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The Localization of Glucose-6 Phosphatase in Developing Rat Liver: A Histochemical and Biochemical Study

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THE LOCALIZATION OF GLUCOSE-6-PHOSPHATASE IN DEVELOPING RAT LIVER: A HISTOCHEMICAL AND BIOCHEMICAL STUDY

A thesis submitted to the Faculty of The Rockefeller University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

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SUMMARY

The distribution of glucose-6-phosphatase activity in rat hepatocytes during a period of rapid cell and membrane differentiation was studied by electron microscopic histochemistry. Livers from animals 4 days before to 1 day after birth were perfused with 2% glutaraldehyde in the presence of 6 mM glucose-6-phosphate. The short fixation time (3 to 5 minutes) allowed retention of 70-90% of the enzyme activity. Slices 50-100 micra thick were incubated in 1 mM glucose-6-phosphate, 2 mM lead nitrate and 50 mM cacodylate buffer, pH 6.6. The slices were post-fixed in osmium tetroxide, oriented during embedding, and sectioned perpendicular to their length so that the complete width of a slice could be examined. Observations were confined to the outermost layers of cells where consistent results implied adequate penetration of both lead and substrate. The concentration of lead used did not inhibit glucose-6-phosphatase or cause non-specific hydrolysis of the substrate. At all stages examined the lead phosphate deposit was localized to the endoplasmic reticulum and the nuclear envelope.

At four days before birth, when the enzyme specific activity is only 9% of the adult level, the enzymatic reaction product was present in only a few hepatocytes. In these cells, a light deposit was seen throughout the entire rough-surfaced endoplasmic reticulum. At birth, with a specific activity approximately equal to that of the adult, nearly all cells gave a positive reaction for the enzyme. Again the lead phosphate was evenly distributed throughout the entire endoplasmic reticulum, nuclear envelope included. By 24 hours post-parturition, all of the rough membranes, and in addition the newly-formed smooth membranes, contained 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 non-synchronously in the hepatocyte population as a whole. In addition, the enzyme appears in the smooth endoplasmic reticulum as the membranes form during the first 24 hours after birth.

To confirm that the pattern of histochemical deposits reflects the actual distribution of enzyme sites, a method to sub-fractionate the rough endoplasmic reticulum was developed, based on the retention of reaction product within fresh microsomes reacted *in vitro*. Lead phosphate increases the density of glucose-6-phosphatase-containing microsomes and thereby makes possible their separation from enzyme-free vesicles by isopycnic centrifugation on a 2-step density gradient.

The procedure was applied to rough microsomes isolated from rats at various stages during hepatocyte differentiation, and the results obtained agree very well with those given by the histochemical experiments *in situ*. Before birth, when only some of the cells react, only a commensurate proportion of the microsomal fraction can be rendered heavy by the glucose-6-phosphatase reaction. Thus, the distribution of the reaction product in the intact endoplasmic reticulum reflects the actual distribution of enzyme sites in the membranes. The enzyme is evenly distributed in closely-spaced sites throughout the endoplasmic reticulum membranes, and there is no regional differentiation within the rough endoplasmic reticulum with respect to glucose-6-phosphatase at this level of resolution. These findings suggest a mechanism of membrane assembly and differentiation involving molecule-by-molecule insertion into a pre-existing structural framework. The membranes formed are mosaics of old and new components and do not contain regions of entirely "new" membrane, in which all components are newly synthesized or newly assembled.

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ABBREVIATIONS

mg - milligram

gm - gram

ml - milliliter

G6P - glucose-6-phosphate

G6Pase - glucose-6-phosphatase

EDTA - ethylenediaminetetra-acetate

ER - endoplasmic reticulum

MP - mitochondrial pellet

NADH - reduced nicotinamide-adenine dinucleotide

NADPH - reduced nicotinamide-adenine dinucleotide-phosphate

Pi - inorganic phosphate

PPi - inorganic pyrophosphate

PLP - phospholipid

PMB - parahydroxymercuribenzoate

PMS - post-mitochondrial supernatant

RNA - ribonucleic acid

TCA - trichloroacetic acid

V-A - veronal-acetate

INTRODUCTION

HISTORY OF THE ENDOPLASMIC RETICULUM

The endoplasmic reticulum (ER) is an interconnected network of membranes which is found within cells. These membranous components divide the cell into two compartments, and possibly act as selective barriers. The topography of the endoplasmic reticulum varies from cell to cell; in the hepatocyte the reticulum is composed of tubular vesicles and flattened cisternae. The endoplasmic reticulum membranes are of two types: those studded on their cytoplasmic surface with ribosomes, called rough ER, and those lacking the ribosomes, called smooth or sometimes tubular ER.

As a structure, the endoplasmic reticulum was unknown to the light microscopists, although sub-microscopic components with an affinity for basic dyes had been identified as early as the late 1900's. This material was known variously as basophilic material, ergastoplasm, Nissl substance and Nebernkern (see Garnier 1900 for review, Garnier 1897, Mathews 1899, Deane 1946).

In early electron microscopic investigations on flattened preparations of whole cells, Porter, Claude and Fullam (1945) and Claude, Porter and Pickels (1947) saw a lacelike network in the cytoplasm of cells. Porter (Porter and Thompson 1948, Porter and Kallman 1952) called the network the endoplasmic reticulum since in the whole mounts it was found in the interior of the cytoplasm, the endoplasm, and was absent from the outer region of the cytoplasm, the cortex or ectoplasm. Technical advances in sectioning and embedding led to the use of sectioned material for electron microscopic studies, allowing much greater resolution. In 1954 Palade and Porter (1954) and Palade (1955b) described the network in sectioned material as a reticulum of membrane-bounded vesicles. The reticulum was found in many cell types (Porter and Palade 1957, Palade and Porter 1954).

In addition to the morphological studies, work on the fractionation of cells provided evidence for the existence of sub-microscopic particles in the cytoplasm of cells. Claude (Claude 1938, 1939, 1940, 1941, 1947-48) isolated a sub-microscopic, small granule fraction which

he later called microsomes. Based on their sedimentation properties, he calculated that the granules were in the range of 50-200 mμ in diameter and he suggested that the particles were of widespread occurrence in cells. When Claude (Claude 1939, 1947-48) and others (see Hogeboom and Schneider 1955, for review) found the fraction to be rich in RNA, Claude hypothesized that the microsomes corresponded to the basophilic material of the light microscopists.

The morphological and cell fractionation observations were brought together in the early fifties. In 1953, after comparing the structure and occurrence of the ER in the electron microscope with the appearance of the reticulum seen in living cells under the light microscope, Porter (1953) postulated that the ER corresponded to the microsome fraction of Claude. In addition, he suggested (Porter 1954) that it was also the "basophilic material" of the light microscopists. Palade (1955a) identified small granules, later called ribosomes, in the cytoplasm of many cell types. He observed a close association between these granules and the membranous components of the endoplasmic reticulum. By comparing the distribution of these small granules to the staining properties of many cells, he concluded that the presence of the small granules imparted basophilia to the cytoplasm. The association of these small granules with the endoplasmic reticulum membranes produced the whorl-like shapes of the basophilic material seen in the ergastoplasm. The particles were found to be rich in RNA (Palade and Siekevitz 1956a) and were thought to correspond to the "macromolecules" isolated by Peterman and her colleagues (Peterman and Hamilton 1952, Peterman et al. 1953, Peterman et al. 1954). The RNA, which is very basophilic, is what caused the granules, and the membranes to which they were attached, to stain darkly with the basic dyes. (For reviews of the history, see Palade 1956 and Palade 1958.)

The two types of ER, the rough- and smooth-surfaced, were identified by several investigators. In 1956, Palade and Siekevitz (1956a, 1956b) undertook parallel biochemical and morphological studies of liver and pancreas cells, and proved conclusively that the microsomes were derived from the ER of the intact cell. They suggested

that when the cells are homogenized, large structures, such as the long and tubular ER, fragment; the vesicles formed are those vesicles found in the microsome fraction. Since most of the isolated microsomes obtained were closed spheres, and when ribosomes were found on vesicles they were on the outer surface, the authors proposed that there was either an active pinching off process by the membranes under the stress of homogenization, or that if the fragmentation were caused exclusively by mechanical means, then the broken ends healed very quickly to form the closed vesicles.

Since its identification as a structure within cells, much work has been done on the endoplasmic reticulum and on microsomes, using biochemical, morphological and histochemical means. A great deal of the biochemical work on the isolated microsomes has been concerned with the identification of the enzyme components. A multitude of enzyme activities has been found concentrated in this fraction, including enzymes with very diverse functions in the metabolism of the cell.

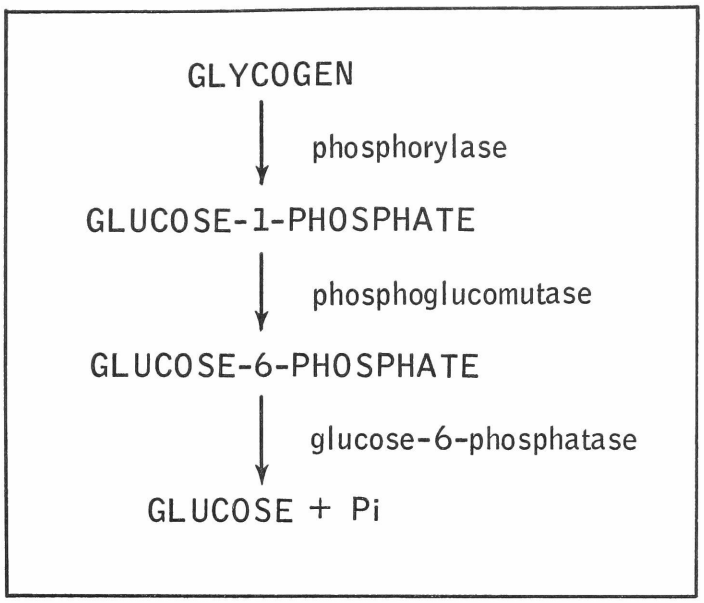
Since there are three parts to the endoplasmic reticulum, there was a question concerning the localization of the enzymes. Were they in the cisternal space, in the membranes, or on the ribosomes? The ribosomes have now generally been dismissed as a possible site of many enzyme activities, except for their predominant role in protein synthesis, and most investigators are concentrating their attention on the enzymatic characteristics of the membrane and the cisternal space (for a review of the enzyme activities found associated with the ER as of 1963, see Siekevitz, 1963).

HISTORY OF GLUCOSE-6-PHOSPHATASE

One of the enzymes found to be concentrated in the microsomal fraction of liver cells was glucose-6-phosphatase (G6Pase) (Hers et al. 1951). The existence of an enzyme that would split either glucose-1-phosphate or glucose-6-phosphate (G6P) was first postulated by Cori and Cori (Cori and Cori 1938, Cori et al. 1939). The enzyme was actually discovered and shown to be a specific phosphatase, different from acid or alkaline phosphatase, several years later by Fantl and Rome (Fantl

et al. 1942; Fantl and Rome 1945). They found the enzyme to be specific for glucose-6-phosphate and named it glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, E.C. 3.1.3.9). The biochemical properties of the enzyme were studied by de Duve and co-workers (de Duve et al. 1949, Hers and de Duve 1950, Hers et al. 1951, Beaufay and de Duve 1954a and 1954b, Beaufay et al. 1954) and by Swanson (1950) almost simultaneously. More recently, attempts at isolation and purification have been made by Ganoza (1964) and Nordlie and Arion (1964). The enzyme has a well-defined pH maximum, between 6.5 and 6.7, which distinguishes it from acid and alkaline phosphatase. It has a high specificity for its substrate, and does not efficiently hydrolyze many phosphate-sugars closely related to glucose-6-phosphate, although even when partially purified it does split ribose-5-phosphate, α-glycerophosphate, and phenyl phosphate (Ganoza, 1964). The enzyme is relatively unstable (de Duve et al. 1949, Stetten and Taft 1964). It has been identified in kidney and intestine in addition to liver, although it is absent from muscle and many other tissues (Hers and de Duve 1950). The enzyme appears to be tightly membrane-bound (Reid 1967; Hultin 1957, Carruthers and Baumler 1962), and it is not easily solubilized or purified from phospholipid. In fact, glucose-6-phosphatase itself is probably a lipoprotein since it appears to require lipid for activity (Beaufay and de Duve 1954b, Ganoza 1964, Duttera et al. 1968). The activity of the enzyme is fairly dependent upon the integrity and the conformation of the membrane as was found by Ernster (Ernster et al. 1962, Ashmore and Nesbett 1955). Since its localization by Hers et al., glucose-6-phosphatase has been widely used as a marker for the endoplasmic reticulum.

The main pathway in which glucose-6-phosphatase participates is shown below. It involves the conversion of stored glycogen into free glucose, which is liberated into the blood stream. The glycogen is first cleaved and phosphorylated by phosphorylase. The glucose-1-phosphate formed is converted into G6P by phosphoglucomutase; there is no appreciable cleavage of the phosphate directly from glucose-1-phosphate. The G6P thus formed is cleaved by the enzyme into free glucose and inorganic phosphate. In addition to the G6P formed from glycogen, some is produced by the reversal of glycolysis. Although



Pathway involving glucose-6-phosphatase.

the enzyme appears to act reversibly and has a binding site for glucose (Ganoza 1964), it does not so function within the cell. The main pathway for the phosphorylation of glucose is *via* a kinase, requiring ATP as a high-energy cofactor. G6Pase itself does not have any external cofactor requirement, although it may be dependent upon internally bound divalent cations (Beaufay *et al.* 1954, Nordlie and Lygre 1966).

Recent work (Stetten and Taft 1964, Fischer and Stetten 1966, Nordlie and Arion 1964, Arion and Nordlie 1964, Nordlie *et al.* 1965) suggests that G6Pase is a multifunctional enzyme, carrying out a hydrolysis of inorganic pyrophosphate (1) and a pyrophosphate-glucose phosphotransferase reaction (2) as well as the hydrolysis of G6P.

(1) $PPi + H_2O \longrightarrow 2\ Pi$

(2) $PPi + glucose \longrightarrow Pi + glucose\text{-}6\text{-}phosphate$

The identification of these as activities of a single enzyme rests on many factors including tissue and intracellular distribution, parallel inactivation and response to inhibitors, and enzyme kinetic data including competition between PPi and G6P. In addition, Nordlie and co-workers

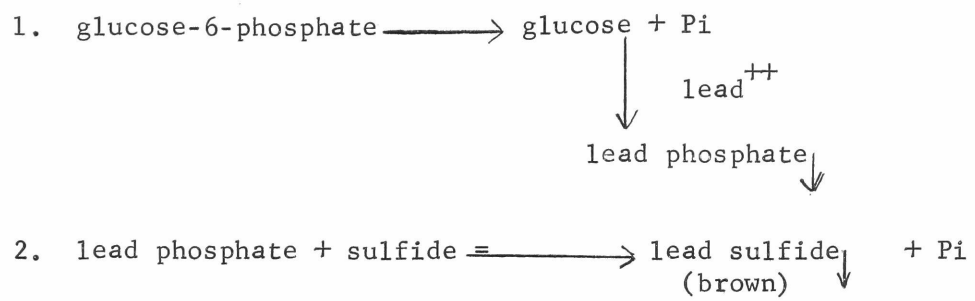
(Nordlie and Arion 1965, Nordlie et al. 1968) believe that the enzyme functions to transfer a phosphate group from nucleoside di- and triphosphates to glucose. Nordlie and Lygre (1966) suggest that the transferase reaction (2) may be important in the phosphorylation of glucose during diabetes when the glucokinase activity is low.

G6Pase activity is influenced by a number of hormones including cortisone, glucagon (Greengard and Dewey 1967), and thyroxine (Greengard and Dewey 1968). Under some conditions increased enzyme activity is due to newly-synthesized enzyme (Weber et al. 1964) while under others, there is an activation of already existing enzyme (Nordlie et al. 1965, Arion and Nordlie 1967). Precocious development of G6Pase activity can be induced in the late rat foetus by injecting glucagon (Greengard 1969). The response of the enzyme to hormonal factors is probably related to the central role it plays in gluconeogenesis (Weber et al. 1964, Dawkins 1963).

HISTOCHEMISTRY OF GLUCOSE-6-PHOSPHATASE

The first to attempt to localize glucose-6-phosphatase histochemically was Chiquoine (1952). He incubated fresh-frozen slices, which were attached to slides, with glucose-6-phosphate and lead ions. By light microscopic investigation, he found the enzyme to be present in liver cells, in the proximal convoluted tubules of the kidney, and in the brush border of the intestine (Chiquoine 1955). The histochemical reaction was refined and modified by Wachstein and Meisel (1956), who introduced the use of buffer to maintain the proper pH and in addition incubated free-floating slices. Both of these changes made the reaction much more reproducible and with these modifications it has been used ever since for the localization of glucose-6-phosphatase.

The reaction is outlined below. Lead ions are included during the reaction to precipitate the released phosphate as lead phosphate. Because the precipitate is white, it is converted to brown lead sulfide by treatment with ammonium sulfide. The lead sulfide can be seen in the light microscope. Tice and Barrnett (1962) used the procedure for electron microscopic observations. They utilized the fact



that the lead nucleus is electron-opaque and efficiently scatters electrons. The lead phosphate precipitate which is formed directly when the reaction is carried out in the presence of lead ions is visible in the electron microscope. Tice and Barrnett examined the liver cells of the rat using this method and localized the enzyme to the endoplasmic reticulum. This histochemical work, together with the biochemical studies which found the enzyme concentrated in the microsomal fraction, helped to confirm that the ER actually gives rise to microsomes upon fragmentation.

The electron microscopic technique has been used by others including Rosen et al. (1966), Orrenius and Ericsson (1966), Pollack and Shorey (1968), Sabatini et al. (1963), and Ericsson (1966).

DIFFERENTIATION WITHIN THE ENDOPLASMIC RETICULUM

Although many enzyme activities have been found associated with the endoplasmic reticulum, the actual function of the ER, aside from the role played in protein synthesis by the ribosomes, remains rather obscure. Since so many different types of enzymes are found in the membranes, it seems possible that the ER may be highly differentiated. In the broadest sense, the ER is differentiated, since two types of membranes, rough and smooth, do exist. These are distinguished from each other on the basis of the presence or absence of attached ribosomes. In addition, the organization of the two types of membranes within cells differs. The rough ER is usually found as long, parallel cisternae, while the smooth ER appears in section as randomly arranged circular profiles. Although the enzymatic composition of the smooth and rough seems to be similar, these two types of ER are found in

varying proportions in cells specialized for different functions. Cells which synthesize protein for export, such as the pancreatic acinar cell, have a very well-developed rough ER (Palade 1956). Cells involved in the production of steroids, on the other hand, such as the interstitial cell of the testis, have very large amounts of smooth ER and little rough ER (Porter 1961, Christensen and Fawcett 1961).

Within the smooth and rough ER there is also differentiation. The Golgi region is morphologically different from the rest of the smooth ER, and plays a unique role in the transport and packaging of material (Jamieson and Palade 1967a, 1967b; also see Beams and Kessel 1968, for review). The nuclear envelope is part of the ER system, but again has a distinct role.

Within the morphologically homogeneous rough ER and within the smooth ER, are there regions specialized to perform specific functions? This question is not easily answered biochemically since there are no good means of separating one type of rough ER from another. Recently, Dallner and co-workers have tried to attack this problem by fractionating the rough ER of liver cells into very small fragments by sonication (Dallman et al. 1969, see also Dallner et al. 1968). In this way they hoped to produce fragments which might differ from each other in enzymatic components. Unfortunately, the only means available to separate these small fragments from each other is by differences in size and density, which have no obvious relationship to the enzymatic components of the vesicles. Upon sub-fractionation of the vesicles, these workers did find some separation of enzymatic activities, with the NADH-linked electron transport chain sedimenting differently from the NADPH-linked chain.

On the other side of the question, Orrenius and Ericsson (1966) have looked at the distribution of glucose-6-phosphatase activity within the ER of rat liver after stimulation with phenobarbital. The drug induces the formation of new smooth ER and also stimulates the synthesis of several enzymes involved in the pathway which detoxifies the drug (NADPH-cytochrome c reductase, cytochrome P-450) (Orrenius et al. 1965, Ernster and Orrenius 1965). At the same time, some enzymes, such as

glucose-6-phosphatase, decrease in specific activity. Orrenius and Ericsson looked for newly-formed smooth ER which might be rich in the newly-synthesized enzymes and poor in glucose-6-phosphatase. No such fractions were found either by biochemical or histochemical means.

So although the question of heterogeneity within the rough and smooth ER is intriguing, there is still no conclusive evidence to support either view. In the second part of this thesis, I will describe a method for the subfractionation of rough microsomes based on the presence or absence of glucose-6-phosphatase. This provides a rational basis for the separation of two fractions of rough ER. Some of the applications of the method will be discussed.

BACKGROUND OF THE PROBLEM

The work of this thesis is concerned with the development of one enzyme associated with the endoplasmic reticulum, during a period of rapid cell and membrane differentiation. During the days immediately before and after birth, the hepatocyte of the rat undergoes striking morphological and enzymatic changes. A number of these changes have been studied by Dallner, Siekevitz and Palade (1966a, 1966b). The amount of glycogen in the hepatocytes increases before birth, and at the time of birth a large store is accumulated in the cell, which is utilized during the first day of life. The amount and organization of the rough ER increases during the last few days of gestation and the membranes become organized into parallel arrays similar to the pattern seen in the adult. The complement of bound and free ribosomes changes. At birth, the number of ribosomes attached to the membranes of the ER is very large, while before and after birth, fewer ribosomes are attached and more are found free in the cytoplasm. Before birth, the cells do not contain a significant amount of smooth ER, although they do have Golgi cisternae. Smooth ER is formed rapidly during the first 24 hours after birth, the membranes appearing in the glycogen regions.

In addition to these morphological changes, the enzymatic activity of the cell as a whole (Burch *et al.* 1963, Greengard 1969, *inter alios*) and of the endoplasmic reticulum changes during this time. There are

three main patterns of development of the ER enzymes. One group, typified by ATPase, has almost reached the adult value in the ER by four days prior to birth. Another group, including NADH-cytochrome c reductase and nucleoside diphosphatase, increases in activity slowly after birth. A third group, including glucose-6-phosphatase and NADPH-cytochrome c reductase, displays a dramatic increase in activity immediately after birth. This dramatic increase after birth is apparently due to the synthesis of new enzyme, since the changes can be prevented by actinomycin or puromycin treatment.

