleling the increase in biochemical activity seen during development, whereas the center of the slices lacked deposits at all stages. Only occasionally were nonspecific deposits seen along the outer edges. Further observa-

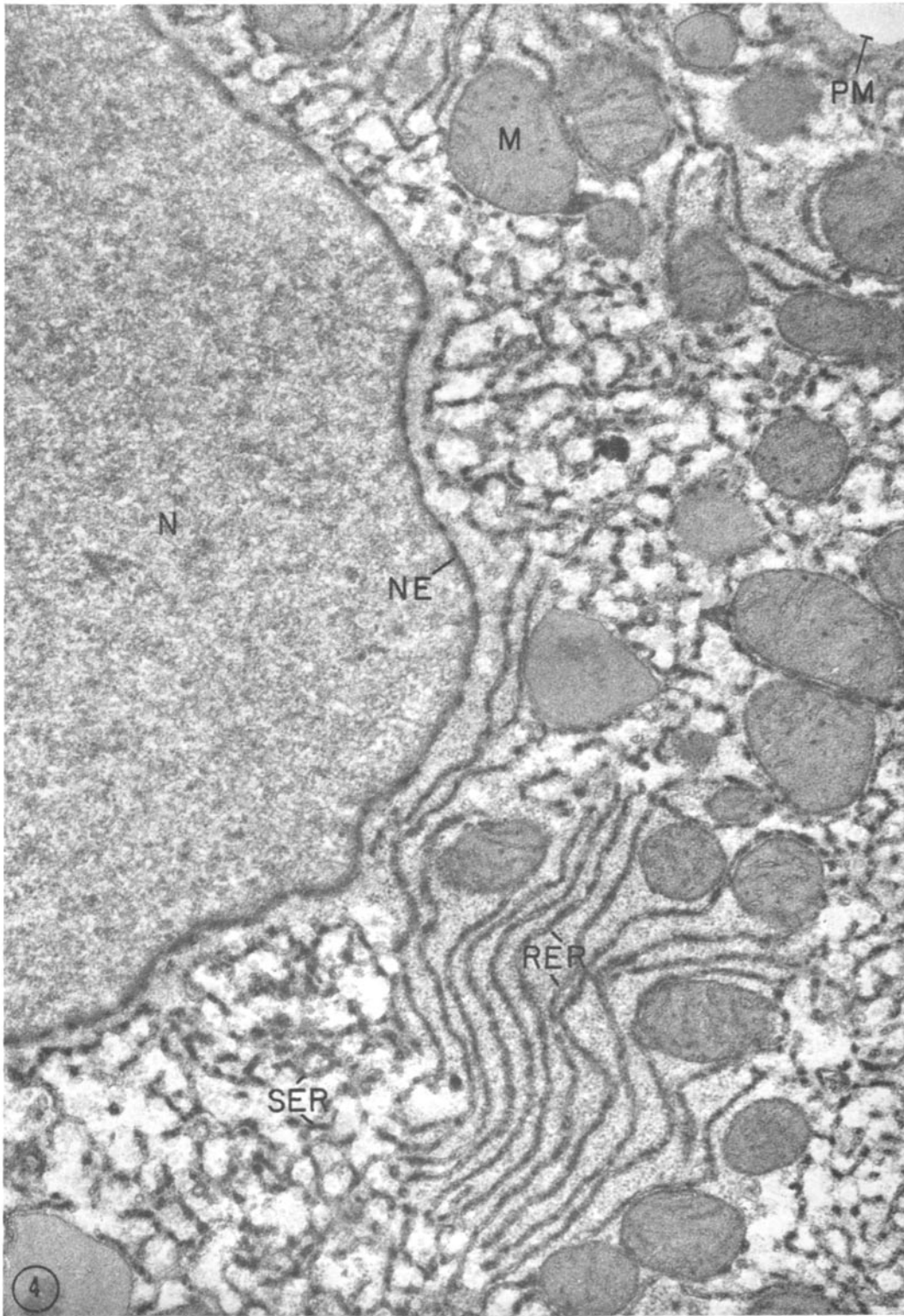
tions were thus always confined to the outermost layers of cells where both lead and substrate were readily available. In this manner reproducible and consistent results were obtained. Our findings on the limited penetration of reagents in the tissue are similar to those already reported by others (33, 34).

CONTROLS: In order to substantiate further the assumption that the activity localized cytochemically is actually G6Pase, a number of controls were conducted on liver taken from animals at all stages of development. Omission of substrate, prior heating of the tissue for 5 min at 80°C, or substitution of β -glycerophosphate for G6P produced little or no observable deposit. Biochemical tests under the same incubation conditions showed that no inorganic phosphate was released. In addition to those parameters so far mentioned, it was found that ether anesthesia produced, if anything, a very slight increase in enzyme activity, and G6Pase was as active in cacodylate as in Tris buffer.

Preservation of Morphology

Fig. 3 shows a typical hepatocyte from adult liver tissue fixed by perfusion but not incubated in the cytochemical medium. The cells of the liver remain intact and retain their normal topographical relationships to each other. At all stages of development the morphological preservation of the tissue is good and is similar to that seen after prolonged fixation. Both the rough and smooth ER are well preserved and are easily recognizable; the Golgi complex (not shown) and other intracellular components retain their accepted morphological appearances. There is no vacuolation of the ER, the nuclear envelope, or mitochondria, although occasionally small myelin figures are seen in the vicinity of the smooth ER or Golgi membranes. The glycogen regions appear light instead of dark, probably because glycogen particles do not stain after fixation in cacodylate buffer. They appear to be stained reproducibly and intensely by lead only after fixation in phosphate buffer. The difference

FIGURE 4 Electron micrograph of a hepatocyte from an adult liver incubated for 30 min for G6Pase activity. The deposits of reaction product are evenly distributed throughout the ER and, with few interruptions, label quasi continuously the entire system. Note the poor definition of the ribosomes and the density of the matrix surrounding the rough ER (*RER*) compared to that surrounding the smooth ER (*SER*). *N*, nucleus; *NE*, nuclear envelop; *M*, mitochondria; *PM*, plasma membrane. $\times 26,000$.



in topography between the rough and smooth ER is well demonstrated; the rough ER is arranged as long parallel cisternae while the smooth ER appears as a network of connected tubules. The rough ER ends at the edges of the glycogen deposits while the smooth ER is found within the glycogen regions. Since the animals were not starved before sacrifice, the glycogen deposits were very large and sometimes the smooth ER did not penetrate them completely.

After reaction in the cytochemical medium, it is often difficult to distinguish between rough and smooth ER solely by the presence of ribosomes on the former since the ribosomes are not clearly visible. This may be due to depolymerization of ribosomal RNA by lead (35) or to the presence of dense lead deposits inside the cisternae which interfere with the observation of ribosomes. After reaction, the identification of the two types of ER relies primarily on differences in their characteristic arrangement and on the electron opacity of the surrounding matrix. Rough-surfaced ER profiles are embedded in relatively opaque matrix made up of free ribosomes and ribosomes attached to membranes obliquely cut by the section, while smooth ER membranes are located in the lighter matrix of the glycogen regions.