The enzymological work of Dallner et al. was done on homogenized liver tissue. It could not, therefore, provide information about the distribution of the newly-formed enzymes within the intact organ or within the cell. Histochemistry is the only tool available for looking at the localization of an enzyme activity within intact tissue, without breaking open cells, fragmenting components and changing topographical relationships. In this respect, it is an important companion to biochemical studies.

Investigating the development of the enzymes of the hepatocyte by histochemical techniques could provide answers to at least four questions not answered by the fractionation work:

1. What is the distribution of the newly-formed enzymes within the cells of the hepatocyte population? Do all the cells begin to synthesize enzyme at the same time or is the population asynchronous, with some cells developing the enzyme ahead of others?

2. How are the enzymes distributed within the ER membranes of each cell? Do only a small number of ER membranes contain new enzymes? Does the ER seem to be homogeneous or heterogeneous with respect to the localization of the enzymes?

3. Are the enzymes found in all of the smooth ER membranes when they are formed after birth, or do only some of the membranes contain enzyme?

4. Are newly-synthesized enzyme molecules inserted into preexisting membrane structures or are they incorporated along with other newly-synthesized membrane components into a totally new portion of membrane?

The topic of this thesis is the application of electron microscopic histochemical techniques to the study of the development of one of the ER enzymes. Glucose-6-phosphatase was chosen since it is very tightly bound to the membrane and is probably an integral part of the membrane structure, because the enzyme shows an interesting pattern of development, and because a histochemical technique for the electron microscopic localization of the enzyme has been reported. The localization of the enzyme during the entire course of development was examined, from 4 days prior to birth, when the enzyme activity is barely detectable biochemically, to 24 hours after birth, when the activity is at its maximum and the smooth ER membranes have started to form. The results obtained histochemically were substantiated biochemically, using a fractionation scheme to separate microsomes having glucose-6-phosphatase from those vesicles lacking the enzyme. This separation procedure was applied to several stages in the development of the enzyme.

MATERIALS AND METHODS

ANIMALS

Adult

Male albino rats of the Sprague-Dawley strain were fed ad libitum on Purina Lab Chow. Animals weighing 150-300 grams were considered to be adult for both histochemical and biochemical experiments. They were not starved previous to sacrifice in order to approach the conditions found in the foetal and newborn rats as closely as possible. In these latter animals, the glycogen content of the liver cannot be reduced by starvation.

Foetal

Female Sprague-Dawley rats, in most cases from Holtzman Co., Madison, Wisc., were received between the 14th and 17th days of pregnancy. These rats have a gestation period of 22 days. At least 4 rats, mated on the same date, were maintained on Purina Lab Chow. One of these animals was sacrificed at the desired stage of pregnancy and at least two rats were kept as controls and allowed to deliver normally so that the actual age of the foetuses from the sacrificed animals could be determined. The rats almost always delivered on the expected date and generally within several hours of each other.

Newborn

For all newborn animals the time of birth was known to within about 1 hour since it takes approximately 1 hour for the mother to complete delivery. For the earliest time point after birth, the young were sacrificed 1-2 hours after the start of delivery and were therefore 1-2 hours old. At this age they have not yet started to nurse.

HISTOCHEMISTRY

Perfusion Fixation

Adult

Rats were anesthetized with ether and the peritoneal cavity was opened. The hepatic portal vein was ligated with surgical thread at the caudal end to prevent profusive bleeding. An incision was then made

proximal to the thread and a cannula of polyethylene tubing was inserted and tied into place. The heart was cut to permit the easy flow of the perfusion medium through the liver. The liver was perfused through the cannula, first with 50 ml cold buffer (0.1 M sodium cacodylate pH 6.6, containing 6 mM glucose-6-phosphate, Sigma Co.), then with 100 ml cold fixative (2% glutaraldehyde, Fisher Co., in 0.1 M cacodylate buffer pH 6.6, containing 6 mM G6P) and finally with another 50 ml of the buffer. The initial perfusion with buffer was designed to wash out blood cells so they would not become fixed in the blood vessels, and the final perfusion with buffer was to wash the fixative away from the cells. Since after perfusion of the first 50 ml of buffer the perfusate leaving the heart was almost colorless, this volume appears to be sufficient to wash the entire liver. The perfusion with fixative lasted about 3-5 minutes. The liver became light pink and solid to the touch, indicating good fixation. After the second perfusion with buffer, the liver was transferred to cold buffer, cut into pieces and kept at 4^{o} C.

In some experiments, the right hand lobe of the liver was ligated with surgical thread after the initial perfusion with buffer and was then immediately removed into cold buffer. No fixative leaked through the cut if the thread was tied tightly, so that a closed system was still maintained for the perfusion of fixative. In this way both fixed and unfixed tissue could be obtained from the same liver. This was especially important in the experiments to determine the extent of enzyme inactivation by the fixation procedure.

Foetal

Female rats at the desired stage of pregnancy were anesthetized with ether, the peritoneal cavity was opened and the foetuses were removed. As each animal was removed it was either anesthetized in an ice bath or decapitated with a scissors. The animal was pinned down onto a board and the peritoneal and pericardial cavities were opened. The heart was cut to provide an exit for the perfusate and the hepatic portal vein was located under a dissecting microscope. An incision was made near the point where the vein enters the liver and 5 ml of fixative

(same as that used for the adult) were carefully pushed through. The perfusion had to be done very slowly and steadily since the liver of the foetal rat is very fragile and tends to break open if too much force is exerted. The initial perfusion with buffer was omitted because of the fragility of the tissue. As the liver is fixed, it hardens and becomes less delicate so that a final wash with 5 ml of cold buffer could be performed. Generally the right hand side of the liver was well fixed. It became lighter pink and could be handled with forceps whereas unfixed tissue tended to fall apart. The well-fixed lobes were transferred to cold buffer and cut into pieces. The perfusion was usually performed on 2 animals of each litter.

Newborn

The ages of the animals were determined by taking the midpoint in delivery as time zero. The newborn animals were treated much like the foetuses, but 10 ml of fixative and then 10 ml of buffer were used. Litter mates were used for the time points between birth and 24 hours in any one experiment.

Slicing, Incubation and Preparation for Microscopy

The rest of the procedure was similar for all ages. Small cubes of liver were transferred to a piece of filter paper which was attached with glue to a plastic disc. The tissue was covered with luke-warm agar which was then solidified by placing the plastic dish in the freezing compartment of a refrigerator for 10-30 seconds. The tissue was chopped into thin slices on the Farquhar slicing apparatus (Smith and Farquhar 1963). The slices were washed with buffer for 1/2 - 1 hour before being incubated in the histochemical medium. The thickness of the slices was estimated to be 50-100 μ by photographing the width of a slice at low magnification in the electron microscope and measuring the width from the plate.

The histochemical procedure was performed at 24-25° C in a water bath. The tissue was placed in approximately 40 ml of histochemical medium in a beaker and was shaken continuously during the incubation. The medium was a modification of that of Wachstein and Meisel (1956)

and contained 1 mM glucose-6-phosphate (dipotassium salt), 2 mM lead nitrate, 50 mM sodium cacodylate buffer pH 6.6. The lead concentration was twice that of the substrate to ensure the presence of adequate lead ions to trap all the released phosphate. This concentration of lead did not produce any precipitate with the glucose-6-phosphate, and the medium did not become cloudy even during a 60-minute incubation. Some batches of cacodylate (Amend Drug Co.) produced a slight cloudiness when added to the lead. Only batches which did not produce precipitate were used for the histochemistry.

After the desired length of incubation (30 or 60 minutes), slices were transferred directly into 2% OsO_4 in 0.1 M cacodylate buffer pH 6.6, to stop the reaction and to post-fix the tissue. This fixation, for 1 hour at 4° C, was followed by several rinses in the same buffer. The tissue was usually left overnight in either cacodylate or veronal-acetate (V-A) buffer (0.28 M, pH 6.8). After several changes in veronal acetate buffer the material was stained for 1 hour at room temperature with 0.5% uranyl acetate prepared in V-A buffer (Farquhar and Palade 1965), washed again with V-A buffer and then dehydrated through graded alcohols (beginning with 70%) and propylene oxide. The tissue slices were left overnight at 4° C in a mixture composed of 50% propylene oxide and 50% Epon 812 (Shell Chemical Co., New York). The propylene oxide was evaporated by rotating the vials and passing cool air over them, and the tissue was then embedded in Epon (Luft 1961). An entire slice was embedded and oriented with its long axis perpendicular to the plane of sectioning.

After at least two days of polymerization at 60° C, 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. This procedure was followed to insure reproducible results, since often the inner areas of the tissue slices displayed no reaction product, implying that either substrate or lead did not penetrate. The blocks were sectioned on a Porter-Blum Servall MT2

automatic microtome with a Dupont or Sugg diamond knife. Gold, silver or gray sections were collected on bar grids (E.F. Fullam Co.) covered with 2% formvar and carbon. The sections were stained with alcoholic uranyl acetate for 1 minute (Watson 1958) and basic lead citrate for 3-5 minutes (Venable and Coggeshall 1965), and were examined in a Siemens Elmiskop electron microscope fitted with double condenser, 400 μ condenser aperture, 50 μ objective aperture, operated at 80 kilovolts.

Light Microscopy

Embedded sections

If the incubated slices were to be used for light microscopic histochemistry, they were washed with 0.05 M cacodylate buffer after incubation, and were then rinsed briefly with dilute ammonium sulfide. Following further rinses with buffer and water, they were dehydrated and embedded as described above. One-half μ sections were cut from the polymerized blocks and were mounted on slides.

Frozen sections

Small pieces of perfusion-fixed liver were sectioned at a setting of 20 μ on an American Optical freezing microtome. After being washed in buffer, the sections were incubated in the same manner as described above. After incubation and treatment with ammonium sulfide, the slices were mounted directly on slides coated with albumin solution.

Pictures

Sections of tissue prepared for light microscopy were examined and photographed in either a Zeiss Ultraphot microscope or a Zeiss photomicroscope equipped with oil-immersion phase-contrast lenses.

Controls for Histochemistry

For all histochemistry, controls were incubated without substrate. For the electron microscopy and the light microscopy on embedded slices, tissue was incubated with 1 mM beta-glycerophosphate replacing the glucose-6-phosphate. In addition, for the electron microscopy, controls included prior heating of the tissue at 80° C for 5 minutes, pre-

incubation of the tissue with 5 x 10^{-4} M parahydroxymercuribenzoate, or inclusion of 5 mM sodium oleate during the incubation.

Morphology

When liver tissue was used solely for morphological studies, it was treated as described for the histochemistry but the incubation in the histochemical medium at 25° C was omitted. The thin slices were transferred directly from cold cacodylate buffer into osmium tetroxide.

Preparation of Glucose-6-phosphate

In order to obtain highly purified glucose-6-phosphate for the histochemical incubation, the barium salt of glucose-6-phosphate was purchased from Sigma Chemical Co., St. Louis, Mo., and was converted to the more soluble di-potassium salt. This conversion was done in either of two ways.

1. Dowex method (C.C. Widnell, personal communication)

Approximately 10 grams Dowex-50 (Dow Chemical Co.), which had been stored in the acid form in 0.1 N HCl, was washed several times in water. A slurry was prepared of 2 grams of barium-glucose-6-phosphate in approximately 10 ml H_2O. To this slurry the washed Dowex-50 was added dropwise with stirring until no white precipitate was visible on the bottom of the beaker. The mixture was allowed to stir for about 20 minutes at 4° C. A column (6 x 1 cm) was prepared from additional washed Dowex and was washed twice with 2 ml water. The mixture of G6P and Dowex was loaded onto the column and the effluent was collected. The column was washed twice with 2 ml water and this wash was added to the effluent (total volume approximately 20 ml). This solution of glucose-6-phosphate (acid form) was neturalized to pH 7.0 with KOH. The concentration of free inorganic phosphate and total phosphate was then determined by the method of Ames and Dubin (1960). The amount of Dowex used had a large enough capacity to convert all of the barium salt of the glucose-6-phosphate to the acid form.

2. Sulfuric acid method

Two grams of the barium-G6P were transferred to a conical centrifuge tube and to this was added several ml of water. Sulfuric acid was added in a concentration somewhat greater than equi-molar with the G6P. The barium sulfate precipitate that formed was spun out and a drop of sulfuric acid was added to the supernatant to check for the complete precipitation of the barium. The clear solution was then neutralized to pH 7.0 with KOH.

The two methods give comparable results in terms of contamination by inorganic phosphate (which is low) but the Dowex method yields greater recoveries. After preparation, the glucose-6-phosphate solution was stored at -20° C.

BIOCHEMISTRY

Preparation of Tissue

Adult male rats were anesthetized with ether and foetal rats were removed from mothers which had been anesthetized. Newborn rats were not exposed to ether at all. When liver tissue was to be used solely for biochemical experiments, the livers were removed from the animals without prior perfusion, and were transferred to cold sucrose, either 0.25 or .88 M. In adult rats, it was possible to do both histochemistry and biochemistry on the same liver by tieing off one lobe as previously described, so that in these experiments the livers were perfused with buffer. The livers of the young animals were too small to use for both biochemistry and histochemistry, so that litter mates were used. Generally 4-5 of these small livers were used for each biochemical experiment, and 5-15 were used in the fractionation experiments. The livers were minced in cold sucrose and were blotted on filter paper before being weighed. Liver from young animals was homogenized directly since it was very soft, but liver from adult rats was generally passed through a tissue press fitted with a stainless steel plate before homogenization. The homogenates (10, 20 or 25% w/v) were prepared by suspending the tissue in sucrose using a motor-driven Potter-Elvehjem homogenizer (Potter and Elvehjem 1936) fitted with a teflon pestle. Preparation of the homogenates was carried out at 4° C.

If biochemical activity of glucose-6-phosphatase was to be assayed in tissue fixed by perfusion with glutaraldehyde, a somewhat more vigorous disruption procedure was necessary since homogenization does not insure complete breakage of fixed cells. After the standard homogenization, homogenates from both fixed and unfixed tissue were examined under the phase microscope and the number of unbroken cells per unit area was counted. The homogenates were then sonicated with a Branson sonifer set at 4-6 Amperes and again were examined. The sonication was continued on both the fixed and unfixed homogenates until there were few unbroken cells in the homogenate from the fixed tissue. The tissue was kept in an ice bath during the sonication and 10-second intervals of sonication were separated by 15 seconds to permit cooling. The loss in activity due to the heat generated during sonication was determined by assaying both the sonicated and unsonicated homogenates (to be discussed in Table 2). Sonication of the fixed tissue insured both an accurate enzyme assay and an accurate protein determination.

Chemical Assays

Glucose-6-phosphatase

The method used was modified from those of Swanson (1950) and de Duve *et al.* (1949). The assay was carried out in a conical centrifuge tube containing 30 mM cacodylate buffer pH 6.6 and 30 mM glucose-6-phosphate dipotassium salt (final concentrations in 1 ml volume). A preliminary experiment indicated that addition of Mg^{++} was not necessary for activity. The reaction was run either at 37° C or 25° C in a shaking water bath for 5 to 60 minutes. For routine biochemical assay, the reaction was run at 37° C for 20 minutes. To determine the amount of phosphate released during the histochemical incubation, the reaction was run at 25° C for 30 or 60 minutes. The reaction was started by the addition of tissue and was stopped by adding 1 ml cold 10% TCA. Occasionally both a tissue and a substrate blank were included but routinely a zero time point blank was used. For this, TCA was added prior to the addition of tissue. The TCA precipitates were kept at 4° C for at least 1 hour and were then sedimented. The supernatants were removed with a

Pasteur pipette and were used for the determination of free inorganic phosphate, while the pellets were suspended in 1 ml 1 N NaOH to be used for protein determination. When protein was determined on homogenates of fixed liver, the TCA precipitates in NaOH were heated in capped tubes for 30 minutes at 90-100° C to solubilize the protein. Routinely, specific activity was determined as μmoles inorganic phosphate released per mg protein per unit time. All samples were run in duplicate.

Protein

Protein was determined by the method of Lowry (Lowry et al. 1951), with bovine serum albumin (BSA) as standard. For samples heated at 100° C for 30 minutes a standard curve was prepared from BSA which had been TCA-precipitated and heated. In determining protein on homogenates from fixed tissue, the Lowry method was checked by assaying for protein nitrogen. The two methods agreed when the homogenates had been sonicated and the TCA precipitates heated in NaOH as previously described.

Phosphate

For most work, inorganic phosphate was determined by the method of Fiske and SubbaRow (1925). Occasionally the method of Ames and Dubin (1960) was used for both inorganic and total (organic plus inorganic) phosphate. K_2HPO_4 was used as a standard.

Phospholipid

Phospholipid was extracted by the Folch procedure (Folch et al. 1957) using 0.73% NaCl as the aqueous phase. The chloroform layer was transferred to acid-washed tubes and evaporated under a stream of warm air. After evaporation, 0.1 ml chloroform was added to the dry tubes, and was swirled around to collect the lipid material in the bottom of the tube. This was then evaporated and the dry material was resuspended in 0.1 ml ethanol and was washed with $MgNO_3$. The residue was assayed by the Ames method for total phosphate. To obtain micrograms phospholipid, micromoles of phosphorous were multiplied by 775.

RNA

RNA was determined by the method of Munro and Fleck (1966). RNA was extracted from a perchloric acid precipitate by heating in 0.3 N KOH at 37° C for 60 minutes. The optical density at 260 mμ was measured. This method was chosen rather than the orcinol method (Mejbaum 1939), since the high concentration of sucrose in some of the fractions interfered with the orcinol reaction.

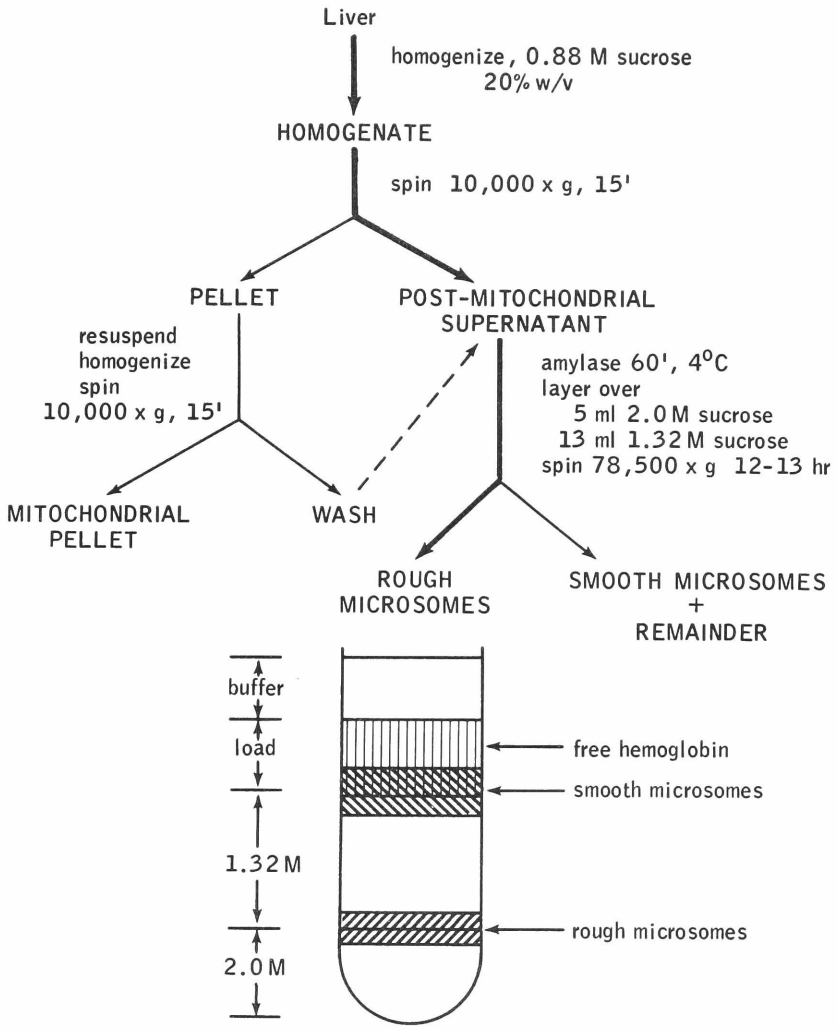
FRACTIONATION OF ENDOPLASMIC RETICULUM

Preparation of Rough Microsome Fraction

The procedure used to obtain a fraction of rough microsomes is a modification of that of Rothschild (1963) and is shown schematically in Figure 1. At all stages, livers were removed in the same manner as described under "Biochemistry". Homogenates, 20% w/v, were prepared by homogenizing the livers in 0.88 M sucrose as described earlier. This concentration of sucrose was used in preference to 0.25 M since it produced a cleaner separation of rough and smooth microsomes. The homogenates were either kept at 4° C for several hours, or 9-12 ml were immediately centrifuged for 15 minutes at 10,000 x g (average) in the Spinco S 40 rotor. The rotor was stopped with the brake and the supernatant was removed with a curved pipette fitted with a rubber bulb. The pellet was washed with 0.88 M sucrose and was recentrifuged. The wash supernatant was added to the original supernatant to form the post mitochondrial supernatant (PMS). This fraction contained microsomes and free ribosomes. The pellet (mitochondrial pellet, MP), containing whole cells, mitochondria, nuclei and some microsomes, was resuspended to 10 ml and was kept at 4° C.

The PMS was stored at 4° until one hour before the rough-smooth fractionation. At that time alpha amylase (hog pancrease, Worthington Biochemical Corp., Freehold, N.J.) was added to the PMS and incubation was continued for 60 minutes at 4° C. The amylase added was in great excess, based on an estimate of the glycogen content, and almost completely digested the glycogen present in the PMS. During the incubation, the solution was homogenized three times and then the PMS was layered

SEPARATION PROCEDURE FOR ROUGH AND SMOOTH MICROSOMES



* all g values are average force at the middle of the tube filled to capacity

Figure 1. Separation procedure for rough and smooth microsomes. The upper part of the figure outlines the centrifugation procedure; for full description see text. The lower part shows the distribution of material on the gradient.

onto the following step gradient: 5 ml 2.0 M sucrose at the bottom, overlayed with 13 ml 1.32 sucrose. An aliquot of the PMS was layered over the 1.32 M sucrose and the remainder of the tube was filled with 0.01 M cacodylate buffer pH 6.6. The gradient was spun in the Spinco S-30 rotor for 12-13 hours at 78,000 x g (average). The gradients were tapped on an Isco Density Gradient Fractionator, using 50% (w/w) potassium tartrate to push up the gradient. Optical density was monitored at 550 mμ and 1 ml fractions were collected into precooled tubes in an ice bath. The fractions containing the rough and smooth microsomes were separately pooled and kept at 4° C. In this gradient, the smooth microsomes remain at the interface between the 1.32 M sucrose and the load zone, while the rough microsomes collect at the interface between the 1.32 and 2.0 M sucrose. Free glycogen and glycogen associated with membranes would pellet under these conditions if any were present. Only a very slight reddish pellet is seen if the PMS is treated with amylase. Polysomes and free ribosomes should be in the 2.0 M sucrose layer or in the pellet.

The pooled rough microsome fraction was gently sonicated in an Acoustica ultrasonic bath for 30 or 60 seconds at 50 milliamperes in order to break up any aggregates that might have formed during the centrifugation.

Histochemistry and Subfractionation of Rough Microsomes

Aliquots of the rough microsome fraction were incubated in the histochemical medium in exactly the same manner as were the tissue slices. The reaction was continued at 25° C for 60 minutes at which time the beakers were transferred to the cold. Aliquots of microsomes were also incubated without glucose-6-phosphate. During the reaction, aggregation of the microsomes occurred. In order to reverse this aggregation, the reacted microsomes were dialyzed against EDTA. Ten ml aliquots of the microsomal suspension were transferred to washed dialysis bags. The washing procedure for the bags included an overnight soak in 0.01 M EDTA, rinsing with distilled water, boiling for 10 minutes in 2% Na_2CO_3, thorough washing with distilled water and a

final 10-minute boiling in distilled water. The washing was necessary since dialysis in unwashed bags caused fragmentation of the microsomes. The microsomal suspension was dialyzed overnight at 4° C against 0.01 M cacodylate buffer pH 7.4 containing 100 μM EDTA. Dialysis volumes were 1:100. Twice during the dialysis the bags were transferred to fresh medium of the same composition and three times during dialysis the bags containing the microsomal suspension were gently sonicated for 9-10 minutes at 50 milliamperes in the ultrasonic bath. The sonication helped to break up the aggregates formed during the incubation and allowed the EDTA to more efficiently bind the free lead ions. It was necessary for the bags to spin freely during the dialysis in order to completely reverse the aggregation. After a third sonication, the microsomal suspension was removed from the dialysis bags and 20 ml were layered over 9 ml of 2.0 M sucrose. The material was spun at 63,600 x g for 60 minutes in the Spinco SW 25.1 rotor. The gradients were tapped on the gradient fractionator, and the absorbance was monitored at 254 mμ. Two ml fractions were collected into precooled tubes in an ice bath and adjacent fractions were pooled. For biochemical analysis, the pellets were resuspended in 0.88 M sucrose and homogenized in a small centrifuge tube with a close-fitting teflon pestle. Aliquots were taken from the pooled fractions immediately, or after freezing, for the determination of protein and phospholipid. When the fractions were assayed for glucose-6-phosphatase activity, this was done immediately after the gradients were collected.

Electron Microscopy of Fractions

For electron microscopy, the pellets from the final gradients were fixed *in situ*. Aliquots of the material at the interface were pelleted as were fractions of the isolated rough and smooth microsomes. The aliquots were diluted to 5.0 ml with 0.1 M cacodylate buffer, pH 6.6, or 0.01 M cacodylate buffer, pH 7.4, and were centrifuged for 45 minutes in the Spinco SW 39 rotor at 125,000 x g (average). All pellets were fixed overnight at 4° C in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 6.6 or 7.4, washed in cacodylate buffer and several changes of V-A buffer, and stained for 1 hour in 0.5% uranyl acetate. The

pellets were dehydrated in the bottom of the centrifuge tubes through 70% to 95% alcohol. While in 95% alcohol they were cut into sectors which included both top and bottom of the pellet. During embedding these sectors were oriented so that a section would include the total thickness of the pellet in the direction of centrifugation. Thus, changes in the composition across the pellet could be examined.

RESULTS

I. DEFINING THE HISTOCHEMICAL SYSTEM

Reliable histochemical localization is notoriously difficult. Numerous artifacts are possible and must be rigorously excluded before meaningful conclusions can be drawn. These artifacts range from inactivation of the enzyme during the fixation process to nonspecific deposition of the reaction product. Many of the sources of artifact were investigated by biochemical and histochemical means and the results of these experiments will be presented in this section of the thesis.

Biochemical Assay

In order to examine the parameters of the histochemical system used to localize glucose-6-phosphatase, a biochemical assay for the enzyme, modified from those of Swanson (1950) and de Duve *et al.* (1949) was used. (The details of the system are described in "Materials and Methods"). Homogenized adult liver was used for most of these experiments.

First, the kinetic properties of the enzyme were examined. The amount of inorganic phosphate released during the reaction is dependent upon the enzyme concentration (Fig. 2A). Generally the equivalent of 10-20 mg wet weight of liver were assayed, which, under the conditions of the assay, makes the reaction 1st order with respect to enzyme. The substrate concentration (30 mM) on the other hand, was chosen to saturate the enzyme (Fig. 2B) and the reaction is zero order with respect to substrate concentration. Thus, the amount of substrate hydrolysed is directly proportional to the activity of the enzyme.

The rate of the reaction at 37^{o} and 25^{o} is shown in Fig. 2C. At both temperatures the rate of release of inorganic phosphate is linear up to 30 minutes. For biochemical assays at 37^{o}, the reaction was routinely run for 20 minutes, at which time the reaction is still linear. The initial rate at 25^{o} is half that at 37^{o}.

Parameters of the Histochemical Reaction

Buffer

The biochemical system was used to check many of the parameters involved in the histochemistry. The first was the selection of a buffer.

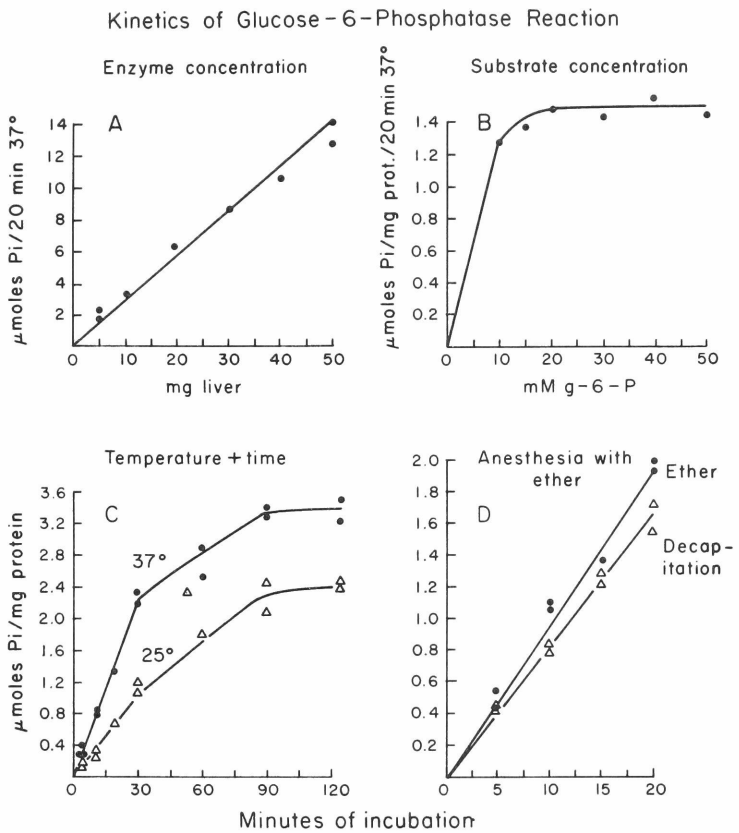


Figure 2. Kinetics of G6Pase reaction in homogenates from adult liver. Assay medium:

A. 30 mM G6P, 30 mM cacodylate buffer, pH 6.6.

B. 30 mM cacodylate buffer pH 6.6, 10 mg liver homogenate.

C. 30 mM G6P, 30 mM cacodylate buffer, pH 6.6, 10 mg homogenate.

D. 30 mM G6P, 30 mM cacodylate buffer, pH 6.6, 10 mg homogenate.

In D, activity in liver of rat killed by decapitation is compared to that in liver of rat killed by anesthesia with ether.

Citrate, the buffer used by Swanson (1950) was reported by Nordlie and Lygre (1966) to inhabit G6Pase. Cacodylate buffer was used by de Duve et al. (1949) in the G6Pase assay, and was also employed by Sabatini et al. (1963) when fixing with glutaraldehyde; it does not interact with the fixative in the way that Tris buffer does. Swanson (1950), however, had reported that arsenate inhibits G6Pase activity although arsenite does not. Since cacodylate is the salt of an arsinic acid in which the arsenic is in the same oxidation state as in arsenate, the effect of cacodylate on G6Pase activity was determined. The activity in the presence of cacodylate buffer was the same as that with Tris-Maleate, and so cacodylate was routinely used for both the biochemical and histochemical assays. The lack of effect of cacodylate on G6Pase agrees with the findings of Nordlie and Lygre (1966).

Anesthesia

In order to perfuse the liver while the rat is still alive, it is necessary to anesthetize the animal. Since ether is a lipid solvent which could alter membrane structure, it might have an effect on the activity of membrane-bound enzymes such as G6Pase. The effect of ether anesthesia on G6Pase activity is shown in Figure 2D. This is one of several experiments which compares the G6Pase activity in the liver of an animal which has been killed by decapitation with the activity found in the liver of an anesthetized animal. The reaction rate is very similar in the two animals. The slight increase in activity after anesthesia which was found in most of the experiments might be significant. Since, however, the difference is so small, and the anesthesia is necessary, all of the histochemical and biochemical experiments using adult rats were performed on rats which had been previously anesthetized. Foetuses were removed from anesthetized mothers and were then decapitated, while newborn animals were merely decapitated.

Fixation

One of the most serious problems in histochemical work is the proper selection of a method of tissue fixation. Two criteria must be met by the fixation technique. First, fine structural localization by

electron microscopic histochemistry requires reliable morphological preservation of the tissue. Thus, many methods of fixation, while adequate for light microscopic work, are not sufficiently good for electron microscopic investigations. Second, the enzyme must not be inactivated during the fixation. Only in this way can one be sure that the activity observed histochemically is representative of the enzyme activity actually present in the cell.

Glutaraldehyde is a commonly used fixative which produces excellent morphological preservation. It has, however, been reported to inhibit G6Pase activity (Sabatini et al. 1963). In the work of Sabatini et al. the tissue was fixed by immersing tissue blocks in the fixative for extended periods of time. We reasoned that a very short fixation might preserve the morphology of the tissue well enough, without inactivating the enzyme. The liver is well suited to a short fixation by perfusion through the portal vein. It is so well permeated with capillaries and sinusoids that the fixative need only diffuse across the thickness of one cell. One other advantage of perfusion fixation is that it permits uniform fixation of the entire liver. Thus, there should be no inactive, overfixed areas, nor regions where, due to under fixation, the reaction product is deposited non-specifically. (See Orrenius and Ericsson 1966 for examples of these types of problems.)

The perfusion fixation method outlined earlier satisfies the two requirements stated. The first, morphological preservation, is good at all stages of development (see plates 2, 8, 12, 15, 22, 26). The cells remain intact and there is no vacuolation of the endoplasmic reticulum or the mitochondria. Other cell organelles also retain their accepted morphological appearance.

The second condition, retention of enzyme activity after fixation, was also met (see Table 1). At all ages, the amount of activity recovered after fixation was 70-80% of that present before fixation. This is a respectable recovery of activity and implies that the histochemical reaction product marks the location of the bulk of the activity present in the unfixed tissue. Since purification of the glutaraldehyde by passage through Norite (Anderson 1967) did not increase the recovery,

TABLE 1

G-6-Pase ACTIVITY AFTER FIXATION BY PERFUSION

Exp. no.	Age	Fixative	Sonication (seconds)	μmoles Pi/mg protein/ 20'/37° *		Per cent recovery
				fixed	control	
1	adult	2% glutaraldehyde unpurified	35	1.18	1.69	70
2	"	2% glutaraldehyde purified	35	1.26	1.66	76
3	"	2% glutaraldehyde unpurified + 6 mM G-6-P	70	1.26	1.60	79
4	"	1% glutaraldehyde purified	30	1.30	1.66	78
5	newborn (2 hours)	2% glutaraldehyde unpurified + 6 mM G-6-P	60	.86	1.04	83

* Values are averages of two determinations.

Each experiment refers to rat liver perfused with different fixation medium. When purified, glutaraldehyde was passed through Norite. Sonication was continued until the number of unbroken cells in the homogenate of fixed tissue was approximately equal to that in the unsonicated homogenate of unfixed tissue. For all experiments on adult liver the specific activity of G6Pase left in the tissue after fixation is compared to that present in the same liver before fixation. In the newborn, the comparison is made to unfixed tissue from a litter mate. All specific activities in the table are from sonicated homogenates.

unpurified fixative was used. Lowering the concentration of the fixative to 1% resulted in poor fixation and had no effect on recovery of activity. Although inclusion of G6P during the fixation procedure did not appreciably increase recovery of activity, it was routinely included during the fixation as an added precaution since the enzyme is relatively unstable (Stetten and Taft 1964, de Duve et al. 1949). This exposure of the tissue to substrate does not contribute any background precipitate detectable either biochemically or histochemically.

Homogenates prepared from both fixed and unfixed tissue were sonicated. Sonication was continued until the fixed tissue was well-dispersed and the cells broken. This sonication breaks up clumps of fixed cells which are very difficult to disperse by homogenization. When clumped, the cells settle during the enzyme assay and thus show a somewhat lower activity. In addition, the sonication is necessary for reliable protein determinations on the fixed tissue. TCA precipitates of the large clumps of cells do not dissolve readily in alkali even when heated and therefore spurious protein measurements result. Table 2 shows the results of several experiments in which losses in enzyme activity due to the heat generated during sonication were determined. The amount of heat produced is very difficult to control and the losses in the unfixed control homogenate varied between 0% and 23%. In the fixed tissue, the activity in the sonicated homogenate was always either the same as or slightly higher than the activity in the original unsonicated homogenate.

Recently, there have been two reports of brief fixation methods which retain histochemically-demonstrable G6Pase. Neither author used biochemical assays to determine the actual percentage of activity retained after fixation. Ericsson (1966) employed a brief perfusion of liver with glutaraldehyde, while Manns (1968) used a brief formalin fixation of frozen sections.

Lead Ions

With the development of an adequate method for fixation, two other important parameters had to be checked. The lead ions present

TABLE 2

EFFECT OF SONICATION ON G-6-Pase ACTIVITY

IN HOMOGENATES FROM ADULT RAT LIVER

Exp.	Conditions	Sonication (seconds)	Specific Activity μmoles Pi/mg Protein/20', 37°					
			Fixed Tissue			Unfixed Tissue		
			unsonicated	sonicated	% increase after sonication	unsonicated	sonicated	% loss after sonication
1	Glutaraldehyde 2% unpurified + 6 mM G-6-P	70	1.22	1.26	3	1.68	1.60	5
2	Glutaraldehyde 2% purified + 6 mM G-6-P	50	.81	.85	5	1.50	1.15	23
3	Glutaraldehyde 1% purified + 6 mM G-6-P	30	1.79	1.79	0	2.40	2.40	0
4	Glutaraldehyde 1% purified + 6 mM G-6-P	30	1.31	1.43	9	1.78	1.62	8

Sonication was continued until the number of unbroken cells in the fixed homogenate was approximately equal to that in the unfixed, unsonicated homogenate. In each experiment, fixed and unfixed tissue was obtained from the same liver; sonicated and unsonicated material was from the same homogenate. The homogenate was kept in an ice bath during sonication and 10 second bouts of sonication were separated by 15 seconds of cooling.

during the incubation have been shown to inhibit some phosphatases (Novikoff et al. 1958, Tice and Engel 1966, Marchesi and Palade 1967). To check the effect of lead on G6Pase activity, tissue was assayed under the histochemical conditions (1 mM g-6-p, 50 mM cacodylate buffer, 25° C) with and without 2 mM lead present, this being the concentration used for the histochemical reaction. After 30 minutes of incubation, aliquots were removed and were precipitated with TCA. Preliminary experiments had shown that 5% TCA completely solubilizes the lead phosphate precipitate and releases it from the tissue so that the assay for inorganic phosphate included the amount of lead phosphate present. The lower part of Table 3 illustrates that this concentration of lead did not inhibit G6Pase at all. Kinetic experiments showed that the rates of reaction in the presence and absence of lead were identical. This concentration of lead, 2 mM, is the highest that can be used without precipitation occurring. Previous investigators (Tice and Barrnett 1962) using higher lead concentrations reported finding such precipitates. Since this precipitation introduces uncertainty in the conditions of the reaction, it was avoided. 1 mM G6P and 2 mM lead were used to ensure that the concentration of trapping agent was greater than that of the substrate without causing precipitation.

Another possible source of artifact in the system is the reported ability of lead ions to hydrolyse various phosphate esters (Rosenthal et al. 1966, Moses et al. 1966, Moses and Rosenthal 1967, 1968, Novikoff 1967). Since under some conditions this non-specifically released inorganic phosphate is deposited preferentially over certain organelles (Moses et al. 1966), interpretation of the histochemical deposit in the presence of such a non-enzymatic hydrolysis becomes ambiguous. The histochemical medium was incubated without tissue, but under the same lead and substrate concentrations used for the histochemical reaction. Aliquots were removed for the assay of inorganic phosphate immediately after mixing, and then after 30 and 60 minutes of incubation. Table 3 (top) illustrates that during the incubation, there is no significant increase in the amount of inorganic phosphate. The amount found can be accounted for by that present in the reagents alone.

TABLE 3

LACK OF EFFECT OF Pb^{++} ON G-6-Pase REACTION

On Substrate	
min. of incubation*	μmoles Pi/ml**
0	.038
30	.025
60	.033
On Enzyme Activity	
incubation conditions*	μmoles Pi/mg prot/30'** (25°)
no Pb^{++}	1.18
+ 2 mM Pb^{++}	1.18

* 1 mM G-6-P, 2 mM $PbNO_3$, 50 mM Na Cacodylate buffer, pH 6.6

** Values are averages of two determinations

Top: G6P and lead ions were incubated together at 25°. Aliquots were removed right after mixing and then after 30 and 60 minutes of incubation, and were immediately assayed for inorganic phosphate.

Bottom: 10 mg of homogenate were assayed with and without 2 mM lead nitrate present.

Inhibitors

In order to find incubation conditions which might be useful as controls for the histochemistry, the biochemical assay for G6Pase was carried out in the presence of a number of inhibitors which had been previously reported to act on the enzyme. Table 4 lists the activity obtained using several conditions which produced partial inhibition of the enzyme. The results are essentially similar to those reported elsewhere (Beaufay et al. 1954, Swanson 1950) but none of the conditions were useful for histochemistry. Since the determination of the histochemical deposit is not quantitative, a 50% inhibition would be difficult to assess. An adequate histochemical control should contain little or no observable deposit. Although β-glycerophosphate does not inhibit the reaction, it is not utilized as a substitute substrate (see Table 5) and in this capacity is a good control for the histochemistry. Table 5 lists several inhibitory conditions under which little or no activity was detectable biochemically. Substitution of β-glycerophosphate for G6P, prior heating of the tissue and omission of substrate were good control incubation conditions. The histochemical results obtained using these conditions will be presented in Results Part III. The inhibition obtained with sodium oleate (Ganoza 1964) is variable. Parahydroxymercuribenzoate may inhibit the reaction by binding to a sulfhydryl group on the enzyme molecule, but since it also precipitates in the histochemical medium, its usefulness as a histochemical inhibitor is limited.

Penetration of Substrate and Trapping Agent

In addition to controlling the histochemical assay conditions one other problem had to be considered. During the 30 or 60 minute reaction, the lead and substrate do not completely penetrate the 50-100 μ thick slices incubated in the histochemical medium (see Holt and Hicks 1961, Essner et al. 1958, Barrnett 1959) for discussions of the problem of penetration). To obtain reproducible results, it is necessary to determine the depth of penetration. The procedure used to observe the penetration was outlined in "Materials and Methods".

TABLE 4

EFFECT OF INHIBITORS ON G-6-Pase ACTIVITY IN

ADULT HOMOGENATES
(partial inhibition)

Exp.	Incubation Conditions	Specific Activity (μmoles Pi/mg protein/ 20', 37°)	Percent Activity Compared to Control
1	Control	2.24	-
	+ glucose 30 mM	.62	28
	+ $PO_4^{\equiv}$ 30 mM	.40	18
	+ β-glycerophosphate 30 mM	2.36	105
	+ NaF 30 mM	1.11	50
	+ linoleic acid 7 mM	1.86	83
	pH 5.0, 30' at 37°	1.61	72
2	Control	1.57	-
	Na caprylate 10 mM	1.36	86

Assay medium: 30 mM G6P, 30 mM cacodylate buffer, pH 6.6, 10 mg homogenate.

The treatment at pH 5.0 included pre-incubating the enzyme for 30 minutes at 37°, pH 5.0, before assaying at pH 6.6. All determinations done in duplicate.