Cytochemistry

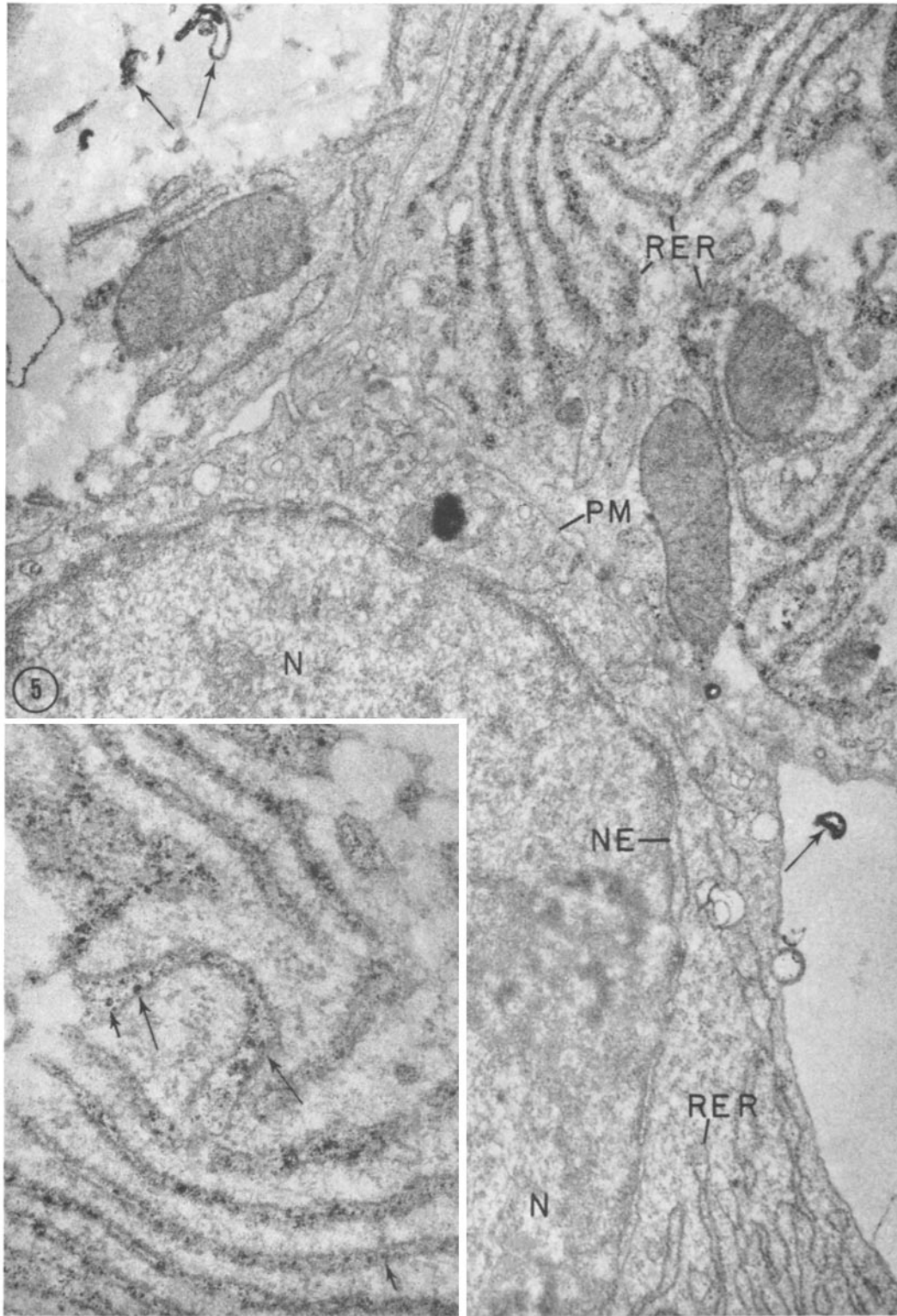
ADULT: The cytochemical reaction in adult tissue incubated for 30 min is illustrated in Fig. 4. The localization of deposit, exclusively within the cisternal space of both rough and smooth ER and within the nuclear envelope, agrees with that originally found by Tice and Barnett (28) and subsequently confirmed by others (36-38). There is no reaction along the plasma membrane, in the elements of the Golgi complex (not shown), or over other intracellular components. The deposit

is heavy and is distributed uniformly throughout the ER of the cell. Although the intensity of the deposit varies from cell to cell, all of the hepatocytes react positively. In light microscope histochemical experiments, designed to observe the intralobular distribution of the reaction, the lead phosphate was found to be evenly deposited across the lobule from the central to the portal veins. In such experiments, 20- μ thick, frozen sections of perfusion-fixed liver were used to insure even penetration of reagents. A uniform distribution across the lobule was also found by Tice and Barnett (28), but other reports (39-41) describe a stronger reaction in the periportal region of the lobule in rats and mice. This discrepancy does not affect our results, but deserves further investigation.

FETUS: At the earliest stages examined, 4 and 3 days before birth, the gross anatomy of the liver is similar to that of the fully adult animal but the microscopic anatomy is extremely different. The cell population of the liver is heterogeneous; at least half of it consists of developing blood cells, mostly erythroblasts, dispersed among hepatocytes and in close contact with them. The hepatocytes themselves are small and irregularly shaped. They contain a small amount of rough ER often with randomly distributed and randomly oriented cisternae. They have essentially no smooth ER, although they have a small Golgi complex.² Glyco-

²The morphology of the Golgi complex in hepatocytes is described in detail in a manuscript in preparation by J. Ehrenreich and G. E. Palade (Studies on the Golgi complex. I. Isolation procedure from rat liver homogenates). For its relations with the ER, especially for the morphology of the Golgi-ER connections described as "GERL," reference 47 should be consulted. Except for the appearance of dense particles, presumably very low density lipo-

FIGURE 5 Electron microscope cytochemistry in hepatocytes of a fetus \sim 4 days before birth. The field, taken near the edge of the tissue slice, shows two adjacent cells, one (upper right) containing lead phosphate deposit within the ER (*RER*), and the other (lower left) lacking deposit in the ER and nuclear envelope (*NE*). Note the large size of the nucleus (*N*) in the lower cell. *PM*, plasma membrane. Incubation, 60 min. The arrows point to myelin figures heavily stained by lead during the incubation; this type of staining is not produced by the G6Pase reaction since it occurs also in controls incubated without G6P. The *inset* shows at higher magnification a small region in the G6Pase-positive cell. The lead phosphate deposits appear as fine, dense particles scattered individually or in small clusters throughout the ER. Some deposits (long arrows) are in contact with the inner aspect of the ER membrane; others (short arrows) appear free in the cisternal content. $\times 25,000$; *Inset*, $\times 46,000$.