TABLE 5

EFFECT OF INHIBITORS ON GLUCOSE-6-PHOSPHATASE ACTIVITY

(whole homogenates)
complete inhibition

Exp.	Incubation conditions	Specific activity (μmoles Pi/mg prot. 37° 20')*	Percent activity compared to control
1	control (adult)	1.34	
	no substrate	0	0
	β-glycerophosphate instead of G-6-P (30 mM)	.03	2
	Na oleate (5 mM)	.05	4
2	control (adult)	2.24	
	heat treatment of enzyme (80° 5')	.03	1
3	control (newborn)	1.84	
	PMB (5×10^{-4} M)	1.21	65
4	control (newborn)	1.84	
	PMB (5×10^{-4} M; preincubated with enzyme 37° 30')	0	0

* Values are averages of two determinations

PMB = parahydroxymercuribenzoate, sodium salt

Reaction was run, except where stated, with 30 mM G-6-P, 30 mM cacodylate buffer, pH 6.6

Essentially it involves embedding the entire incubated slice so that the edges can be easily examined. By trimming and sectioning the slice to include two opposite edges exposed during the reaction, one can see outer regions of the slice, where the deposit is specific and reproducible, and inner areas where there is no deposit at all. Qualitatively, the deposit at the edges increased in a manner paralleling the increase in biochemical activity, whereas the center of the slice, lacked deposit at all stages. If slices are not oriented but are sectioned in random planes, then the presence or absence of deposit depends upon the area through which the section passes. If it passes through the outer areas of the slice, reaction produce is seen, whereas if it passes through the interior of the slice deposit is absent. Plate 1 shows a light micrograph of a slice from adult liver which was processed for light microscopy and then embedded in the manner just outlined. The preparation has not been stained so that contrast is imparted to the tissue solely by the reaction product. The reaction is best at the edges of the slice where the deposit is heavy and is limited to the cytoplasm. The amount of product decreases as one moves into the slice. Only occasionally were non-specific deposits seen at the edges of the slices. Further observations 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.

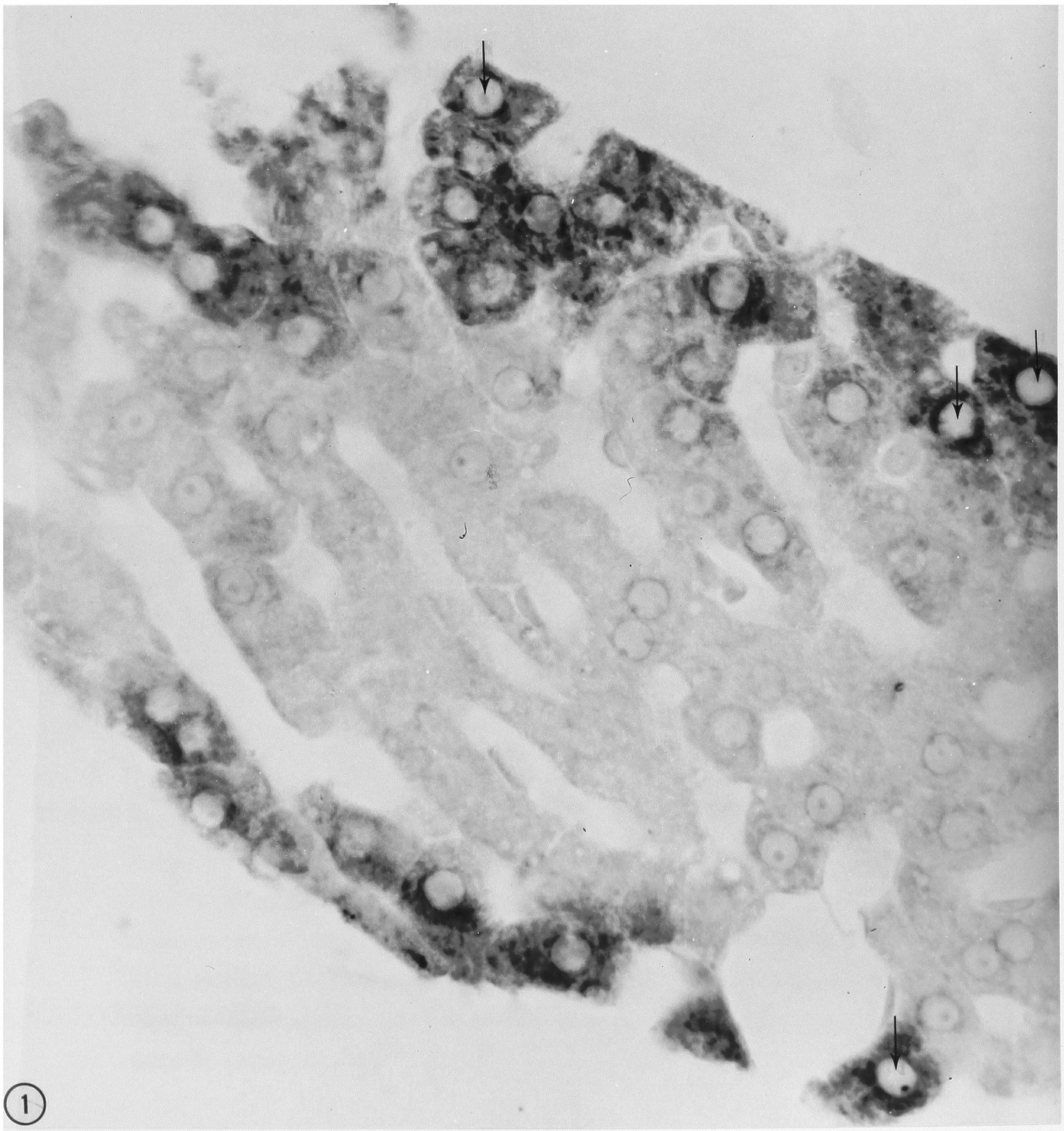
II. THE DEVELOPMENT OF GLUCOSE-6-PHOSPHATASE ACTIVITY

Figure 3 illustrates the development of G6Pase activity in whole homogenates prepared from animals ranging in age from four days before to 72 hours after birth. Enzyme specific activity is presented as a percent of that found in the adult liver. To obtain an average value for the adult, the activities measured in 6 experiments were averaged. Duplicate points at the same stage of development represent different litters. Generally livers from 4-5 animals of a litter were pooled. The specific enzyme activity increases slowly before birth reaching a value approximating that of the adult at the time of birth. Then, during the first 24 hours after birth, the enzyme specific activity increases rapidly, overshooting the adult value by about three-fold.

Plate 1. Light micrograph of histochemical reaction for G6Pase in adult liver slice. Slices of perfusion-fixed liver were incubated with lead and G6P for 30 minutes, processed for light microscopic observation and embedded in Epon. This slice is approximately 90 μ across. The section, 0.5 μ thick, is unstained. Typical hepatic "cords" of cells are visible.

The reaction product is clearly present in the outermost layers of cells and the intensity of the deposit decreases towards the interior of the slice. Note the localization of deposit in the cytoplasm of the hepatocytes and the lack of deposit in the nuclei (arrows).

Magnification: x 1350.


1

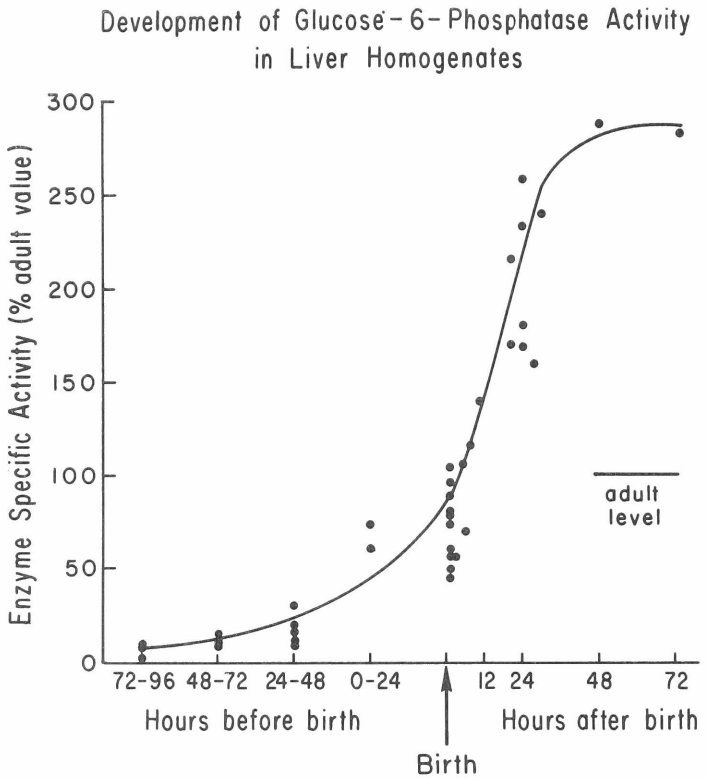


Figure 3. Development of G6Pase activity in liver homogenates. Assay medium: 30 mM G6P, 30 mM cacodylate buffer, pH 6.6. Adult value is average of 6 experiments. All determinations done in duplicate. Each point is from homogenate prepared from combined livers of several animals in a litter. In a single experiment, litter mates were used for all time points after birth.

Similar curves have been reported by Burch et al. (1963), Dallner et al. (1966b), Greengard and Dewey (1967) inter alios, although the time and degree of overshoot vary somewhat from one report to another. Dallner et al. (1966b) have shown that the activity drops off several days after birth and slowly decreases to the adult level. Greengard and Dewey (1968) have postulated that the slow accumulation of G6Pase activity during the last few days of gestation reflects the increased metabolic activity of the foetus caused by the functioning of the thyroid which starts at about this time. The dramatic increase right after birth is probably a response to post-natal hypoglycemia (Dawkins 1963, Greengard and Dewey 1967). The increased enzyme activity facilitates the conversion of stored glycogen into free blood glucose.

Figure 4 illustrates the kinetics of the reaction at several stages in development. At all stages the rate is linear up to 30 minutes of incubation.

G6Pase activity was also measured in rough microsomes which were isolated from animals at several stages of development. In Table 6 this activity is compared to that found in isolated rough microsomes from the adult rat. The increase in activity in the rough microsomes is similar to that found in the whole homogenate. This suggests that the changing composition of the liver during this time does not account for the shape of the curve. Since the vast majority of rough microsomes are derived from hepatocytes, the activity of these vesicles represents the enzyme activity of the hepatocytes, and there is little non-hepatocyte protein in the fraction to decrease the specific activity (which is calculated on a protein basis). In addition, by 1 day before birth, when the activity becomes appreciable, most of the developing blood cells are no longer in the liver. The composition of the liver does not change drastically after that time.

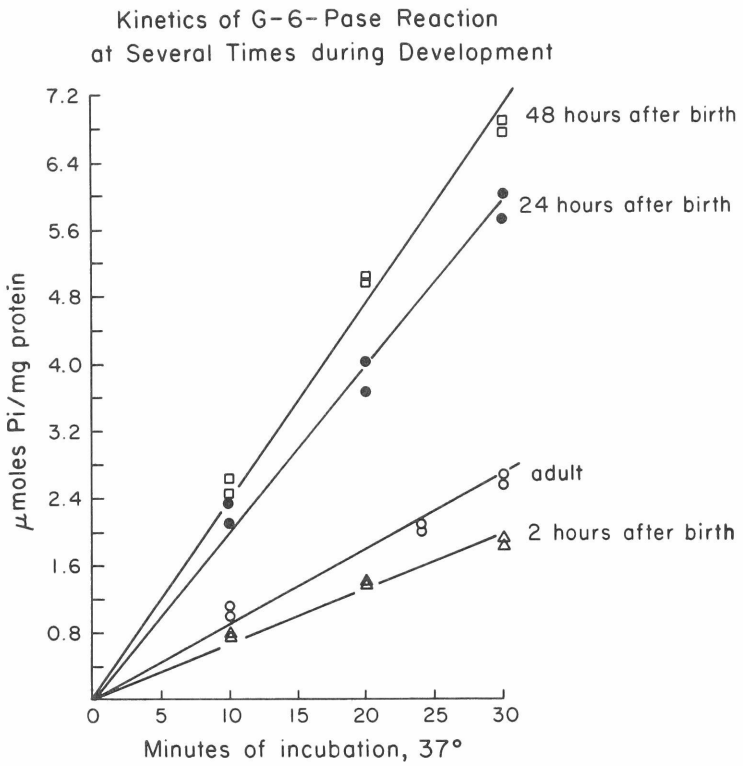


Figure 4. Kinetics of G6Pase reaction at several times during development.

Assay medium: 30 mM G6P, 30 mM cacodylate buffer, pH 6.6. Duplicate points on each curve represent two determinations on the same sample.

TABLE 6

DEVELOPMENT OF G-6-Pase ACTIVITY IN WHOLE HOMOGENATES AND ROUGH MICROSOMES

Age	Specific Activity Rough Microsomes*	Percent of Adult	Specific Activity Homogenate*	Percent of Adult
-3 Days	.18	6	.19	11
-2 Days	.61	19	.38	13
Birth	3.24	100	1.42	82
Adult	3.26	-	1.73	-

* Specific Activity = μmoles Pi/mg protein/20', 37°

Values are averages of duplicate determinations. Assay medium: 30 mM G6P, 30 mM cacodylate buffer, pH 6.6. Microsomes prepared from same homogenate as that assayed. Homogenate kept at 0° overnight and assayed simultaneously with microsomes.

Summary of Parts I and II

The biochemical assay used to detect G6Pase meets the criteria for reproducible and systematic enzyme determination. The reaction rate varies linearly with enzyme concentration, and the enzyme is saturated with substrate under the conditions used. The reaction has linear kinetics for 30 minutes of incubation at both 25° C and 37° C, although it proceeds more slowly at the lower temperature.

The conditions used for the histochemical analysis do not produce appreciable inhibition of the enzyme. Perfusion fixation of the liver for a short time allows retention of approximately 80% of the original enzyme activity in both the adult and newborn. The lead concentration used in the histochemical reaction does not inhibit the enzyme nor hydrolyze the substrate non-specifically.

A number of conditions were found which almost completely inhibit G6Pase and are thus useful as controls to check the histochemical specificity of the enzyme.

Oriented embedding of the entire tissue slice permits controlled examination of the penetration of substrate and lead into the slice.

The biochemical activity of G6Pase in both whole homogenate and isolated rough microsomes was determined at a number of the stages during the development which will be examined histochemically.

III. HISTOCHEMISTRY

The material to be discussed in this chapter includes the results of histochemistry done on liver from animals ranging in age from 4 days prior to birth, when G6Pase activity is low, to 24 hours after birth, when the activity is at its highest.

Adult

To examine the intracellular distribution of the reaction product and compare its localization to that obtained by others, the G6Pase histochemistry was first performed on adult tissue.

Morphology: A typical hepatocyte from adult liver is shown in Plate 2. The material was fixed by perfusion but was not incubated in the histochemical medium. The morphological preservation of the tissue is good and is similar to that seen after prolonged fixation (Porter and Bonneville 1963). There is no vacuolation of the ER, the nuclear envelope or the mitochondria. Both the rough and smooth ER are well preserved and easily recognizable. The Golgi complex (Plate 3) and the other cell organelles retain their accepted morphological appearances. The cells are intact and also retain their normal topographical relationships to each other (see Plates 1 and 7). Occasionally myelin figures are seen in the vicinity of smooth ER or Golgi membranes. The glycogen regions appear light instead of dark due probably to fixation in cacodylate rather than in phosphate buffer. The glycogen in Plate 3 is better preserved than that in Plate 2. Plate 4 shows a higher magnification view of the edge of a glycogen region. The adult hepatocyte contains many ER membranes of both the rough and smooth surfaced variety, and points of continuity between the two types of ER are visible. The difference in topography between the rough and smooth ER is well demonstrated; the rough is arranged as long parallel cisternae while the smooth appears as a connected network of tubules. The rough ER ends at the edges of the glycogen deposit while the smooth penetrates into it. These animals have not been starved and the glycogen deposits are so large that sometimes the smooth membranes do not completely permeate them.

After reaction in the histochemical medium it is often difficult to distinguish between rough and smooth ER solely by the presence of ribosomes on the former. The ribosomes are not clearly visible. This may be due to the incubation at room temperature for 30 minutes, the presence of dense lead deposits inside the cisternae which obscure the ribosomes or the depolymerization of the RNA by the lead (Farkas 1968). After reaction, it is necessary to identify the two types of ER by their characteristic morphology, their relationship to other cell organelles and the density of the surrounding matrix. Rough surfaced vesicles are embedded in a dense matrix made up of free ribosomes and ribosomes attached to membranes which are out of the plane of section. Smooth

Plate 2. Electron micrograph of hepatocyte from adult liver fixed by perfusion with glutaraldehyde. The tissue was not incubated in the histochemical medium. None of the cell organelles is distended or vacuolated. The parallel arrangement of the elongated, flattened rough ER cisternae (rm) contrasts with the tubular network of smooth ER membranes (sm). The glycogen regions (gly) appear extracted and are much less electron dense than after fixation in the presence of phosphate buffer. Note the difference in electron opacity between the material surrounding the rough and smooth ER.

ne - nuclear envelope

m - mitochondria

mb - microbody

Magnification: x 25,000.

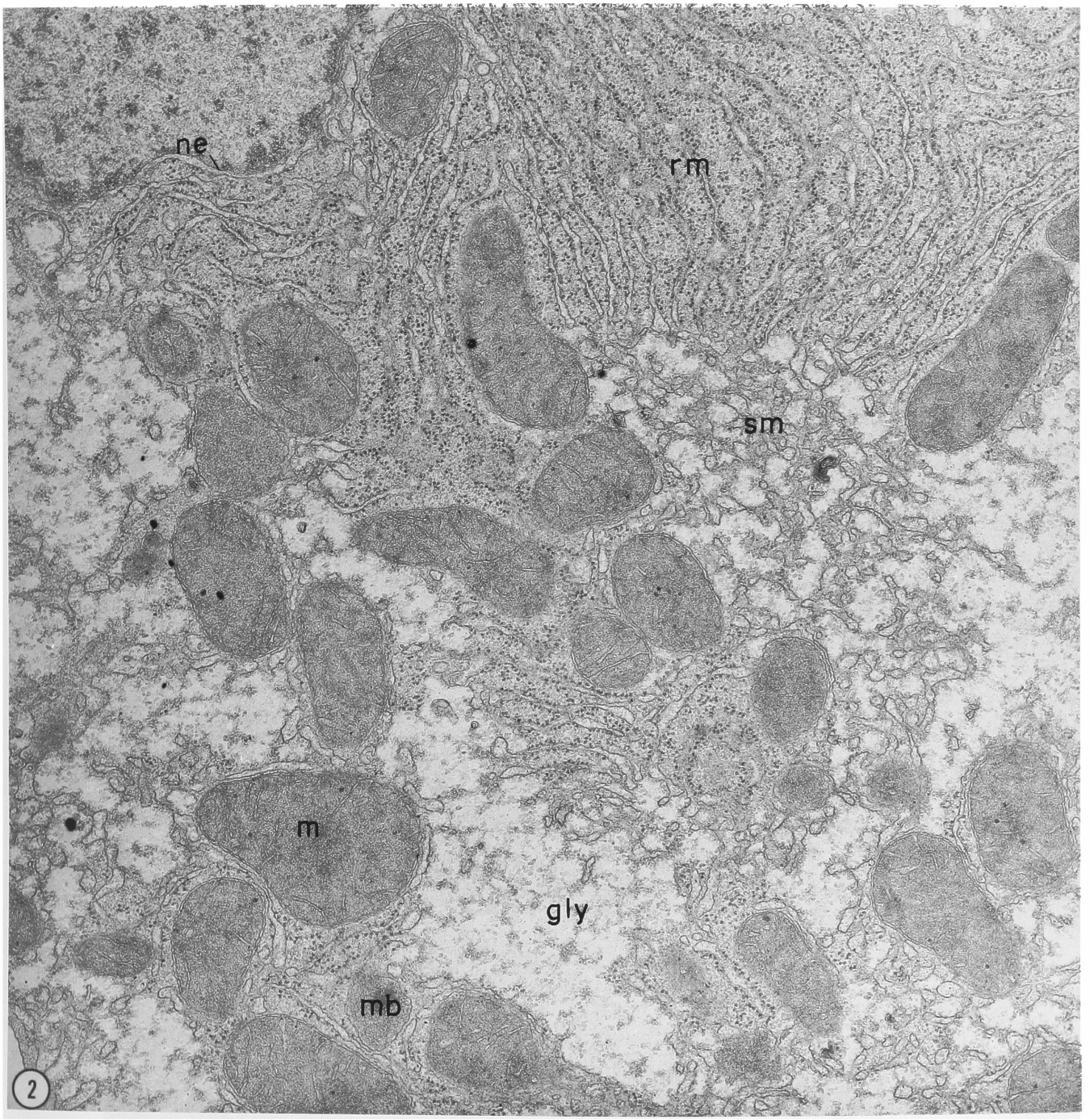

ne
rm
sm
m
gly
mb
2

Plate 3. Electron micrograph of hepatocyte from adult liver fixed by perfusion with glutaraldehyde. In this cell the glycogen areas (gly) are better preserved. An interconnected network of smooth ER (sm) is seen enmeshed with glycogen particles. The Golgi complex (G) is well preserved. Tissue was treated as in Plate 2.

rm - rough ER.

Magnification: x 24,000.