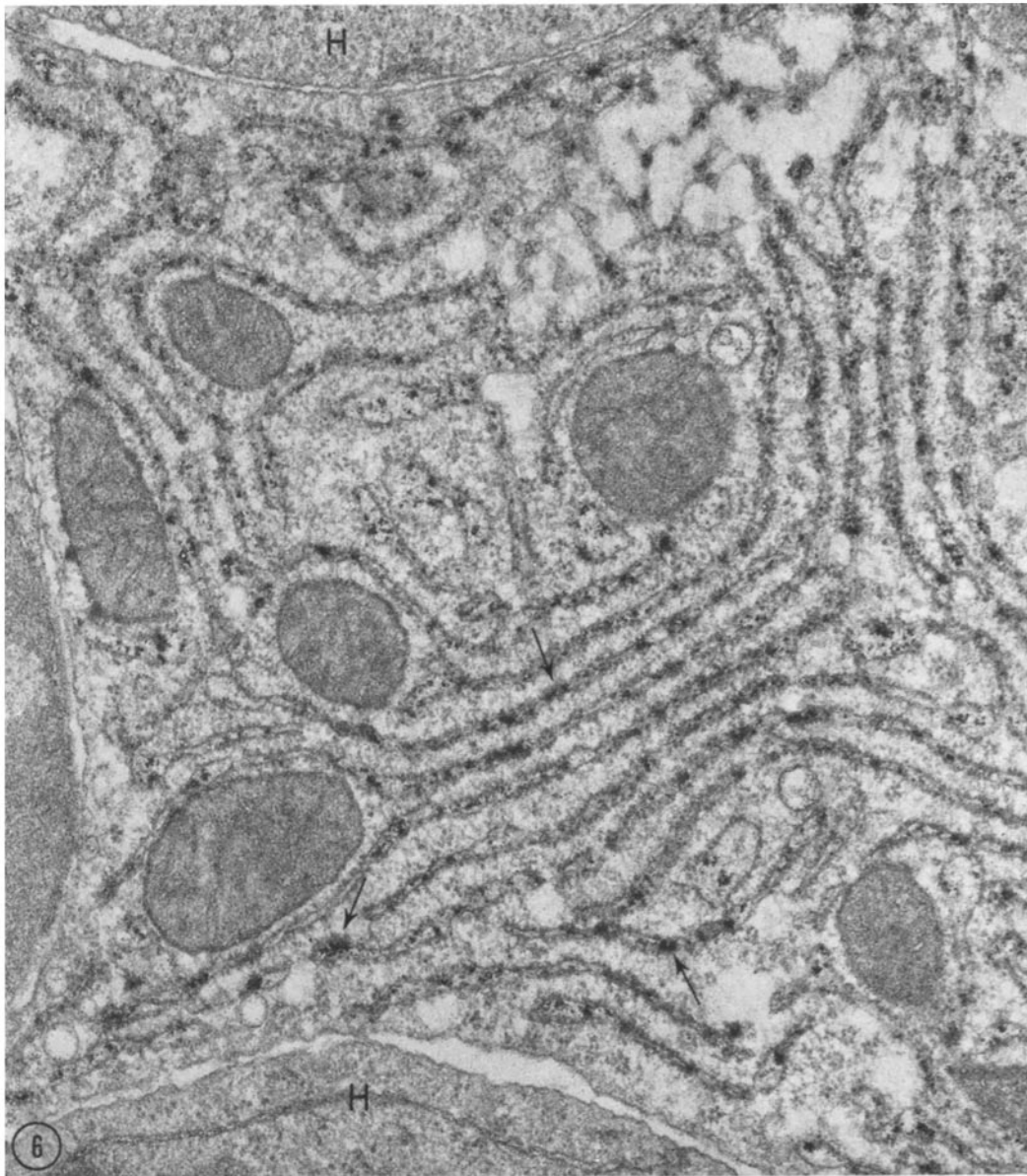


FIGURE 6 Electron micrograph of a hepatocyte of a fetus ~ 4 days before birth. Specimen incubated 60 min for G6Pase activity. The field is near the edge of the tissue slice. Particulate deposits of lead phosphate are scattered throughout the entire ER; the arrows mark areas filled by heavy deposits. *H*, hematopoietic cells. $\times 31,000$.

gen has begun to collect in the cells but the deposits are still much smaller than at birth.

proteins, in Golgi elements soon after birth, changes in the organization of the Golgi complex during development are much less striking than the changes incurred by the ER.

At these stages the biochemically determined G6Pase specific activity is ~5% of that in the adult. Within the population of hepatocytes, however, this activity is very heterogeneously distributed. After cytochemical incubation, most of the cells do not contain any visible lead deposit,

while a small number of cells show a light but positive reaction. A heterogeneous distribution of the G6Pase reaction within the hepatocyte population was also seen when incubated tissue slices were processed for light microscopy. Fig. 5 is an electron micrograph which illustrates the two types of cells directly adjacent to one another. One cell has no deposit in either the rough ER or the nuclear envelope, while the other has deposit specifically located in the ER. In each ER profile, the reaction product appears scattered as free dense particles within the content of the cisternal space and on the inner aspect of the limiting membrane. The relative frequency of the two types of association is difficult to ascertain in sections, but the presence of deposits in the content (without apparent connection with the membrane) is supported by the fact that the reaction product is found evenly distributed within the cisternal space of dilated, normally sectioned cisternae irrespective of the total amount of deposits present; i.e., the association with the membrane does not become predominant or exclusive in specimens reacted for a short time or in specimens with inherently low G6Pase activity. The distribution of the reaction product in the ER content and on the inner aspects of the ER membrane remains unchanged throughout the differentiation process (cf. Figs. 6 and 7). Within a cell which has enzyme activity, the reaction product is found in all of the ER elements present in that cell, as is shown in Fig. 6. Even after the 60 min incubation the deposit is much lighter than that found in the adult after a 30 min incubation. Thus, while G6Pase activity develops asynchronously in the hepatocyte population, it appears simultaneously within all the ER elements of every cell that acquires this activity.

By 2 days before birth, the proportion of hematopoietic cells has decreased. The hepatocytes have increased in size and have acquired larger glycogen deposits. The ER, which is still exclusively rough surfaced, has increased in amount and its organization more closely resembles that of the adult. At this stage, the number of hepatocytes reacting positively for G6Pase has increased, although they are still in the minority, and the G6Pase specific activity is ~20% of that of the adult. The deposits seen in reacting cells have become heavier, are present in the rough ER and nuclear envelope, and are absent from the elements of the Golgi complex and from the plasma membrane. Again the deposit is spread

throughout the ER of every cell that gives a G6Pase-positive reaction.

By one day before birth, the morphology within the liver lobule begins to resemble that typically seen in adult liver. The hepatocytes have further increased their stores of glycogen, the areas of which are still completely devoid of ER membranes. The ER is still exclusively rough surfaced and is partly organized into parallel cisternae. At this time the enzyme specific activity has increased to ~70% of the adult value. A larger proportion of the cells react positively for the enzyme and the cytochemical deposit, which is heavier than at -2 days, is easily visible in the ER and nuclear envelope (Fig. 7). The Golgi complex² is conspicuously free of deposit (Fig. 8), as are the plasma membrane, the mitochondria, and the microbodies (peroxisomes) (Figs. 7 and 8). The localization of deposit is the same as in the adult, except that no smooth ER elements are as yet present in the cells. Within individual cells, the deposit is uniformly distributed in the rough ER, and within individual ER elements it is restricted to the inner aspect of their limiting membrane and to the content of the cisternal space (Fig. 7, inset).

NEWBORN: At the time of birth, the liver cells are large and contain huge deposits of glycogen that displace other subcellular components. The parallel arrays of rough ER are similar to those seen in adult liver. The ER cisternae are filled with material of relatively high electron opacity already visible one day before birth, and the outer surfaces of the ER membranes are densely covered with ribosomes. By this stage, most of the hepatocytes react positively for G6Pase, although the amount of deposit in the ER varies from cell to cell. The reaction product is again found restricted to the rough ER and nuclear envelope, and is evenly distributed throughout all of the ER of each cell.

The smooth ER begins to develop in hepatocytes after birth. This process is asynchronous in the hepatocyte population, so that at any given time cells can be found at various stages of smooth ER proliferation. Fig. 9 shows one of the earliest stages, in a cell 6 hr after birth. Smooth ends of the rough-surfaced cisternae are visible at the edges of the glycogen deposits. Fig. 10, showing a cell 24 hr after birth, illustrates a slightly later stage in the development; rough-surfaced ER elements ring the glycogen areas and completely surround them while smooth-surfaced ER profiles penetrate into these areas. Cytoplasmic "bridges" and "islands"

containing mitochondria and rough ER cisternae separate the regions occupied by glycogen deposits. A later stage in the proliferation of smooth ER is seen in Fig. 11 which is also from material 24 hr after birth. The smooth-surfaced ER has increased in amount and is visible within the glycogen areas (regions of low electron opacity). A comparison of Figs. 3 and 11 shows that at this stage the relation of the smooth ER to the glycogen begins to be similar to that found in the adult hepatocyte. At an even later stage, the glycogen regions are greatly reduced in size and are completely penetrated by the newly formed smooth ER.

By 6 hr after birth, the cytochemical deposit is heavy and quasi continuous within the ER cisternae (Fig. 12, incubated for 30 min). It is still specifically localized in the ER and nuclear envelope and is well distributed within all of the ER elements of the cell. Views of mitochondria ringed by ER clearly show the dense deposits to be present in the ER and absent from the mitochondrial membranes. All of the rough-surfaced membranes encircling the glycogen areas are filled with deposit (Fig. 13), as are the ER elements (mostly smooth ER) penetrating the glycogen regions.

By 24 hr after birth, when G6Pase activity is very high ($2\frac{1}{2}$ times the adult value), cells incubated for 30 min contain heavy deposits of reaction product on the inner aspect of the membrane and within the cisternal content of both the rough and the newly proliferated smooth ER (Fig. 14). Even at this stage of high enzyme activity, the reaction is specific to the ER; the plasma membrane is unreactive, as is the Golgi complex (not illustrated).

DISCUSSION

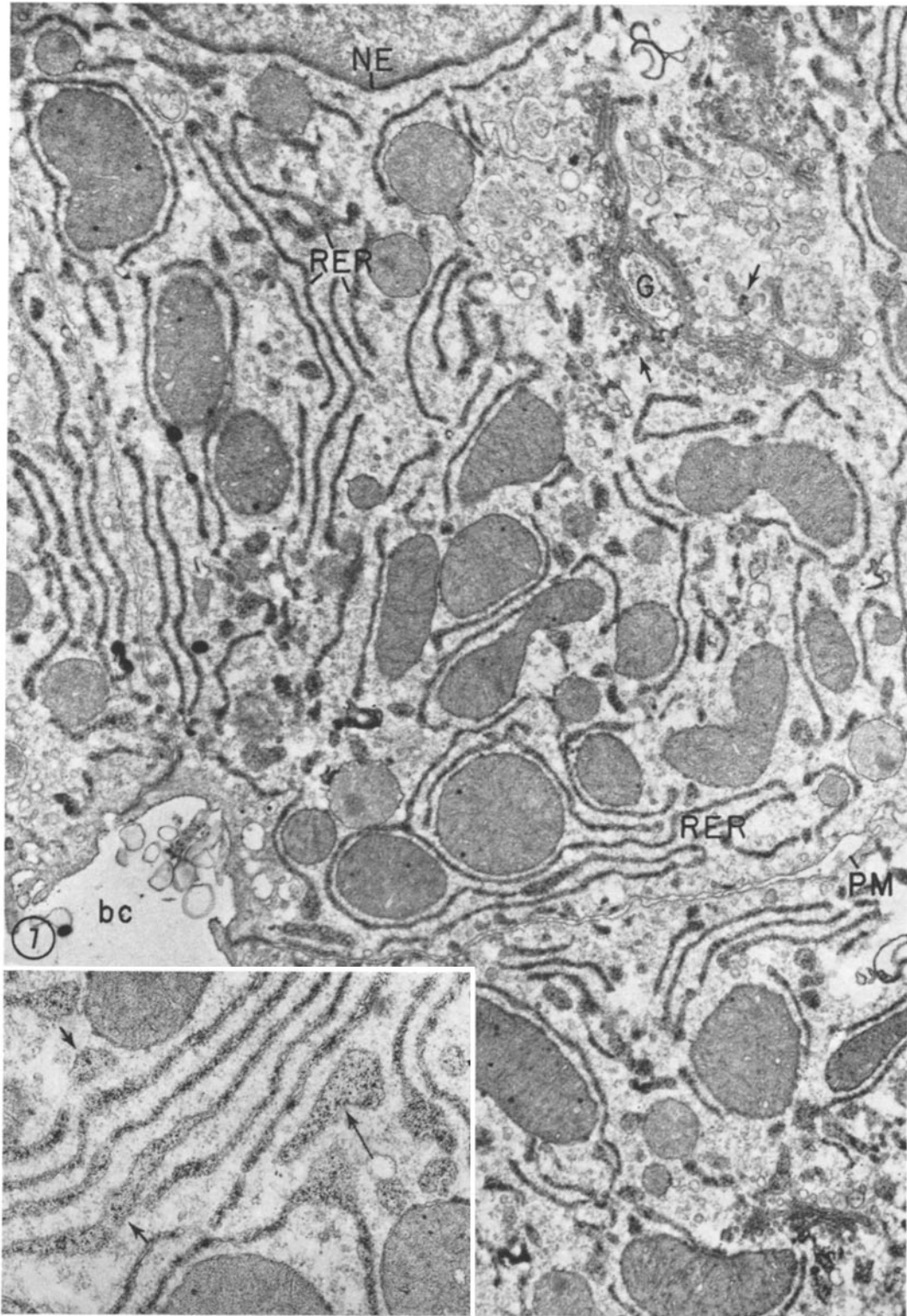
The cytochemical localization of enzymes is subject to numerous artifacts which must be excluded before meaningful conclusions can be drawn. The procedure employed should provide adequate morphological preservation of the tissue, while

preserving a large proportion of the original enzyme activity and eliminating nonspecific reactions. In the present study these conditions were met. There was only slight inactivation of the enzyme by the fixation procedure, which nevertheless allowed good morphological preservation. Sufficient lead ions were present to trap all of the released phosphate and there was no nonspecific breakdown of the substrate or inhibition of the enzyme by any of the components in the incubation mixture. We thus believe that the results obtained are a valid reflection of the situation in unfixed tissue.

In addition to this work, there have been few other reports (36, 37, 42) describing fixation procedures which retain cytochemically demonstrable G6Pase activity. G6Pase is less stable to fixation than many other enzymes (21, 28, 39), but in the liver, with its widely distributed portal system, a very short perfusion fixation preserves both enzyme activity and ultrastructural morphology. It is important that quantitative measurements of enzyme activity remaining after fixation be made. Conclusions concerning the intracellular distribution of enzyme molecules are valid only if the activity visualized cytochemically is representative of a large proportion of that enzyme. The cytochemical deposit might otherwise be produced by a small amount of protected and possibly specifically localized enzyme.

Our results provide information on the distribution and development of G6Pase at two levels of analysis. First, G6Pase activity appears *asynchronously* within the population of developing hepatocytes; some cells contain enzyme activity at a time when others have no detectable G6Pase. Thus, the small amount of G6Pase activity measured in liver homogenates early in development is a result of enzyme activity in a small number of cells, while liver G6Pase activity at birth and in the newborn rat appears to be contributed by all of the hepatocytes. Second, the enzyme activity develops *simul-*

FIGURE 7 G6Pase activity in hepatocytes \sim 1 day before birth. The reaction product is present in all of the rough ER (RER) of these cells, but is absent from the stacked cisternae, large vacuoles, and most small vesicles of their Golgi complexes (G). A few small vesicles at the Golgi periphery (arrows) seem to be marked, however, by reaction product. Their relationship to the Golgi complexes and to the adjacent G6Pase-positive elements of the ER has not been systematically studied. PM, plasma membrane; bc, bile capillary; NE, nuclear envelope. Incubation, 60 min. *Inset*: Localization of the reaction product within rough ER cisternae. Lead phosphate deposits occur on the inner aspect of the limiting membrane (long arrow) and in the cisternal content (short arrows). $\times 17,000$; *Inset*, $\times 53,000$.