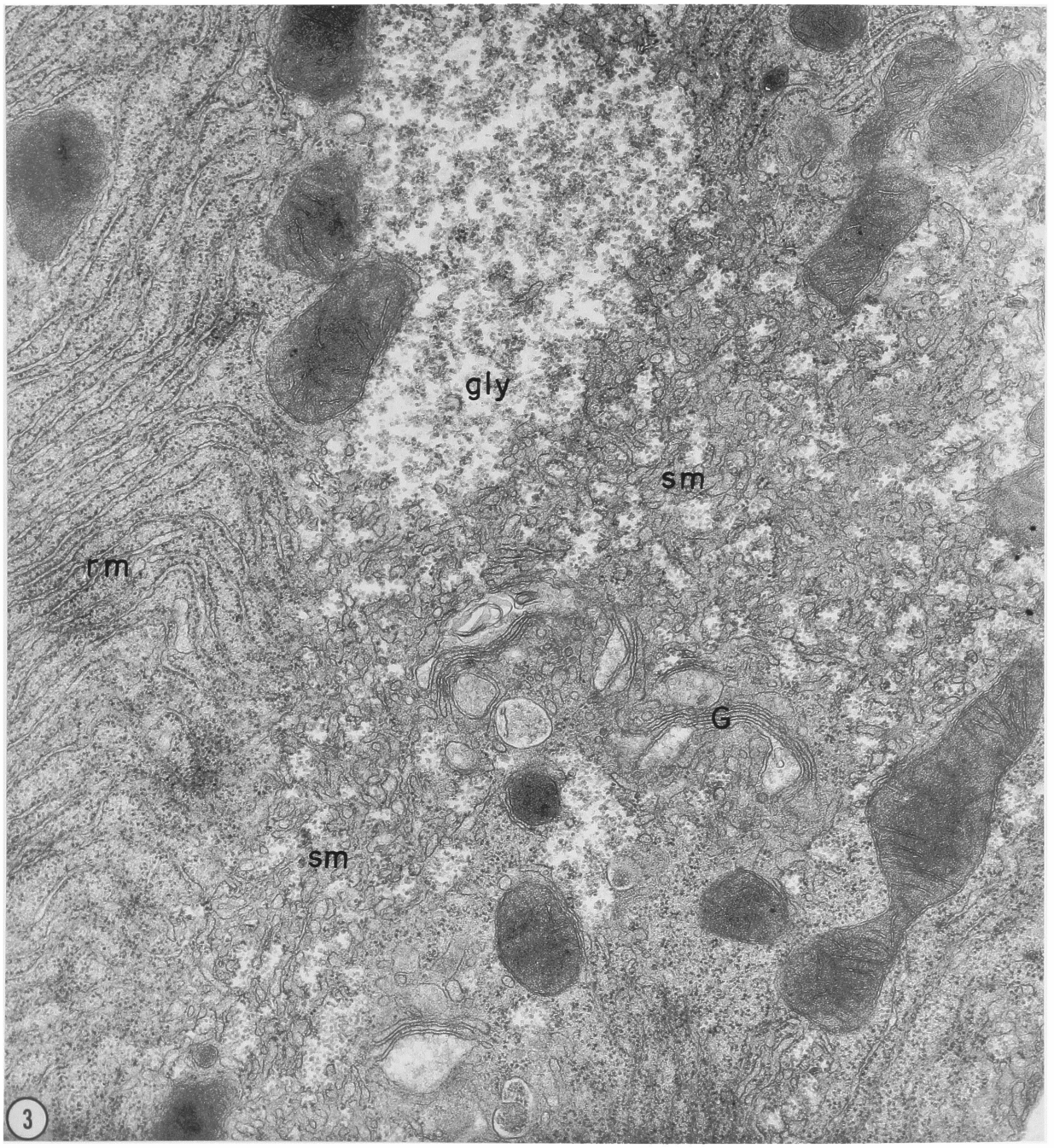
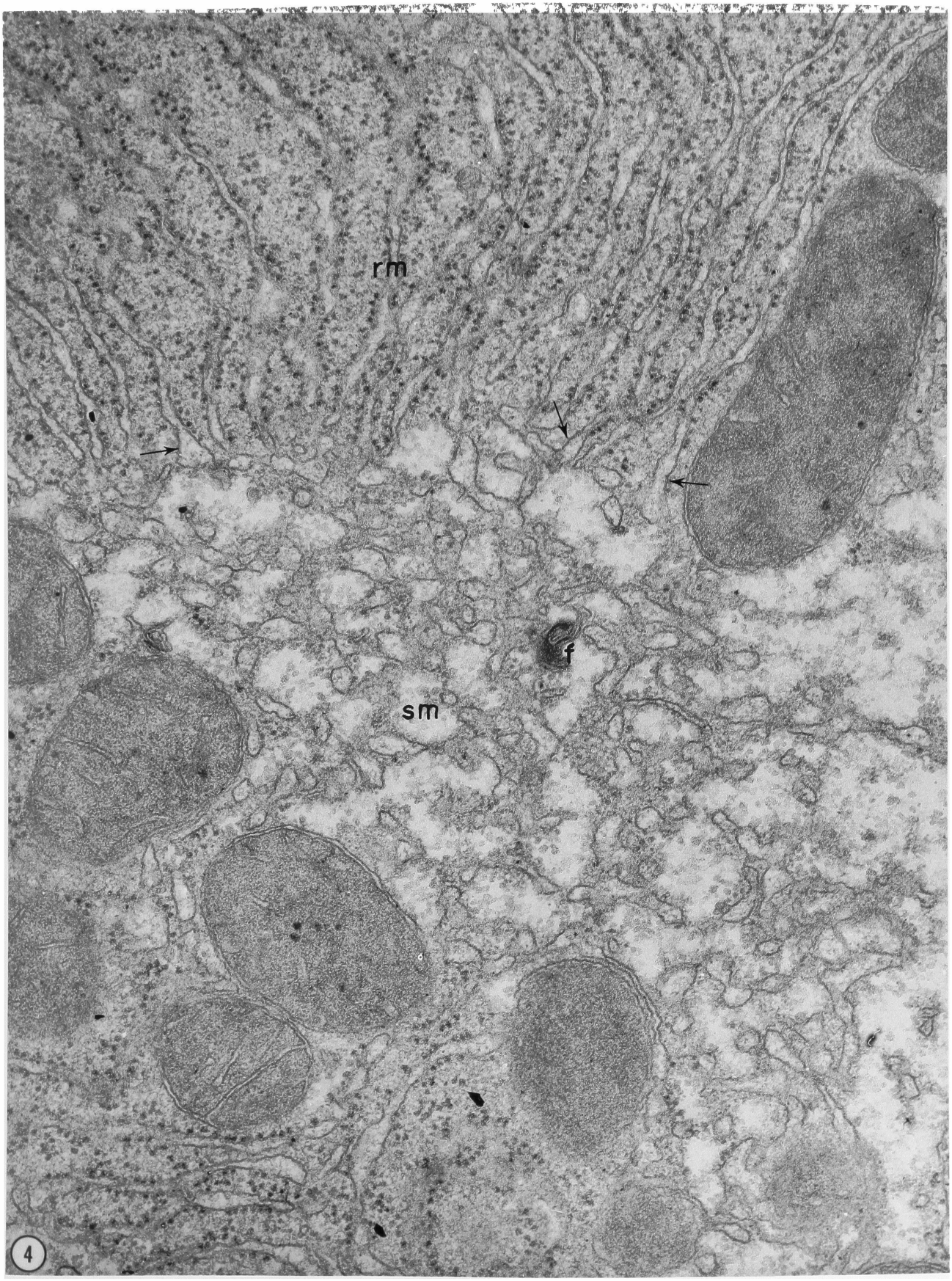

gly
sm
rm
G
sm
3

Plate 4. Enlargement of Plate 2. Arrows indicate points of continuity between the rough (rm) and smooth (sm) ER. A small myelin figure (f), probably formed during aldehyde fixation, is seen in the region of the smooth ER. Tissue was treated as in Plate 2. Magnification: x 53,000.


rm
f
sm
4

membranes are located in the glycogen regions which are areas of low electron density. In order to facilitate the identification of the two types of ER, pictures of tissue which has not been incubated in the histochemical medium are included at most stages during development.

Histochemistry. Plate 5 shows the localization of the reaction product in adult tissue which has been incubated for 30 minutes. The deposit is found exclusively in the cisternal space of both the rough and smooth ER and within the nuclear envelope. There is no reaction along the plasma membrane, in the elements of the Golgi complex (which are not shown) or over other cell organelles. This localization agrees with that found by Tice and Barrnett (1962) and Orrenius and Ericsson (1966). The deposit is heavy and is distributed uniformly in the ER of the cell. Note the poor definition of the ribosomes. Although the intensity of deposit varies from cell to cell, all of the hepatocytes react positively.

The section seen in Plate 6 was not stained with either uranyl acetate or lead citrate although the tissue was treated in block with uranyl acetate; the liver slice was incubated in the complete histochemical medium for 30 minutes. The details of the morphology are clear and the lead deposit is easily visible in the ER membranes. Much of the contrast in the section is provided by the non-specific binding of the free lead ions, present in the incubation medium, to the tissue. This exposure to lead is equivalent to staining the section with lead for an extended period of time at room temperature. In contradiction to this finding Essner and Novikoff (1961) state that incubation of tissue in lead containing medium does not increase contrast. The very fine punctate deposit which is caused by the non-specific lead binding is easily distinguished from the coarse, dense lead phosphate deposit produced by the activity of the enzyme. The faint background deposit is also seen in tissue which has not been stained in block with uranyl acetate. In addition, the fine deposit is visible in control material which has been incubated without substrate. It is not seen in the tissue prepared purely for morphological observations and not exposed to lead.

Plate 5. Electron micrograph of G6Pase histochemistry in adult hepatocyte incubated for 30 minutes in the histochemical medium. This cell is near the edge of the tissue slice. The lead phosphate deposit is present in the nuclear envelope (nm) and in the rough (rm) and smooth (sm) ER. There is no reaction product over the nucleus (N), mitochondria (m) or plasma membrane (pm). Note the density of the matrix surrounding the rough ER compared to that surrounding the smooth ER. The ribosomes are not easily distinguished (cf. Plate 2).

Magnification: x 30,000.

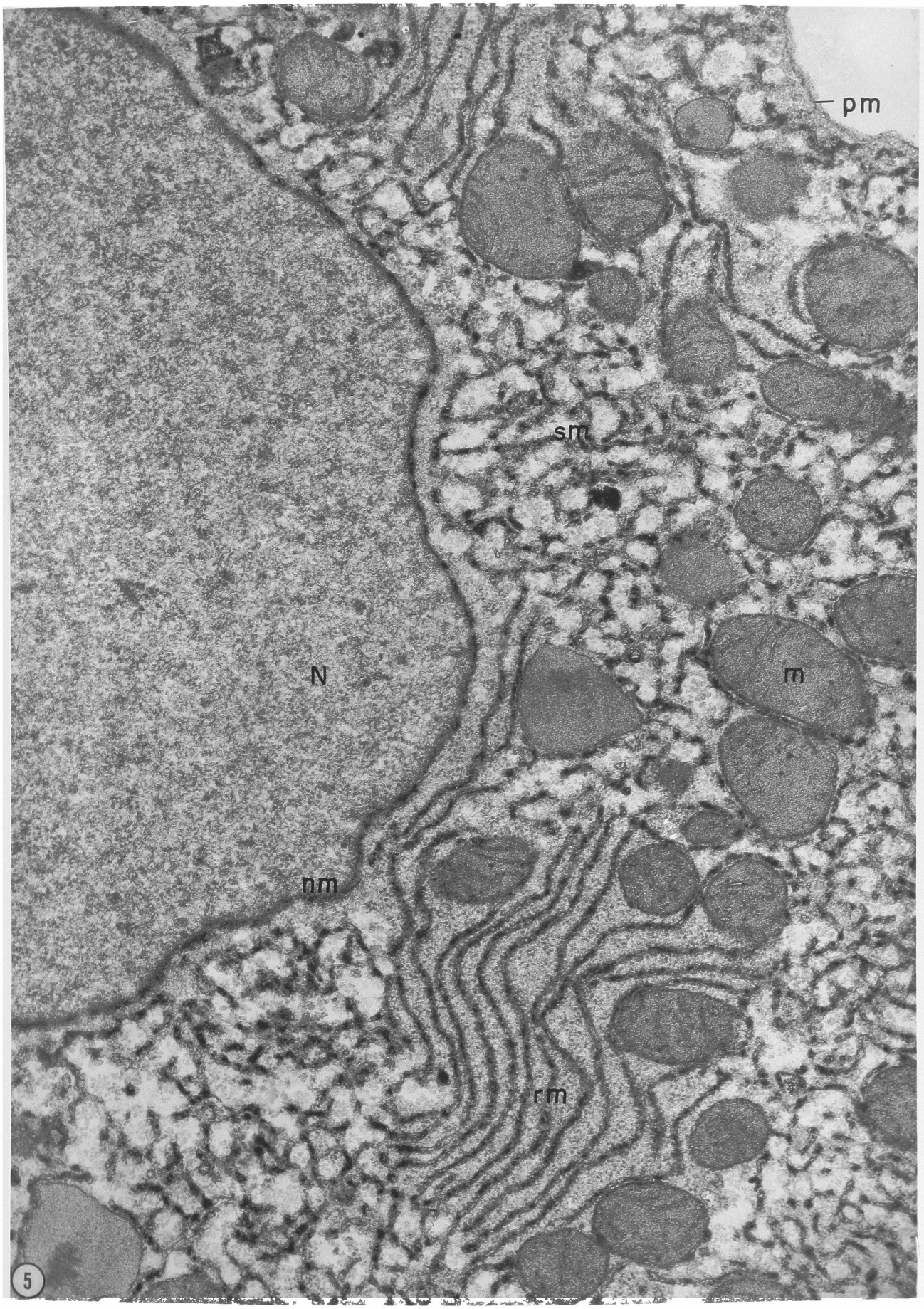
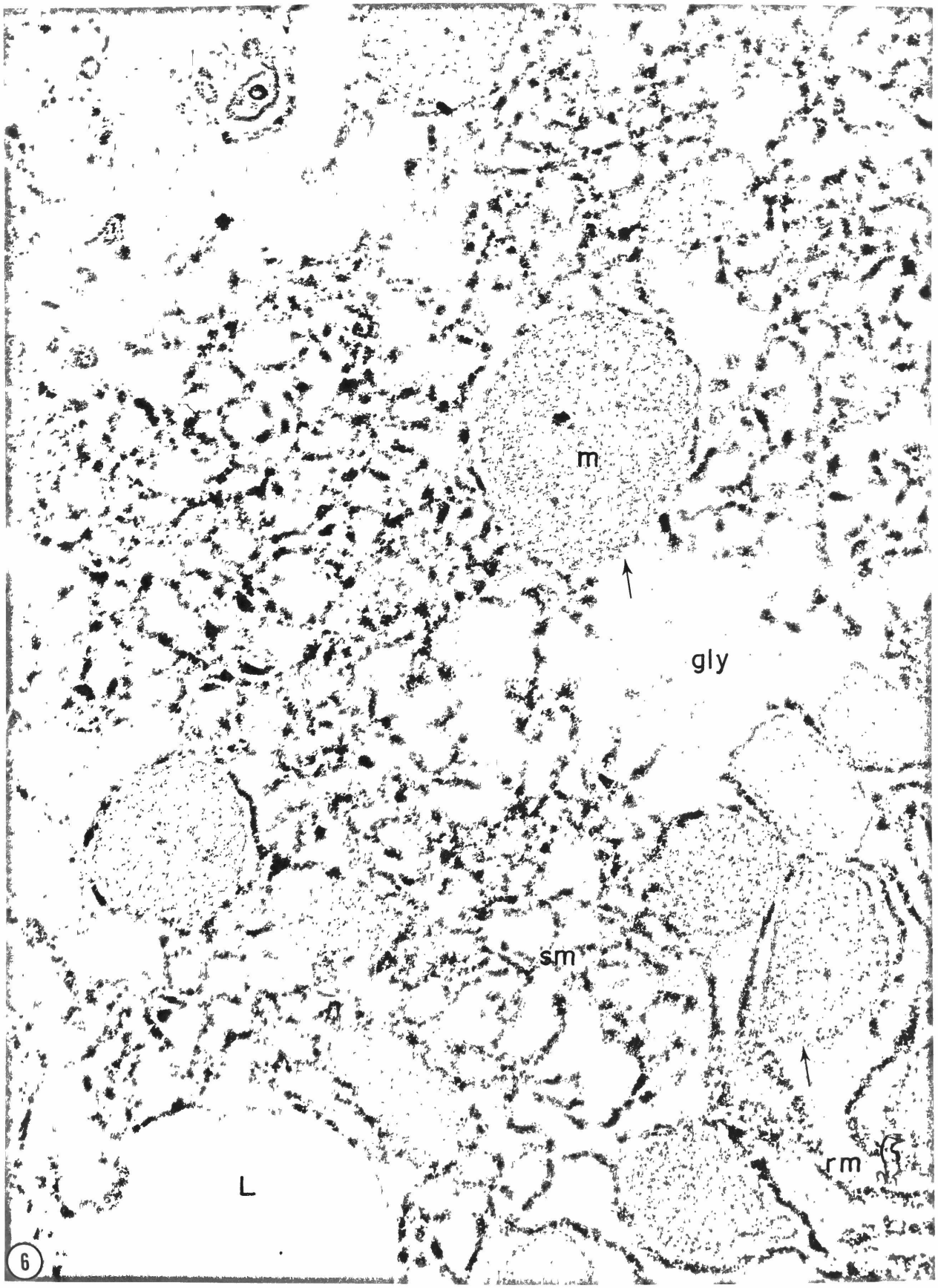

pm
sm
N
m
nm
rm
5

Plate 6. Histochemistry in a section of an adult hepatocyte. The tissue was incubated in the complete histochemical medium for 30 minutes, stained in block with uranyl acetate, but the section was not additionally stained. Much of the morphological detail is visible due probably to the staining effect of the lead present during the histochemical incubation. Note the fine background deposit (arrows) over the mitochondria (m). This punctate deposit is found over many cell organelles and is probably due to non-specific binding of the lead ions to the membranes. The background deposit is readily distinguished from the lead phosphate deposit seen in the rough (rm) and smooth (sm) ER.

gly - glycogen area

L - lipid droplet

Magnification: x 40,000.


m
gly
sm
rm
L
6

The distribution of reaction product within the lobule of the adult rat liver was examined on frozen sections cut from perfusion-fixed liver. Plate 7 illustrates such a preparation which has been processed for light microscopy. The reaction product is evenly distributed throughout the lobule. The cells are unstained and are visible only because they contain lead sulfide deposit. This even intralobular distribution makes it unnecessary to confine observations to a particular part of the lobule. Tice and Barrnett (1962) similarly report an even intralobular distribution of G6Pase while Novikoff (1959) and Manns (1968) find heavier reaction product in the peri-portal areas.

Foetus

-4 and -3 Days

Morphology. These early stages will be considered together since they present virtually identical pictures. In the foetus of this age, the gross anatomy of the liver is similar to that of the fully mature animal but the microscopic anatomy is extremely different. In fact, it is difficult to recognize the typical liver morphology at all. The cell population of the liver is very heterogeneous; at least half of the cells belong to the hematopoietic series (see Plate 8). These blood cells are dispersed between hepatocytes and are in close contact with them. The typical adult "cords" of hepatocytes do not exist at these stages. The entire liver is very loosely packed. This loose structure aids the penetration of substrate and lead during histochemical incubation and often reacted cells are seen in the center of slices.

The hepatocytes are very small and irregularly shaped. They contain a small amount of rough ER which is often not organized into parallel cisternae as in the adult. There is essentially no smooth ER although there are Golgi saccules. Glycogen has started to collect in the cells although the deposits are still much smaller than at birth. The morphological preservation of the tissue fixed by perfusion is good despite the fact that the perfusion of these small livers is extremely difficult. Since the cells at this stage are so small and irregular in shape, it is difficult to find thin sections which show large areas of the cytoplasm. It is more common to find cytoplasmic extensions as in Plate 11.

Plate 7. Light microscopic histochemistry in a fixed-frozen section of adult liver. This 20 micra frozen section of perfusion fixed liver was incubated in the complete histochemical medium for 30 minutes, treated with ammonium sulfide and then mounted in glycerin jelly. The section is unstained so that the contrast is due solely to the brown lead sulfide precipitate in the hepatocytes. The field shows one lobule and the reaction product is evenly distributed across it.

h - cords of hepatocytes

c - central vein

Magnification: x 120.