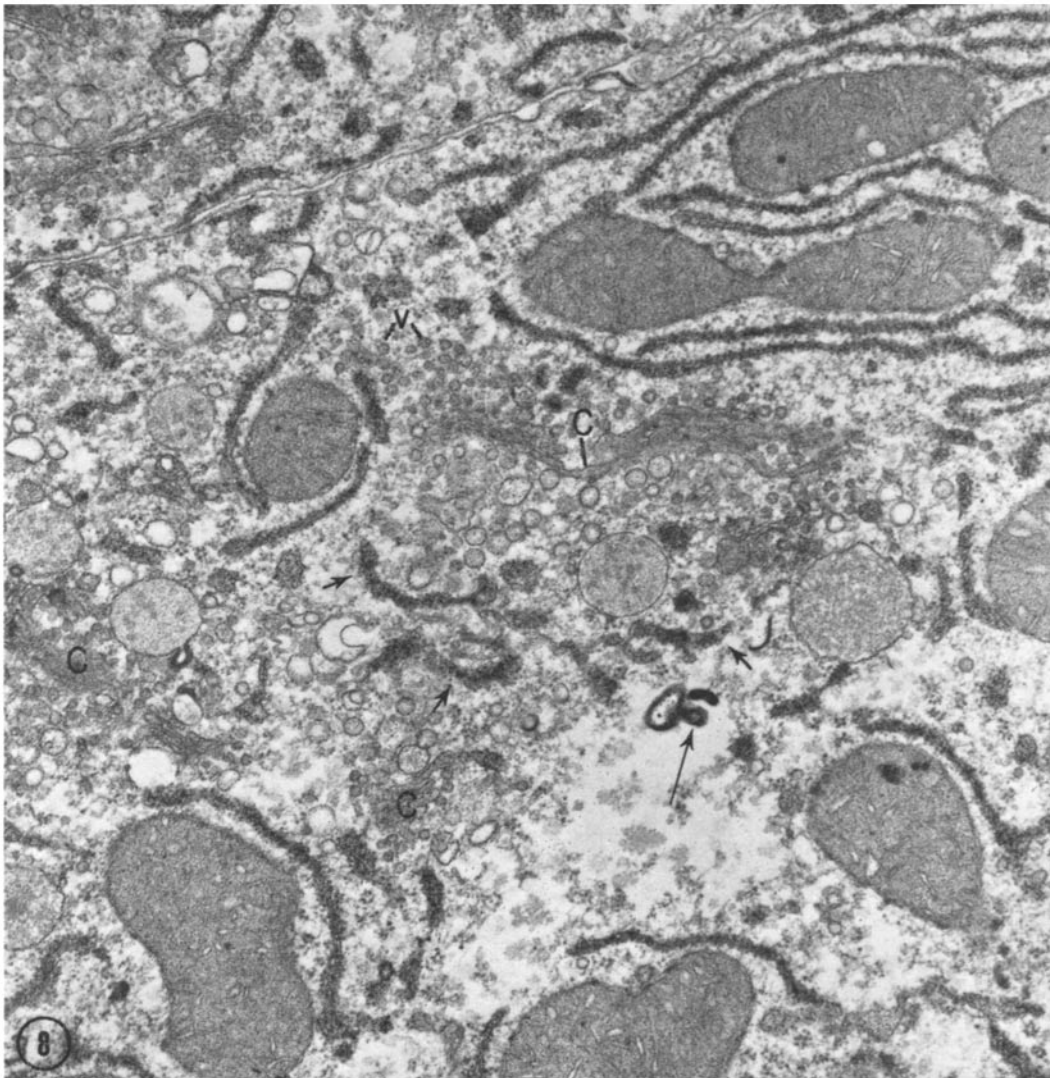


FIGURE 8 Hepatocyte ~ 1 day before birth. Specimen incubated 60 min for G6Pase activity. This higher power view of the Golgi region clearly shows the absence of reaction product in small Golgi vesicles (*v*) and in flattened Golgi cisternae (*C*), and its presence in rough ER elements surrounding, or interposed between (short arrows), the stacks of Golgi cisternae. The interposed ER elements may correspond to the "GERL" units described in liver cell by Novikoff et al. (47). The long arrow points at a stained myelin figure. $\times 53,000$.

taneously within all of the rough ER membranes of individual cells, at least within the temporal resolution of our experiments. At no stage are cells seen which contain deposit in only some cisternae of their rough ER. These findings indicate that at the level of resolution achieved by our electron microscope cytochemistry there is no regional

differentiation with respect to G6Pase within the rough ER. In addition, the enzyme appears in all of the smooth ER as these membranes form during the first 24 hr after birth, suggesting that the smooth ER too, is undifferentiated with respect to this enzyme.

Cytochemical reaction product was never found

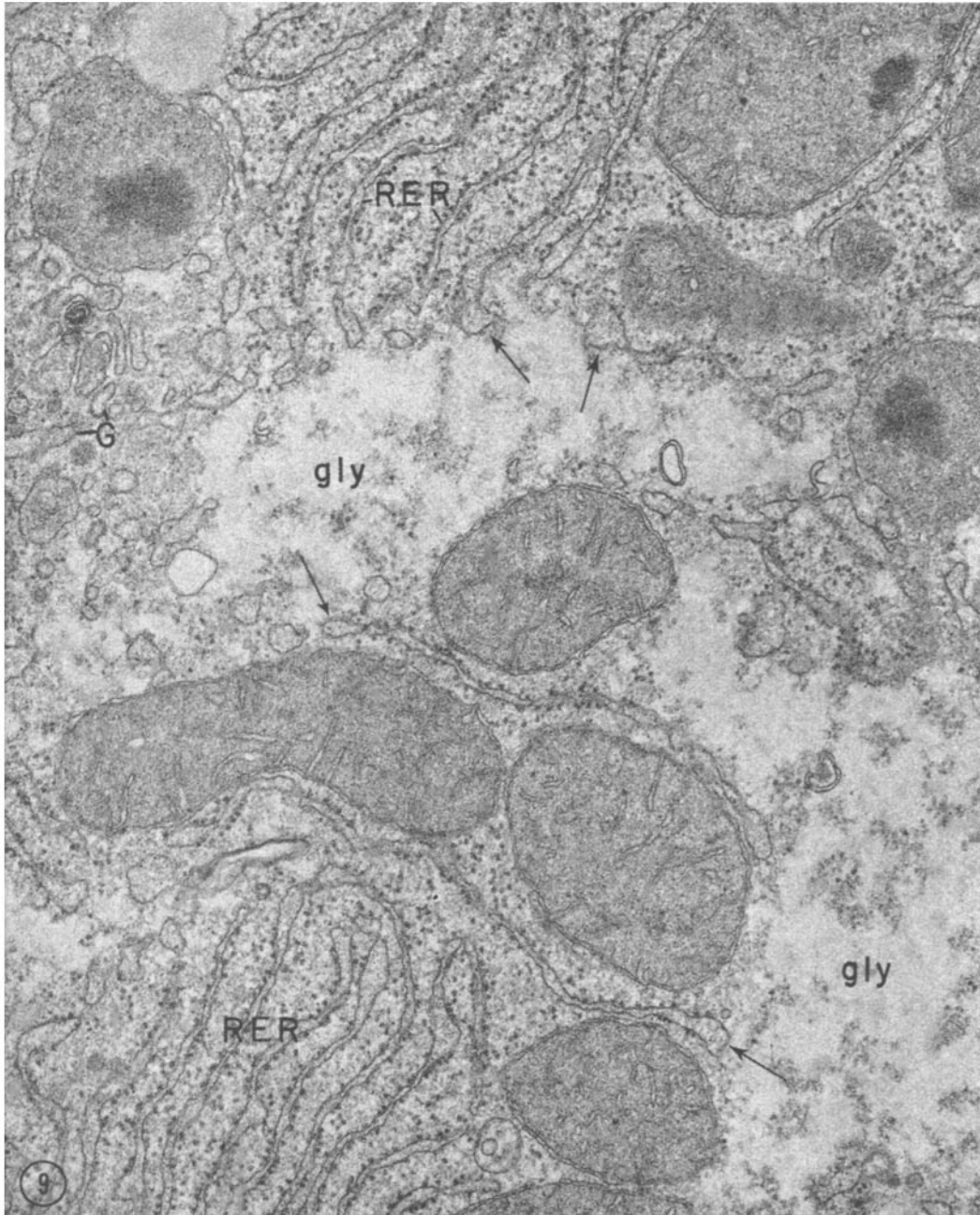


FIGURE 9 Hepatocyte 6 hr after birth; perfusion-fixed, unincubated tissue; early stage in the formation of the smooth ER. Smooth-surfaced ends (arrows) of rough-surfaced cisternae (*RER*) are present along the edges of the glycogen deposits. Most of the glycogen regions (*gly*) are still free of smooth ER profiles. *G*, elements of the Golgi complex. $\times 30,000$.

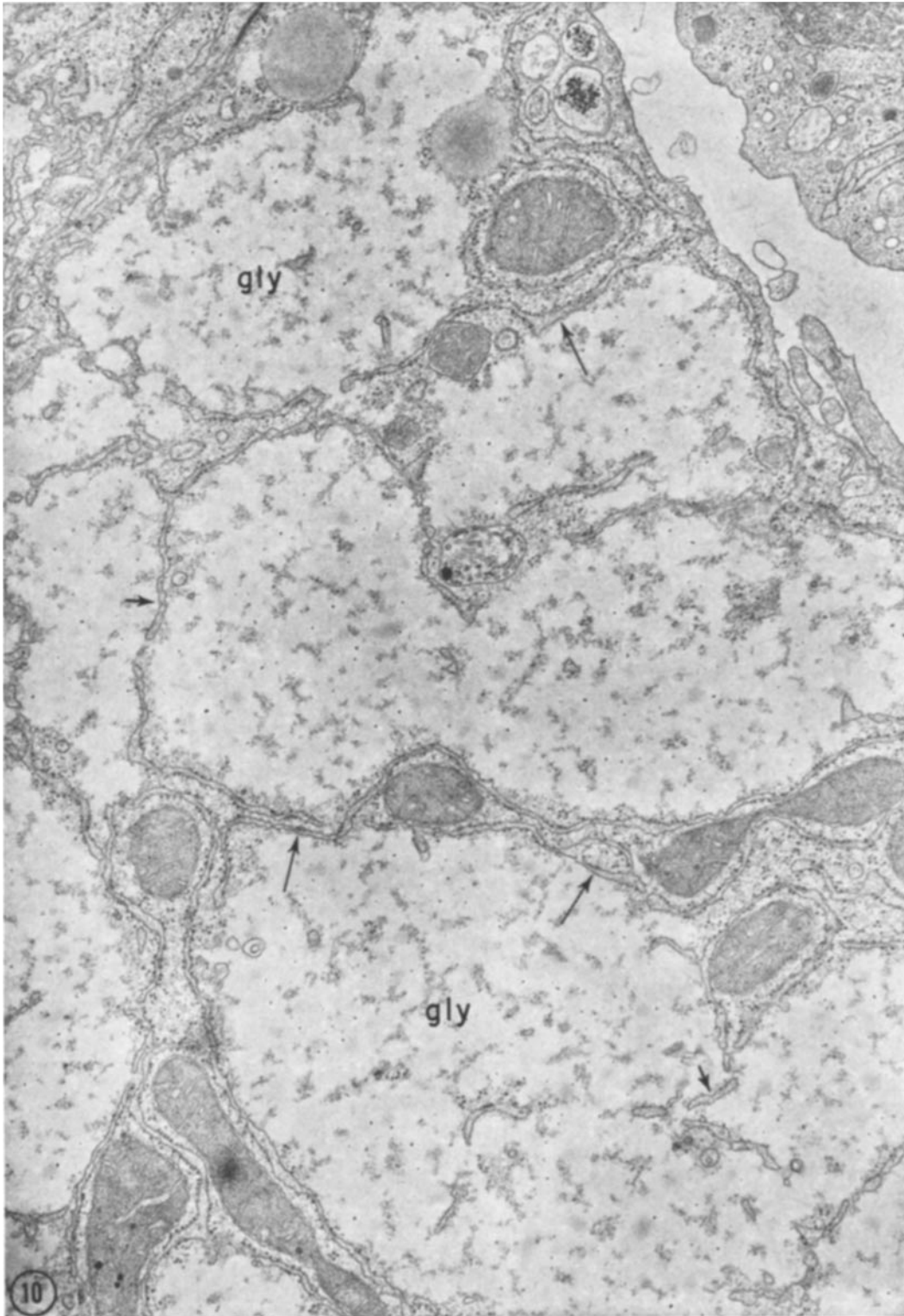


FIGURE 10 Hepatocyte 24 hr after birth; perfusion-fixed, unincubated tissue; later stage in the formation of the smooth ER. ER encircles portions of the glycogen (*gly*) deposits. Areas along the ER surrounding the glycogen deposits appear free of ribosomes (long arrows); smooth-surfaced extensions penetrate the edges of the glycogen masses (short arrows). $\times 22,000$.

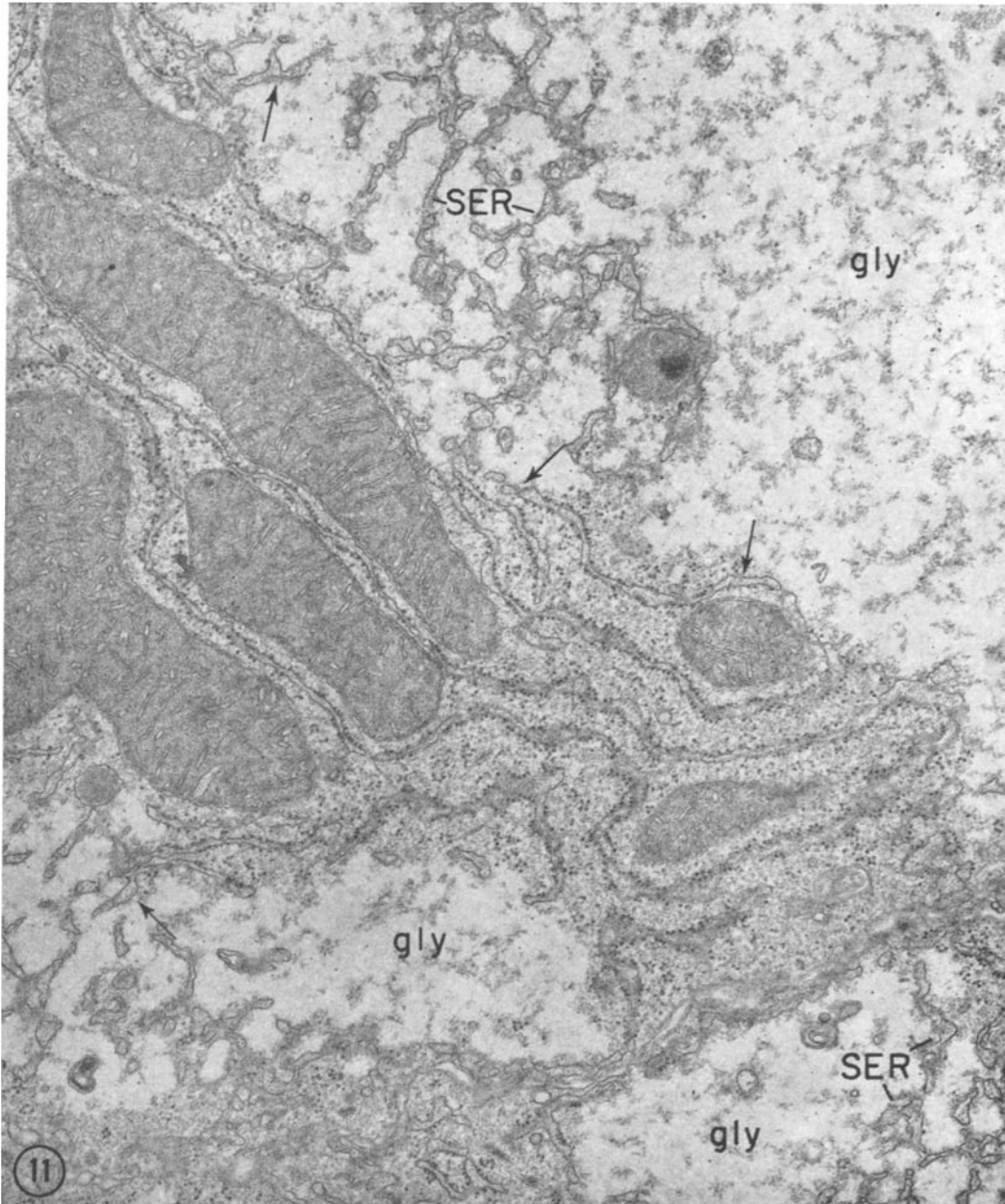


FIGURE 11 Hepatocyte 24 hr after birth; perfusion-fixed, unincubated tissue. Tubular smooth ER (*SER*) is present in the glycogen regions. Arrows indicate points of continuity between rough and smooth ER. *gly*, glycogen regions. $\times 22,000$.