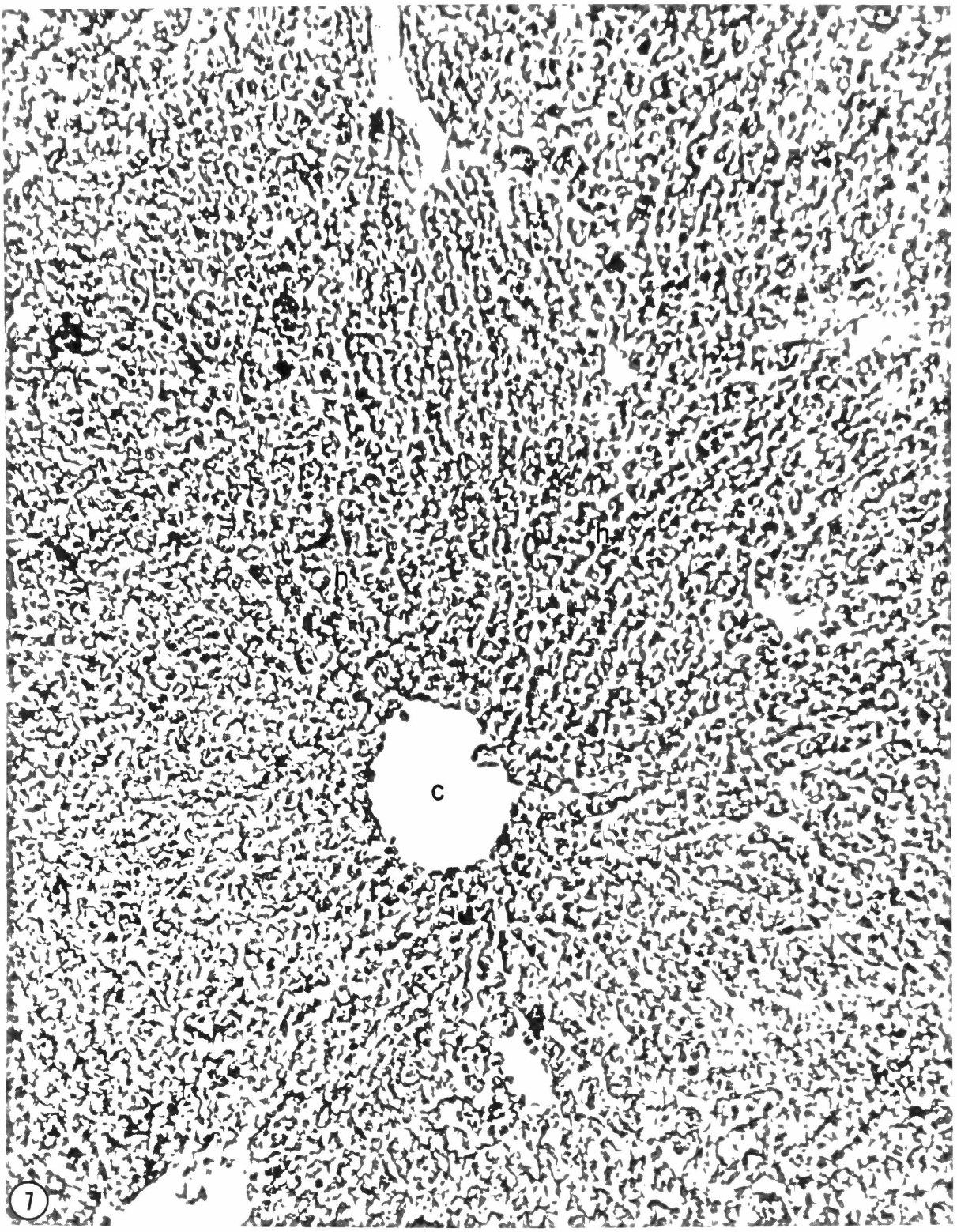
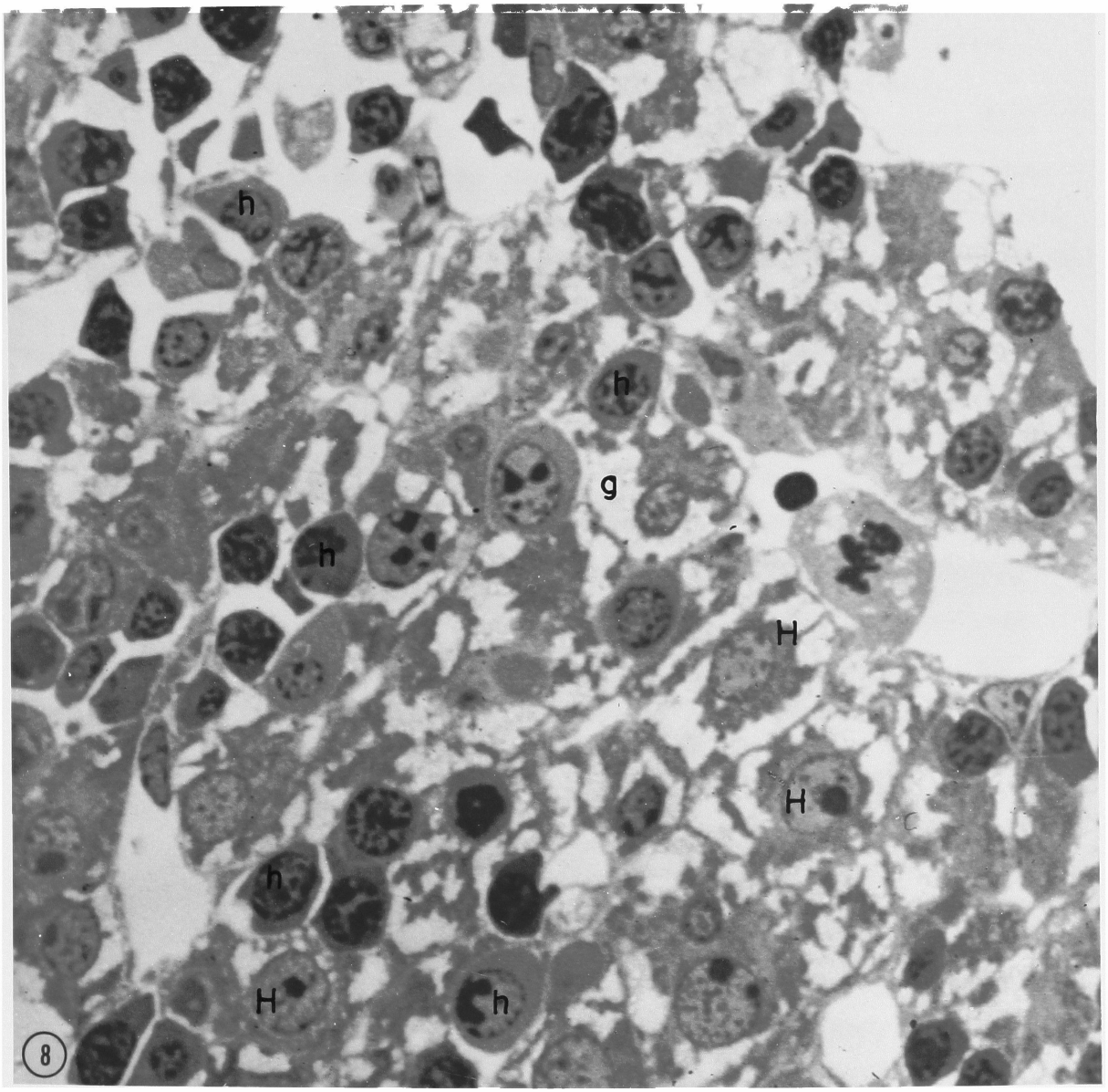

h
h
C
7

Plate 8. Light micrograph of a section of perfusion-fixed liver from an animal 3 days prior to birth. This section illustrates the heterogeneous cell population in the liver at this stage. The section (0.5 μ thick) was stained with toluidine blue. The hepatocytes (H) contain glycogen deposits (g) which are light in color and appear distinct from the other cell organelles (darker areas in hepatocytes). Interspersed among the hepatocytes are numerous hematopoietic cells (h). Several blood vessels are visible in the section but the tissue has not yet differentiated into its adult form (cf. Plate 7).

Magnification: x 1800.


h
h
g
h
H
H
h
H
h
8

Histochemistry. At these stages the G6Pase specific activity is less than 10% that of the adult. Within the population of hepatocytes, this activity is distributed very heterogeneously. Most of the cells do not contain any lead phosphate deposit after histochemical incubation while a small number of cells show a light but definite deposit. These two types of cells can occasionally be found directly adjacent to one another as can be seen in Plate 9. One cell has no deposit in either the rough ER or the nuclear envelope while the other has deposit specifically located in the cisternae of the rough ER. The insert more clearly shows the location of the deposit inside of the membranes. Note the large size of the nucleus in the unreacted cell compared to the small amount of surrounding cytoplasm. Incubation of this material was carried on for 60 minutes. Even after this long incubation the deposit is much lighter than that found in the adult after only 30 minutes of incubation. If this tissue is incubated for 30 minutes, the deposit is so light that it is often difficult to distinguish at all. The heterogeneous reaction within the population of hepatocytes was also seen when incubated tissue slices were processed for light microscopy (see Plate 10). A hepatocyte containing reaction product is next to one which completely lacks deposit.

A part of another reacting cell (Plate 11) illustrates the widespread distribution of the deposit in all of the rough ER of the cell. The deposit is found within the cisternae. It often appears that cells which react positively for the enzyme at this stage have more highly organized ER than unreactive cells. More often long profiles are seen in the ER of the reacting cells and some of the cisternae line up in parallel arrays.

The development of the cells in the hepatocyte population is thus asynchronous. This asynchrony is seen throughout differentiation right up to the last stages studied when the smooth ER forms after birth. In the cells which contain enzyme, however, the reaction product is found in all of the ER membranes present in that cell.

Plate 9. Electron microscopic histochemistry in a liver slice from an animal 4 days before birth. This field, taken near the edge of the tissue slice, shows the heterogeneous reaction between adjacent hepatocytes. In the lower right is a part of a cell which has a light deposit in the rough ER (arrows), while the cell in the upper left has no deposit in either the nuclear envelope (nm) or rough ER (rm). There is no deposit along the plasma membrane (pm) between the two cells. Note the large size of the nucleus (N) in the upper cell compared to the surrounding cytoplasm. The tissue was incubated for 60 minutes.

Magnification: x 20,000.

Insert (9A). This field shows an enlarged portion of the cell seen in the lower right. Note the location of the deposits within the cisternae of the rough ER (arrows).

Magnification: x 39,000.

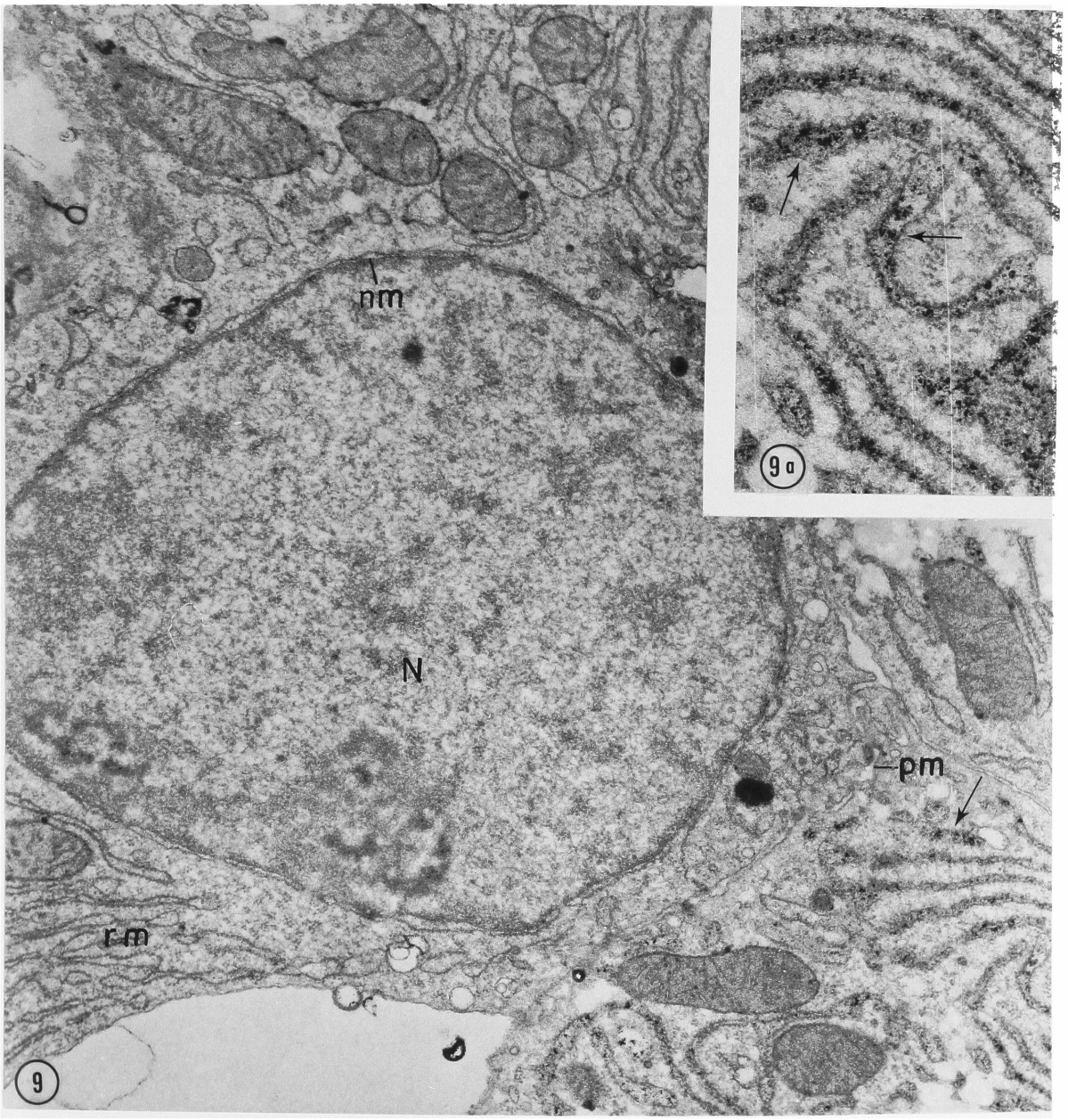

nm
N
pm
rm
9
9a

Plate 10. Light micrograph of G6Pase histochemistry in liver slice from a rat 3 days before birth. Two hepatocytes (H) are included in this field. One contains reaction product in the nuclear envelope and cytoplasm (arrow), and the other completely lacks deposit. This field is near the edge of the slice. The tissue was reacted for light microscopic histochemistry and was then embedded in Epon. This 0.5 μ section is unstained. The picture was taken using a phase-contrast oil-immersion lens.

h - hematopoietic cells.

Magnification: x 4,000.

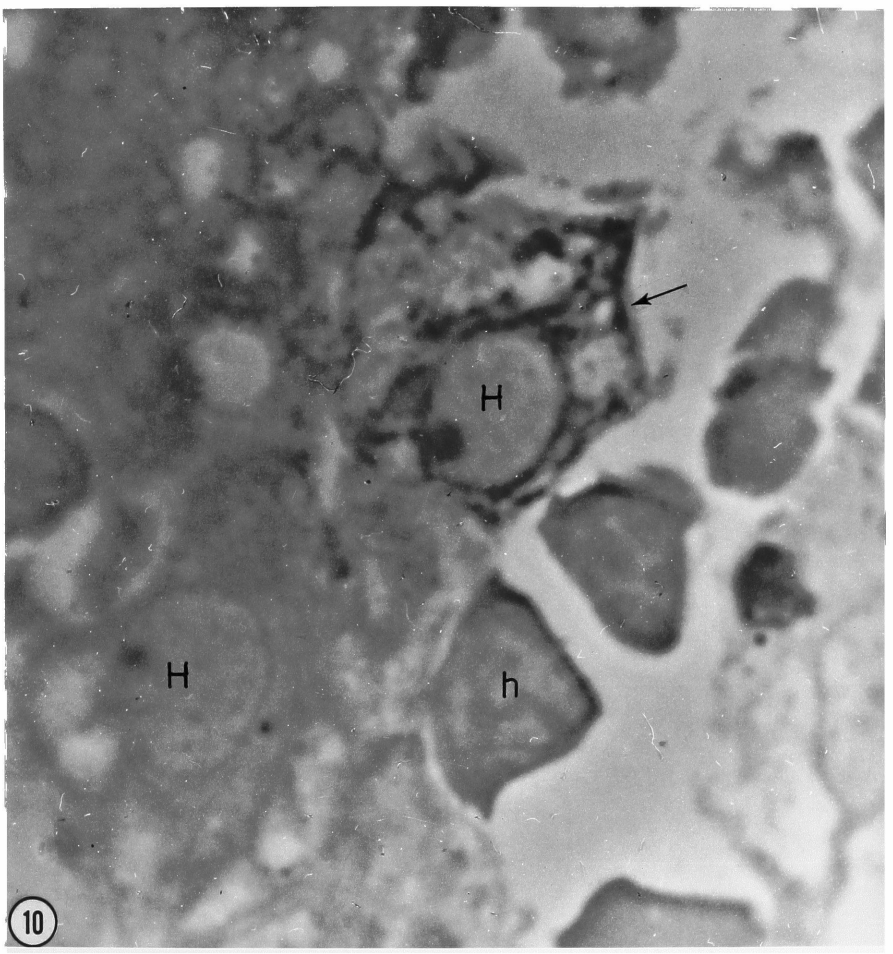
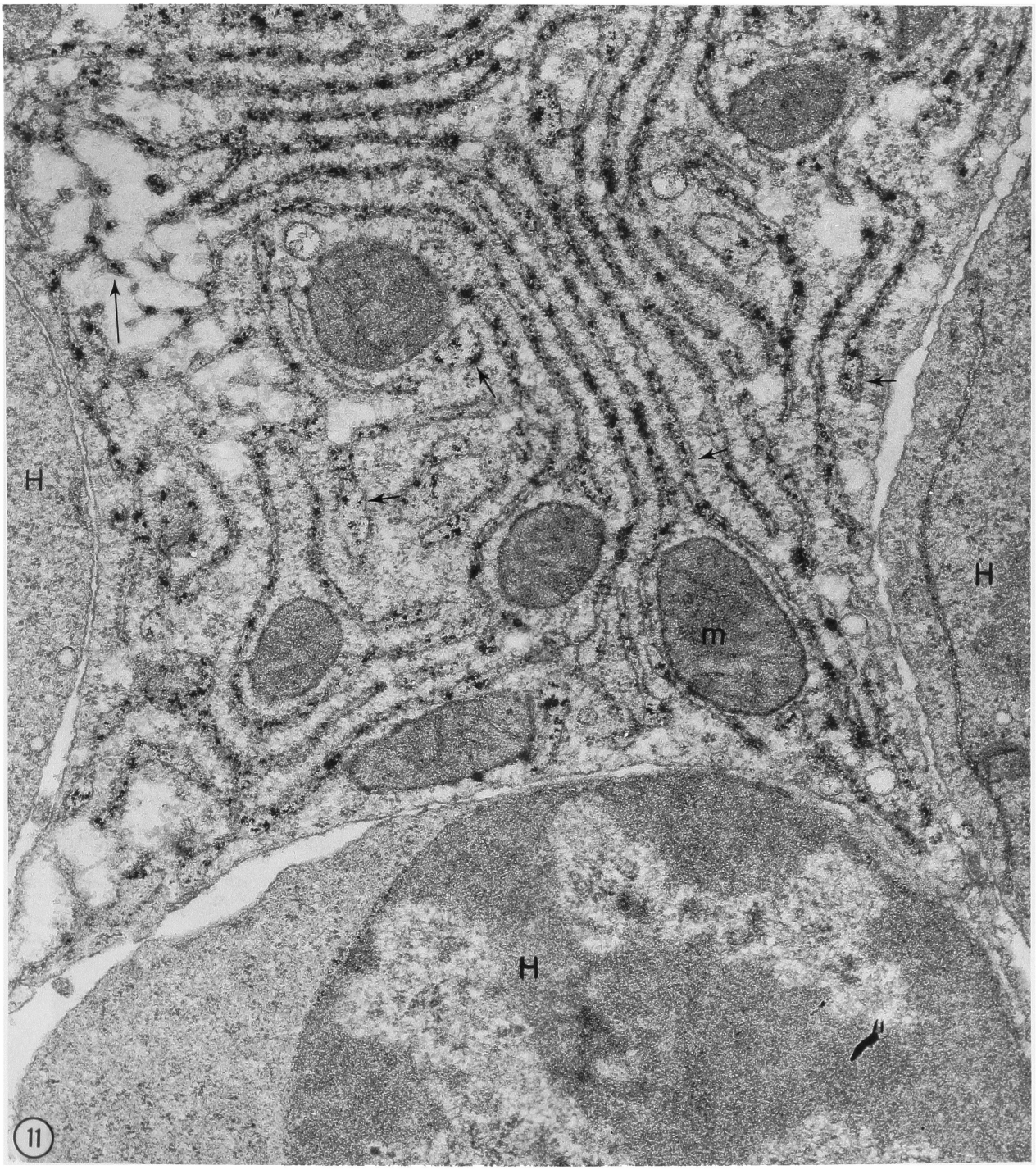

H
H
h
10

Plate 11. Electron micrograph of G6Pase histochemistry in hepatocyte from an animal 4 days before birth. The lead phosphate deposit (short arrows) is uniformly distributed within the cisternal space of all of the ER visible in the cell. In the glycogen region in the upper left some ER containing deposit (long arrows) is present. Because of the location within the glycogen region and the tubular appearance, these are probably smooth ER membranes. The field is near the edge of the tissue slice.

H - hematopoietic cell

m - mitochondria

Magnification: x 32,000.


H
H
m
H
11

-2 Days

Morphology. By two days prior to birth, the cells have increased in size and have acquired larger glycogen deposits (see Plate 12). The ER, which is still exclusively rough surfaced, has increased in amount and its organization more closely approaches that of the adult. The rough ER ends at the edges of the glycogen deposits as it does in the adult. The proportion of developing hematopoietic cells in the population has decreased.

Histochemistry. The number of hepatocytes reacting positively for G6Pase has increased but they are still in the minority. At this stage the biochemical G6Pase specific activity is approximately 20% of that of the adult. The histochemical deposits seen in a reacting cell after 60 minutes of incubation have become heavier (see Plate 13). They are present in the rough ER and nuclear envelope but are absent from the Golgi elements and the plasma membrane. The deposit is widespread in all of the ER of positive cells as is well illustrated in this picture. In Plate 14 a higher magnification view of the deposit is shown. Note its location in the cisternal space. The background, punctate deposit caused by the non-specific binding of the lead is also visible in this section.

-1 Day

Morphology. Plate 15 shows a junction of three cells from the liver of an animal within approximately 24 hours of birth. The morphology within the lobule is approaching that typically seen in adult liver. The hepatocytes have increased still further their stores of glycogen, the areas of which are still completely devoid of penetrating membranes. The ER is quite well organized and now contains rather dense material within it. The preservation of the perfusion-fixed tissue is good and all cell organelles retain their normal structures and normal relationships to each other.

Histochemistry. By 1 day before birth the enzyme activity has increased to 70% of the adult. The histochemical deposit is heavier than at -2 days and is easily visible in the ER and nuclear membrane (Plate 16 and 17). The elements of the Golgi complex are conspicuously free of

Plate 12. Morphology of hepatocyte two days before birth. The tissue was not incubated in the histochemical medium. Note the large glycogen regions (gly) which appear to be extracted. The rough ER (rm) ends at the edges of the glycogen deposits which are free of elements of the smooth ER (cf. Plate 2). The ER cisternae are filled with a moderately electron-dense material. Several empty vesicles (v) are seen in this section. These vesicles are encountered frequently in tissue from young animals. At the upper left is a hematopoietic cell (H). Such cells are still common at this stage.

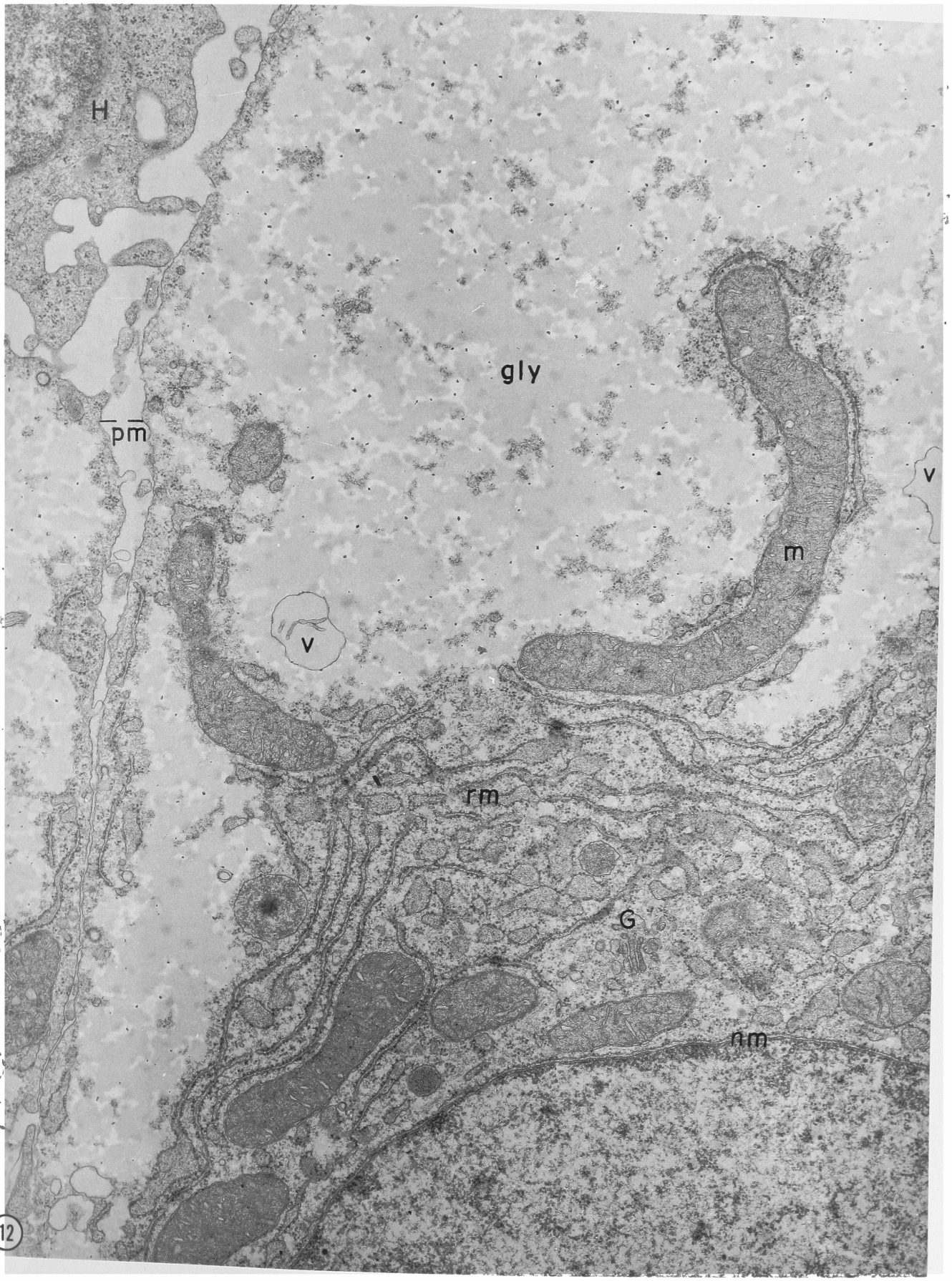
m - mitochondria

nm - nuclear envelope

pm - plasma membrane

G - Golgi region

Magnification: x 20,000.