in the cytoplasmic matrix or on the cytoplasmic side of the ER membranes; it appeared restricted to the inner aspect (intracisternal side) of these membranes and to the cisternal content, as seen convincingly in the normal sections of dilated ER

cisternae, especially in preparations in which the amount of reaction product was small (short incubations, or low inherent activity at the beginning of the differentiation process). The finding suggests that the phosphate released by G6Pase is preferen-

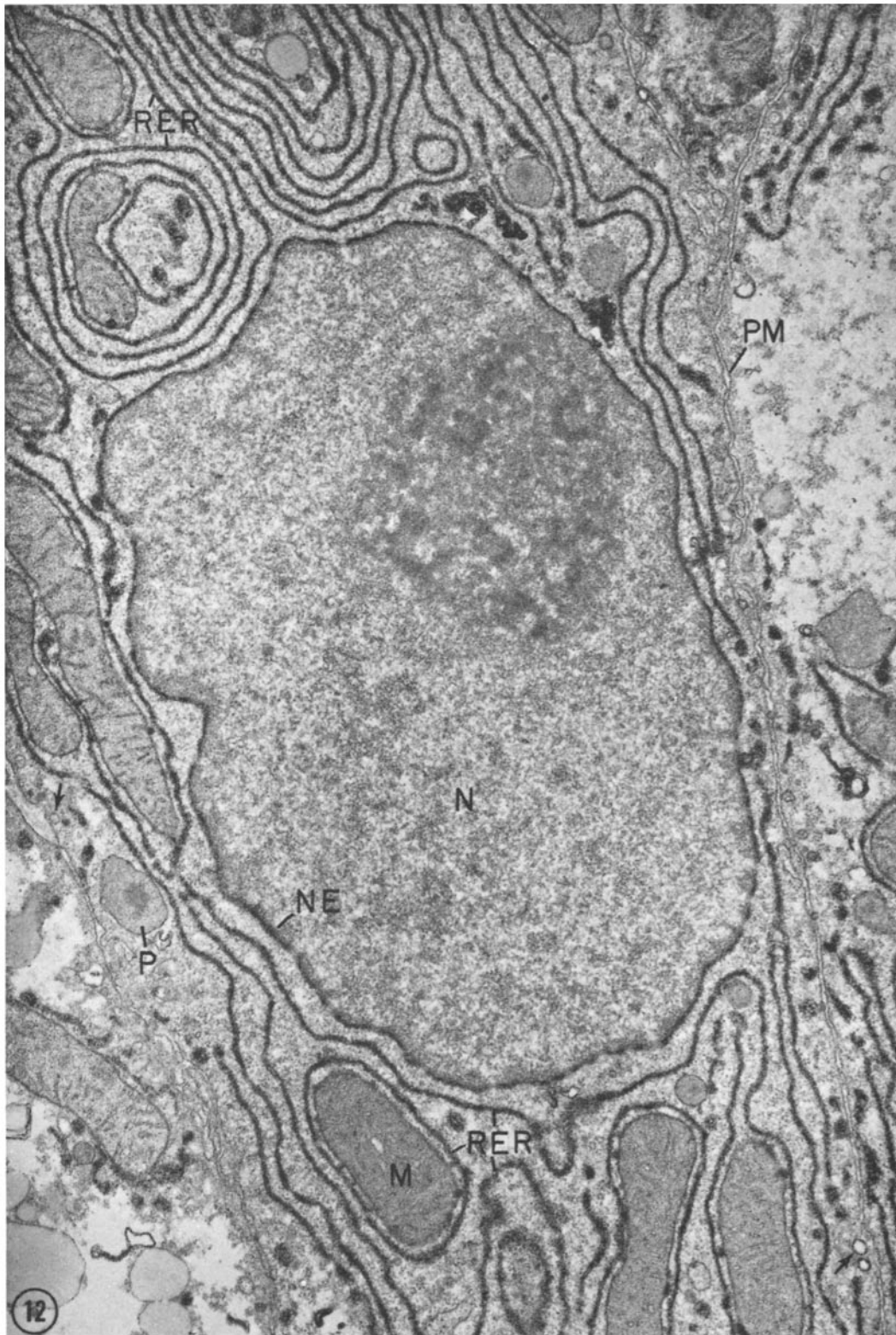


FIGURE 12 G6Pase reaction in a hepatocyte 6 hr after birth. The reaction product is clearly visible in all of the rough ER (*RER*) and in the nuclear envelope (*NE*). Deposit is absent from the plasma membrane (*PM*), some associated plasmalemmal vesicles (arrows), nucleus (*N*), mitochondria (*M*), and peroxisomes (*P*). Incubation, 30 min. $\times 15,000$.