12

Plate 13. G6Pase histochemistry in a hepatocyte 2 days before birth. The tissue was incubated for 60 minutes in the complete histochemical medium. Lead phosphate deposit is widespread in all of the rough ER cisternae (rm). There is no deposit over the nucleus (N) or in the elements of the Golgi complex (G). The faint background deposit caused by the non-specific lead binding can be seen over the mitochondria. This cell is from the edge of a section as are all of the following pictures of tissue incubated in the histochemical medium.

gly - glycogen deposit

nm - nuclear envelope

Magnification: x 19,000.

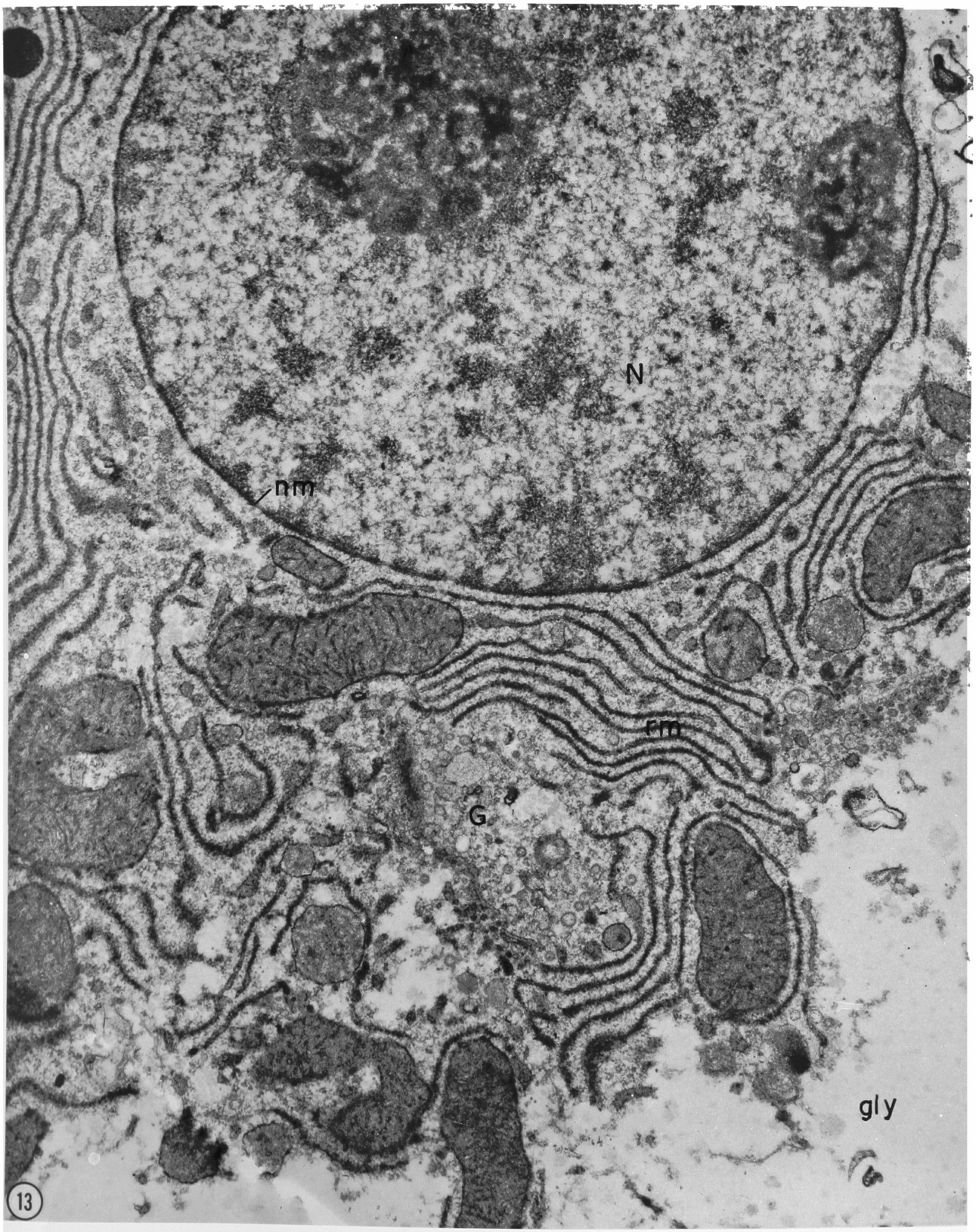

N
nm
rm
G
gly
13

Plate 14. Higher magnification view of a portion of a reacted cell from an animal 2 days before birth. The lead phosphate deposit (short arrows) is visible in the nuclear envelope (nm) and within the cisternal space of the rough ER (rm). The reaction product is seen in all of the ER profiles. In this field the background non-specific deposit (long arrows) is clearly visible over the mitochondria (m) and Golgi saccules. Ribosomes can be seen along the rough ER membranes and between them, although they are not as distinct as in unincubated tissue.

N - nucleus

Magnification: x 51,000.

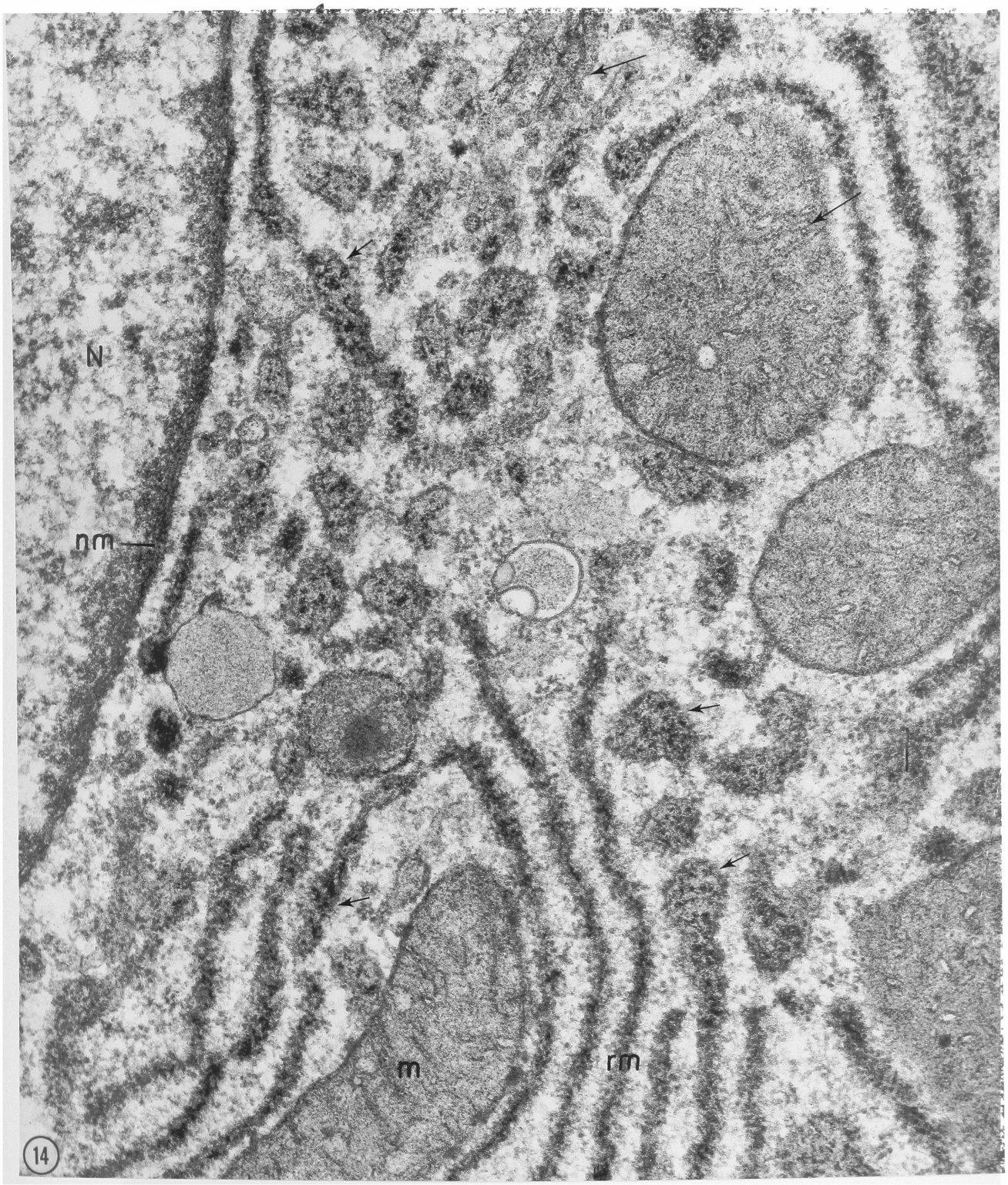

N
nm
m
rm
14

Plate 15. Hepatocyte 1 day before birth. Ribosomes are clearly visible along the rough ER (rm), the cisternae of which are filled with a moderately dense material. The rough ER ends at the edges of the glycogen deposits (gly) which are free of membranous profiles.

G - Golgi region

m - mitochondria

nm - nuclear envelope

pm - plasma membrane

Magnification: x 22,000.

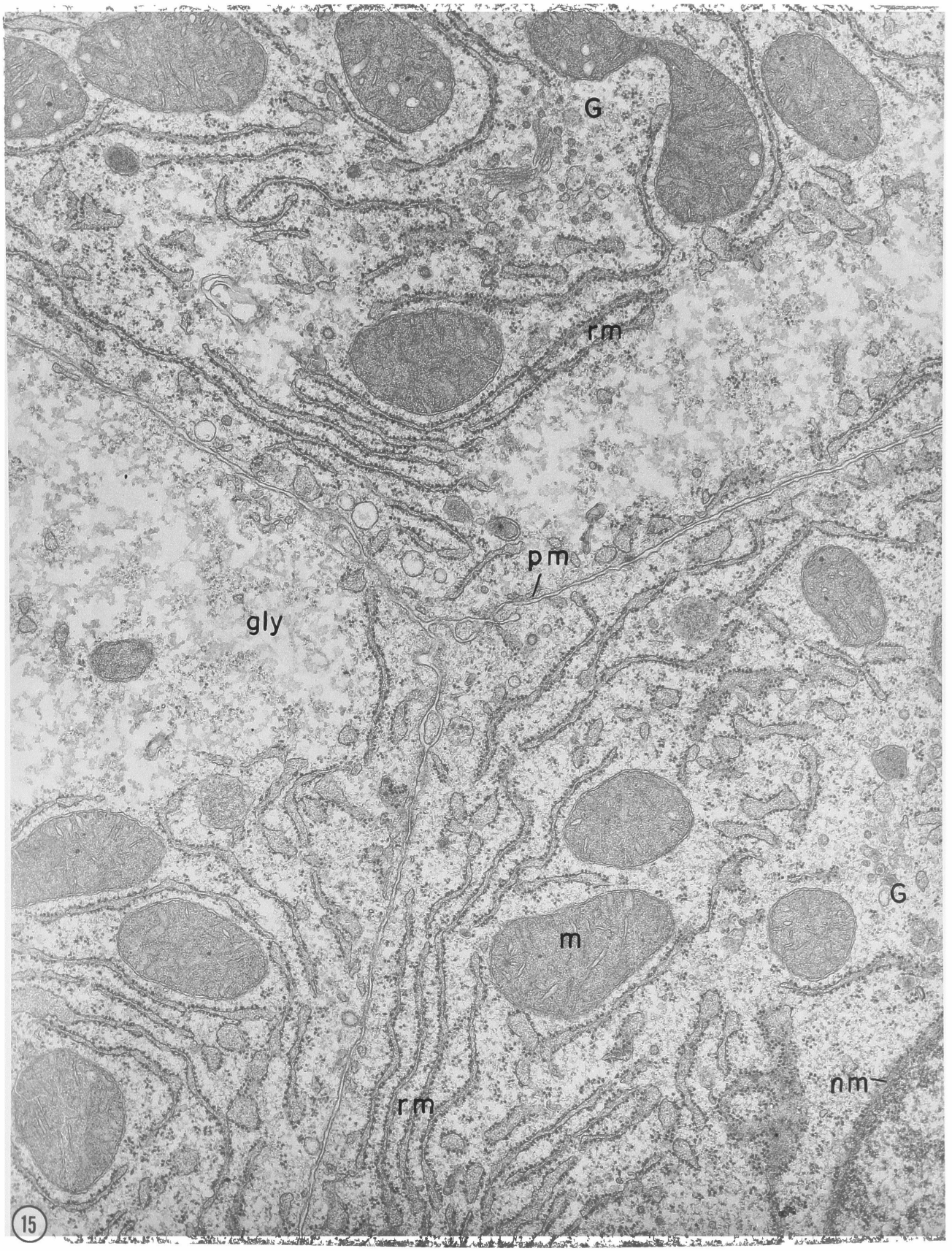

G
rm
pm
gly
G
m
nm
rm
15

Plate 16. G6Pase histochemistry in hepatocytes 1 day before birth. The reaction product is visible in all of the rough ER (rm) of these cells. It is present in the nuclear membrane (nm) but is absent from the elements of the Golgi complex (G). There is no deposit over the nucleus (N) or along the plasma membrane (pm). At the edge of the bile canaliculus (bc) there is a light deposit which is seen even in tissue incubated without substrate (arrows). A large vacuole (v) of unknown origin is seen at the right of the field. The tissue was incubated for 60 minutes in the histochemical medium.

Magnification: x 17,000.

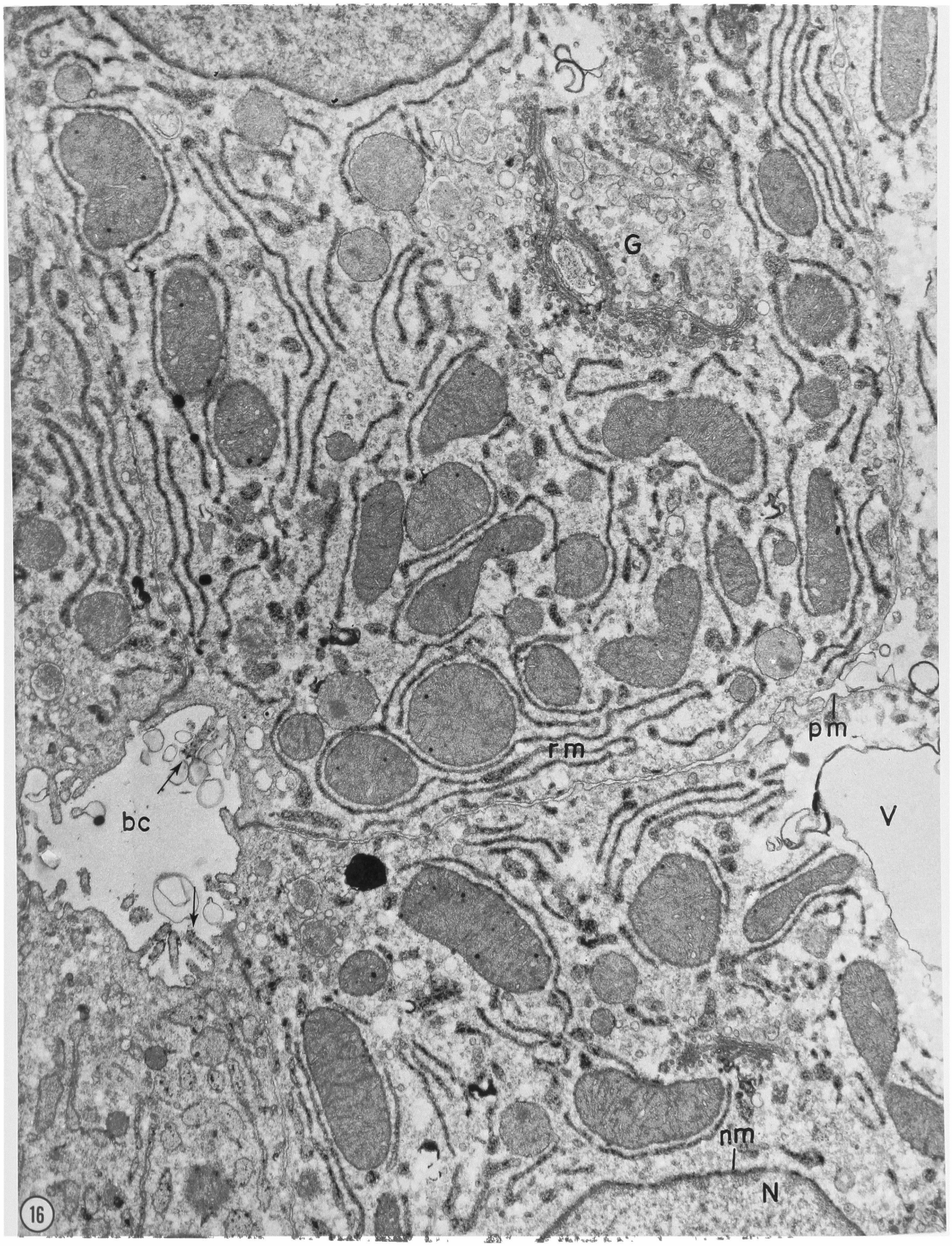
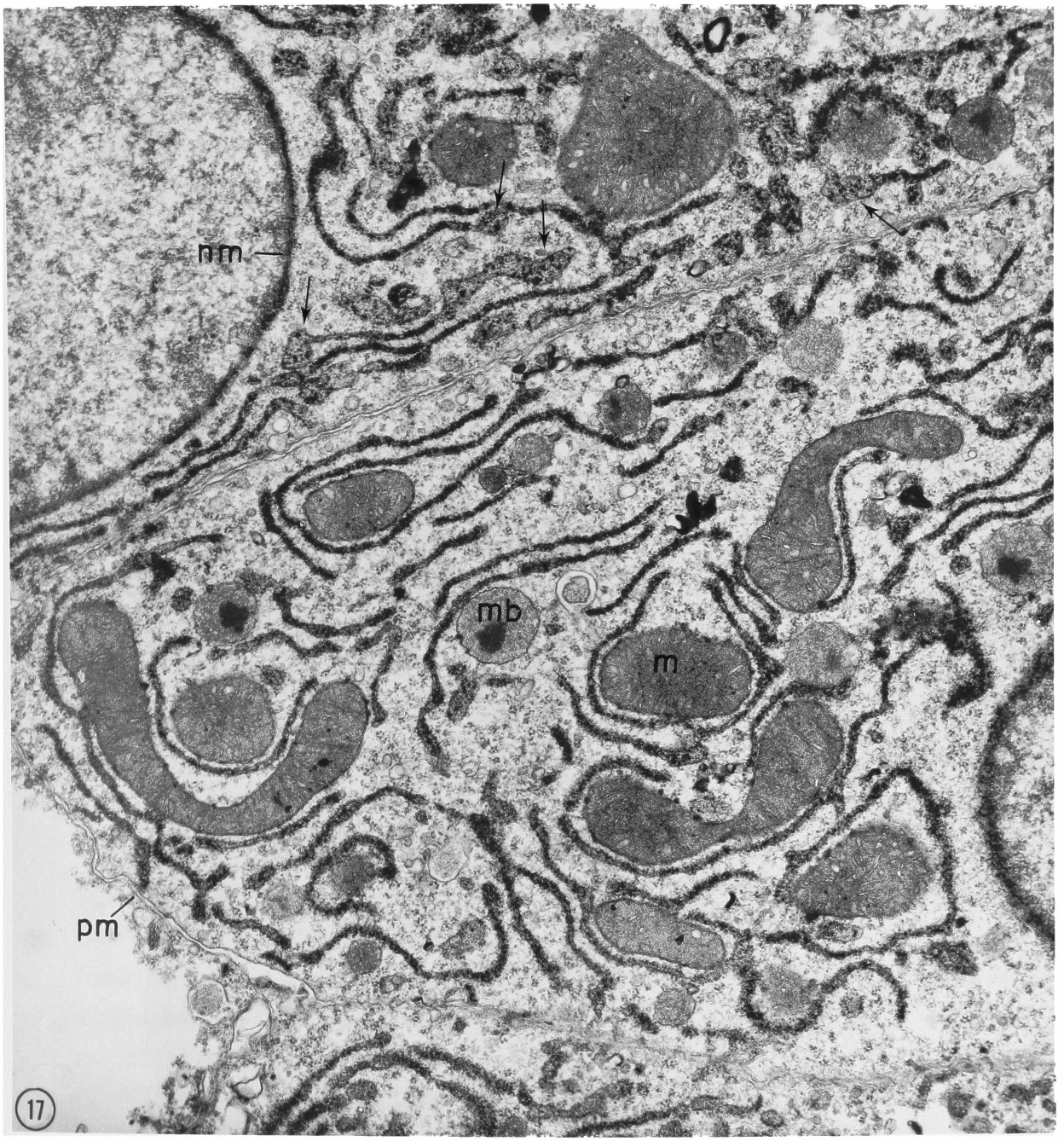

G
pm
rm
bc
V
nm
N
16

Plate 17. G6Pase histochemistry in hepatocytes 1 day before birth. The histochemical deposit again fills all of the ER elements present in the cells, including the nuclear envelope (nm). The localization of the deposit within the cisternal space is clearly visible in some areas (arrows). No deposit is present along the plasma membrane (pm). The edge of the section is at the lower left.

mb - microbody

m - mitochondria

Magnification: x 19,000.


nm
mb
m
pm
17

deposit, as is the plasma membrane (Plates 16, 17 and 18), the mitochondria and the microbodies. In general the localization of deposit is the same as in the adult except that no smooth ER membranes are yet present in the cells. Within a cell, the deposit is uniformly distributed in the rough ER, and the heterogeneity in the population of hepatocytes has decreased. Several reacting cells are seen next to one another in these pictures. Plate 17 well illustrates the location of the deposit within the cisternae of the rough ER.