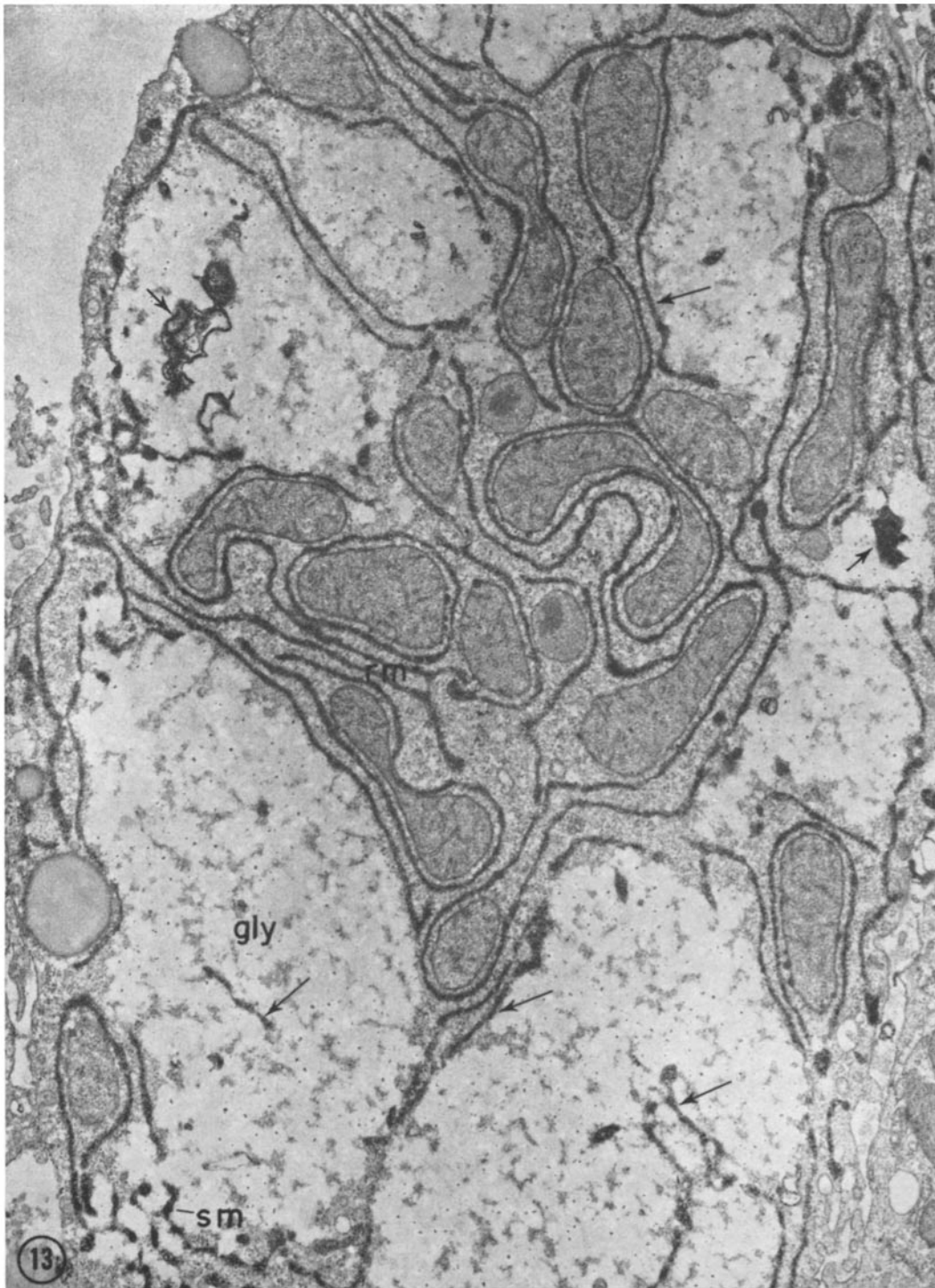


FIGURE 13 Hepatocyte 6 hr after birth. Specimen incubated 30 min for G6Pase activity. Area similar to that in Fig. 10. Lead phosphate deposit is present along the length of the ER cisternae surrounding the glycogen deposits (*gly*) and in those ER elements within the glycogen regions themselves (long arrows). The short arrows mark myelin figures stained nonspecifically by lead. At lower left is a region with reacted membranes arranged in a tubular network. Comparison with unincubated tissue suggests that this is newly formed smooth ER. *rm*, rough endoplasmic reticulum; *sm*, smooth endoplasmic reticulum. $\times 17,000$.

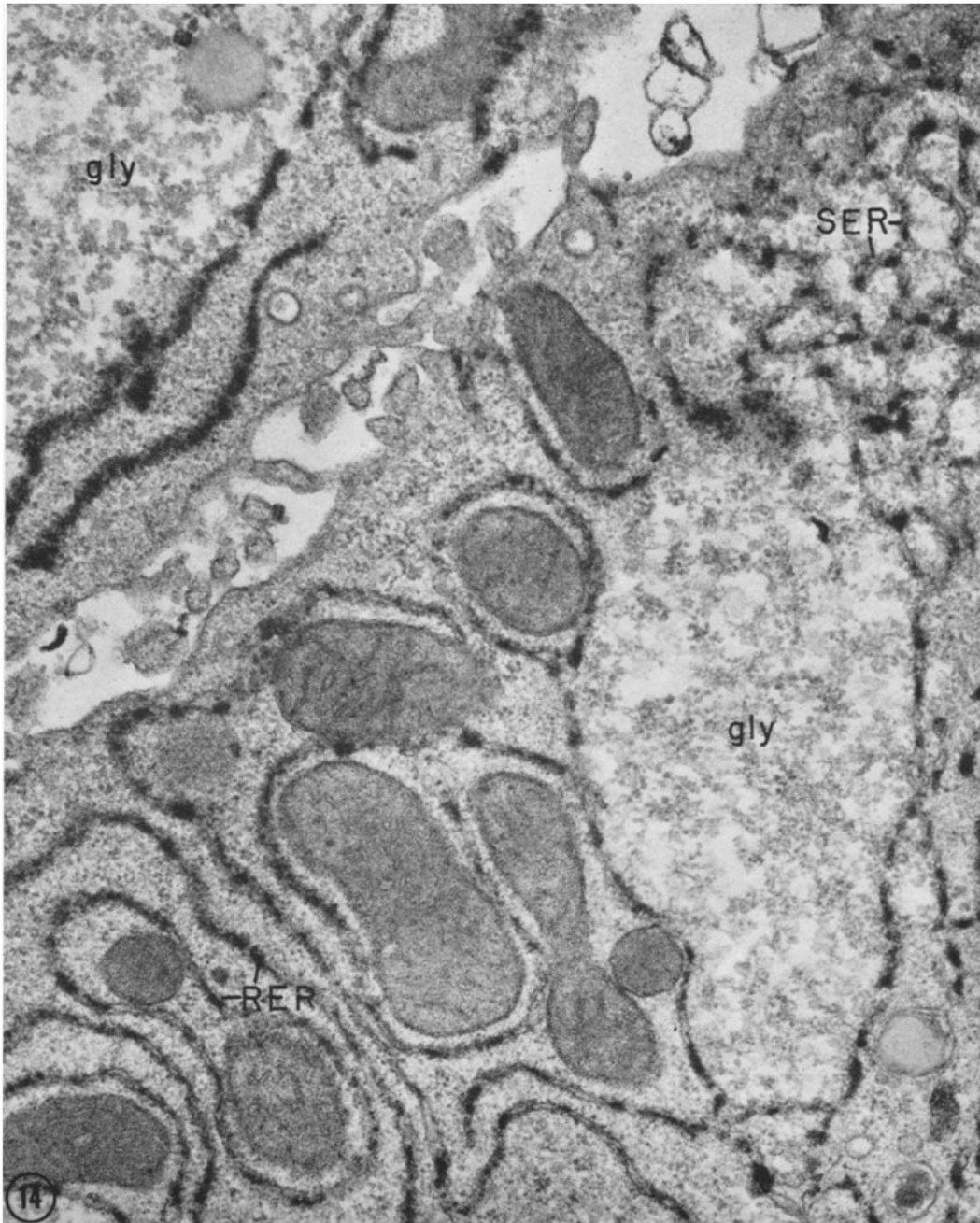


FIGURE 14 Hepatocyte 24 hr after birth; specimen incubated 30 min for G6Pase activity. Parts of two glycogen regions (*gly*) ringed by ER are seen. In the upper right is an area of smooth ER (*SER*). Cytochemical deposit is heavy in all ER elements (*RER*, *SER*). $\times 31,000$.

tially transferred to the cisternal space. This point will be enlarged upon in the companion paper (5).

In interpreting the results of cytochemical studies, a distinction should be made between sites of enzyme activity and sites of deposition of the corresponding reaction product, since these sites are not necessarily identical. In our case, the widespread deposit of lead phosphate seen along the length of each and every ER membrane can be explained by the existence of G6Pase sites similarly distributed uniformly along these membranes. Alternatively, since the ER is a continuous system of interconnected tubules and cisternae (43), the same reaction pattern could be accounted for by an uneven distribution of enzyme molecules if the released phosphate diffuses rapidly within the cisternal space prior to its precipitation by lead ions. This problem is considered in the companion paper (5) which describes a cell fractionation procedure involving the fragmentation of rough ER into microsomes before incubation in the cytochemical medium. The phosphate released by the action of G6Pase cannot diffuse from one vesicle to another, and the distribution of reaction product within the population of vesicles gives an independent analysis of the distribution of G6Pase sites. The results of that study strongly support the first explanation advanced for our cytochemical findings in the intact cell; that is, the enzyme is distributed evenly, in closely spaced sites within the rough ER of the hepatocytes.

G6Pase is a constitutive component of ER membranes in rat liver cells, and is tightly membrane-bound (23, 44-46). Its even and widespread distribution within the ER suggest that these membranes are not composed of large, functionally distinct patches each containing specific enzymes. However, this does not exclude the possibility that other membrane-associated enzymes may exhibit distinctive spatial distributions.

The relevance of these findings to the problem of membrane assembly will be discussed in the companion paper (5).

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