Newborn

2 Hours After Birth

Morphology. At the time of birth the cells are large and contain such huge deposits of glycogen that they displace all of the other cell organelles. Plate 19 shows the edge of such a deposit which is still free of ER profiles. The rough ER is arranged in parallel arrays similar to the pattern seen in the adult liver. The ER cisternae are filled with material of high density which was also visible one day before birth. The Golgi complex is large at this stage and now contains vacuoles with lipoprotein granules, a typical finding in Golgi vacuoles of the adult liver. The amount of rough ER is still less than in a fully mature liver and the membranes are densely covered on their outer surface with ribosomes. The cytoplasm between membranes is also filled with ribosomes. Lipid droplets, which are seen in the cytoplasm of the cells from newborn animals, have started to appear at this stage.

Histochemistry. At birth the specific enzyme activity of G6Pase is approximately equal to its adult value. Plate 20 shows the reaction in tissue incubated for 30 minutes. The deposit is visible within the rough ER and nuclear envelope and is absent from the Golgi vesicles and plasma membrane. The reaction product is evenly distributed throughout the ER of each cell; by this stage most of the hepatocytes react positively for G6Pase, although the amount of deposit in the ER varies from cell to cell. Plate 21 shows the appearance of the deposit after 60 minutes of incubation.

Plate 18. G6Pase histochemistry in a hepatocyte 1 day before birth. This higher power view of the Golgi complex (G) clearly shows the absence of reaction product from the flattened Golgi cisternae, and the presence of the product in the long cisternae of the rough ER (rm). The small vesicles and larger vacuoles of the Golgi complex are also negative.

gly - glycogen region

pm - plasma membrane

Magnification: x 54,000.

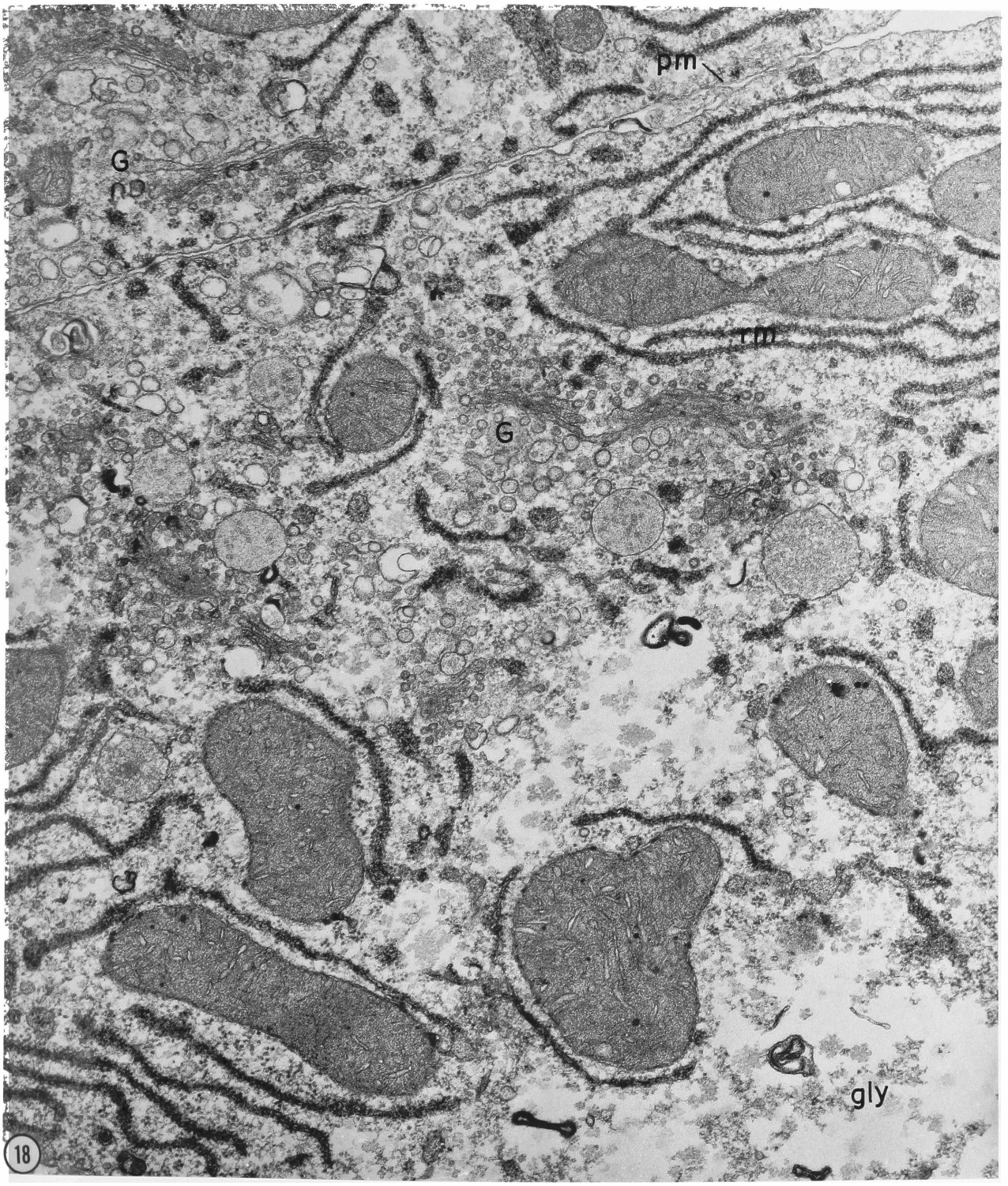

pm
G
rm
G
gly
18

Plate 19. Hepatocyte at birth. This unincubated tissue is from an animal approximately 1 hour old. The glycogen region (gly) at the left is extremely large and displaces all other cell components. These deposits are free of associated ER elements, a situation typical of cells at birth. The rough ER is very densely covered with ribosomes and numerous ribosomes are also free in the cytoplasm. The rough ER cisternae are filled with material of intermediate electron-opacity. The Golgi complex (G) is extremely large and is comprised of elongated cisternae, large vacuoles, and small vesicles. The number of microbodies, several of which are seen in this field (mb), increases at about this time in development. The grazing section through the nucleus reveals several nuclear pores (short arrows) and ribosomes arranged in rosettes (long arrows) on the nuclear envelope.

Magnification: x 25,000.

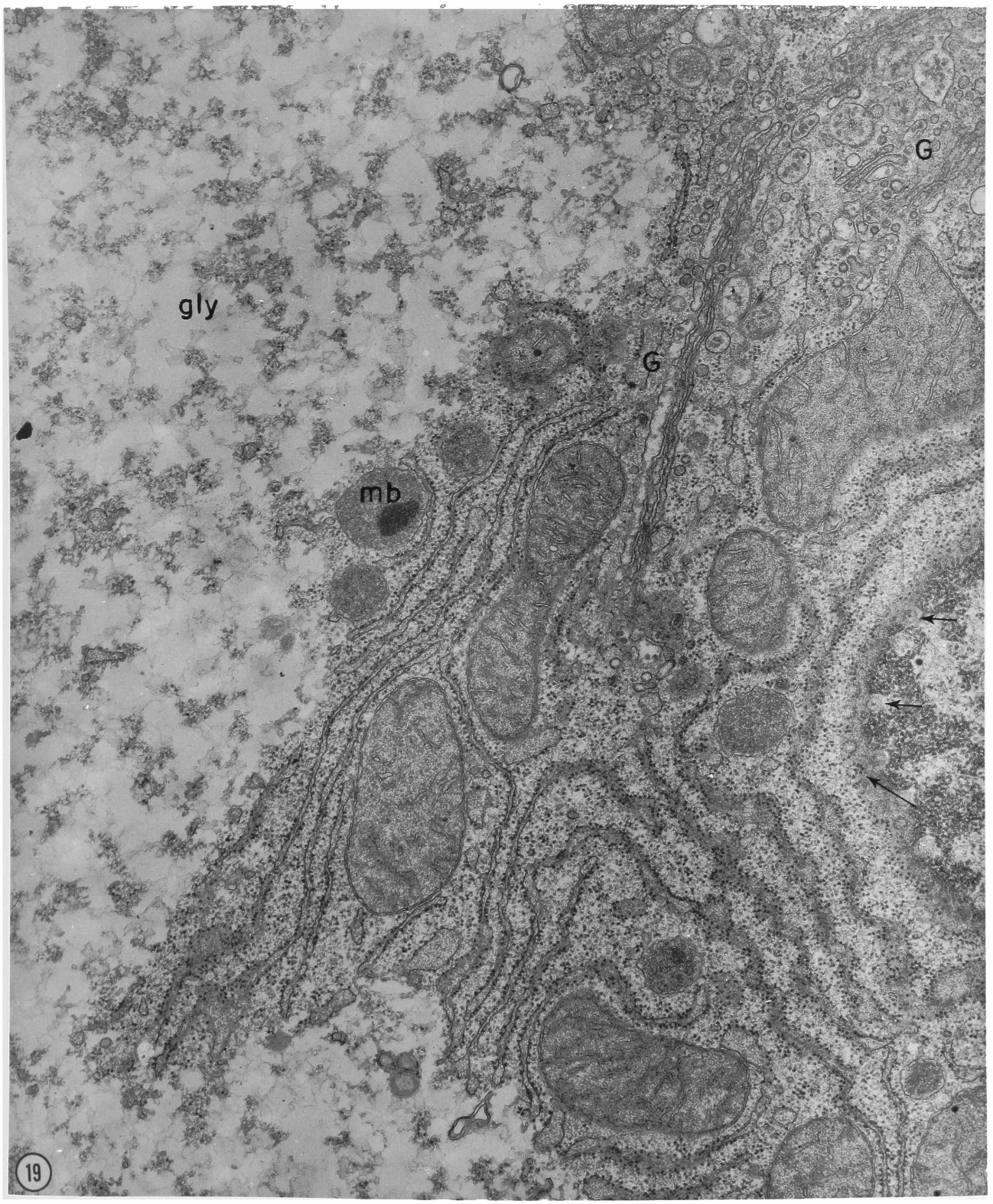

G
gly
G
mb
19

Plate 20. G6Pase histochemistry in hepatocyte from an animal 1 hour old. The histochemical reaction was continued for 30 minutes. The reaction product is seen in all of the rough ER cisternae (rm) and in the nuclear envelope (nm). The elements of the Golgi complex (G), plasma membrane (pm), nucleus and mitochondria are free of deposit. At the right is an empty vacuole (v) and several myelin figures probably produced during fixation.

gly - glycogen region

Magnification: x 18,000.

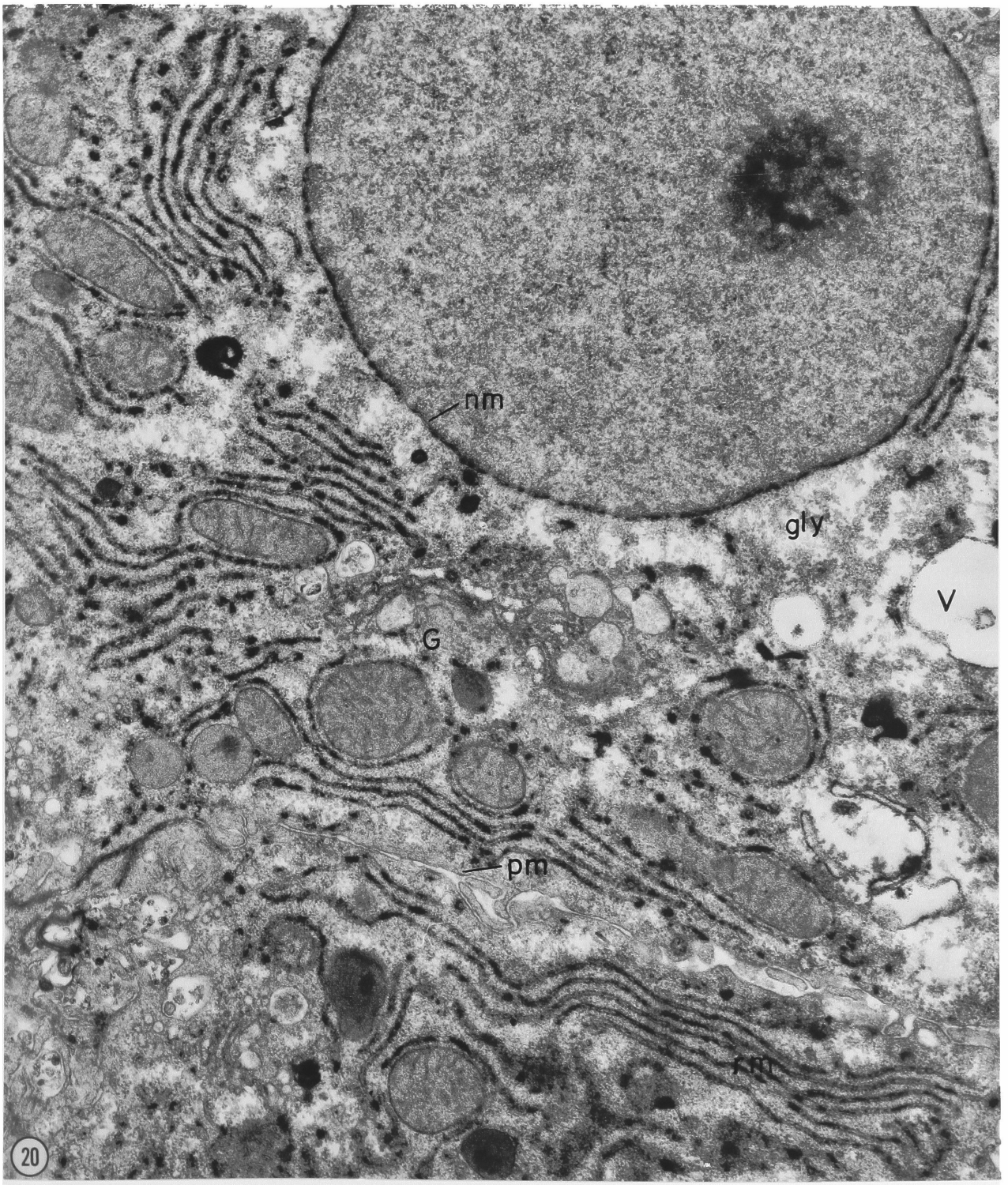
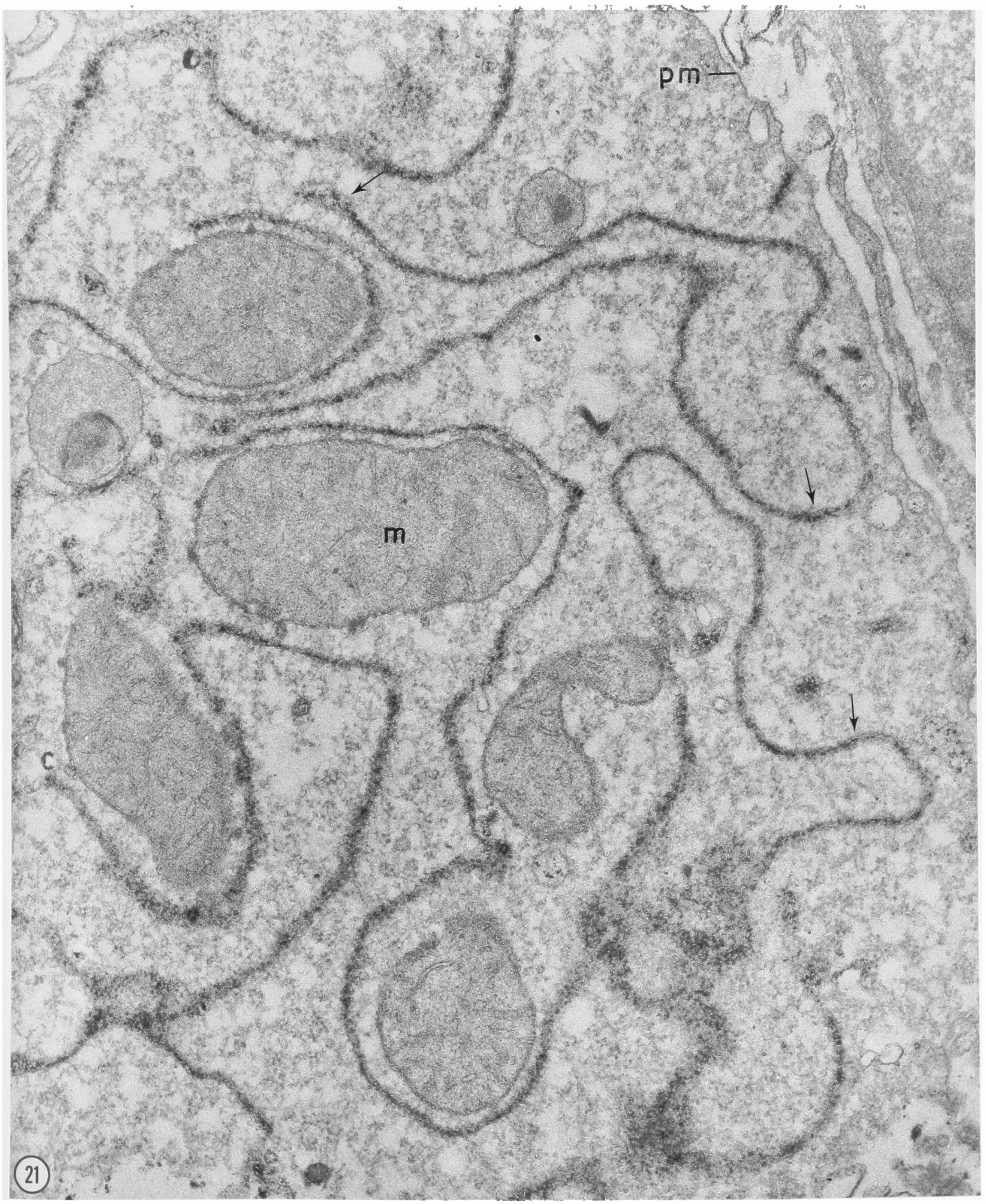

nm
gly
V
G
pm
rm
20

Plate 21. G6Pase histochemistry in hepatocyte at birth. In this tissue the reaction was continued for 60 minutes. The deposit is uniform in all of the ER and is more continuous than after 30 minutes of incubation (cf. Plate 20). Arrows mark regions where the deposit is clearly located within the cisternal space. In this field the ER cisternae are not disposed in parallel arrays but form a continuous, winding network. Note how difficult it is to distinguish ribosomes (cf. Plate 19); free ribosomes appear as gray material between the reacting rough ER cisternae.

m - mitochondria

pm - plasma membrane

Magnification: x 36,000.


pm
m
c
21

6 and 24 Hours After Birth

Morphology: Formation of Smooth Endoplasmic Reticulum. The smooth ER begins to develop in the hepatocyte after birth. The process is asynchronous in the hepatocyte population so that at any one time after birth, cells can be found at various stages of this proliferation. Plate 22 shows one of the earliest stages in a cell 6 hours after birth. Smooth ends of the rough-surfaced cisternae are visible at the edges of the glycogen deposits. Plate 23 is a cell 24 hours after birth. This is another early stage in the proliferation of smooth ER. Rough-surfaced membranes ring the glycogen areas and completely enclose them. Smooth-surfaced tubules appear and penetrate the glycogen regions. Mitochondria are trapped between cisternae of rough ER and these cytoplasmic "islands" separate regions of glycogen. Note the decreased ribosome density on the rough ER compared to that at birth. Plate 24 shows a cell 6 hours after birth which has already reached this stage. In this reacted cell it is difficult to distinguish rough and smooth ER, but the pattern of glycogen areas ringed by ER membranes is obvious.

A slightly later stage in the proliferation of smooth ER is seen in Plate 25, which is also material 6 hours after birth. The smooth-surfaced vesicles have increased in number and are visible in the glycogen areas (regions of low electron-density). Lipid droplets, which can be seen in great numbers by 24 hours after birth, are already visible in the glycogen regions. Comparison of Plate 25 and Plate 4 shows that the smooth ER and its relation to the glycogen is similar to the pattern in the adult. Plate 26, 24 hours after birth, shows an even later stage of development. The glycogen regions have become drastically reduced in size and are completely penetrated by smooth ER. Areas of continuity between rough and smooth ER are easily visible at this stage. Again note the relative sparseness of ribosomes on the ER. This picture also shows a large Golgi complex which is characteristic of many of the stages in development (also see Plate 19 of a cell at birth). As at birth, the lipoprotein containing vacuoles of the Golgi comples are present.

Plate 22. Hepatocyte 6 hours after birth. This field shows an early stage in the formation of the smooth ER. Smooth-surfaced ends (arrows) of rough-surfaced cisternae (rm) are seen along the edges of the glycogen deposits (gly). Most of the glycogen region is still free of membranous profiles. The rough ER has fewer attached ribosomes than immediately after birth and the cisternal content is of lower electron-opacity (cf. Plate 9). Lipid droplets (L) begin to form at about this stage. The tissue was not incubated in the histochemical medium.

G - Golgi complex.

Magnification: x 30,000.

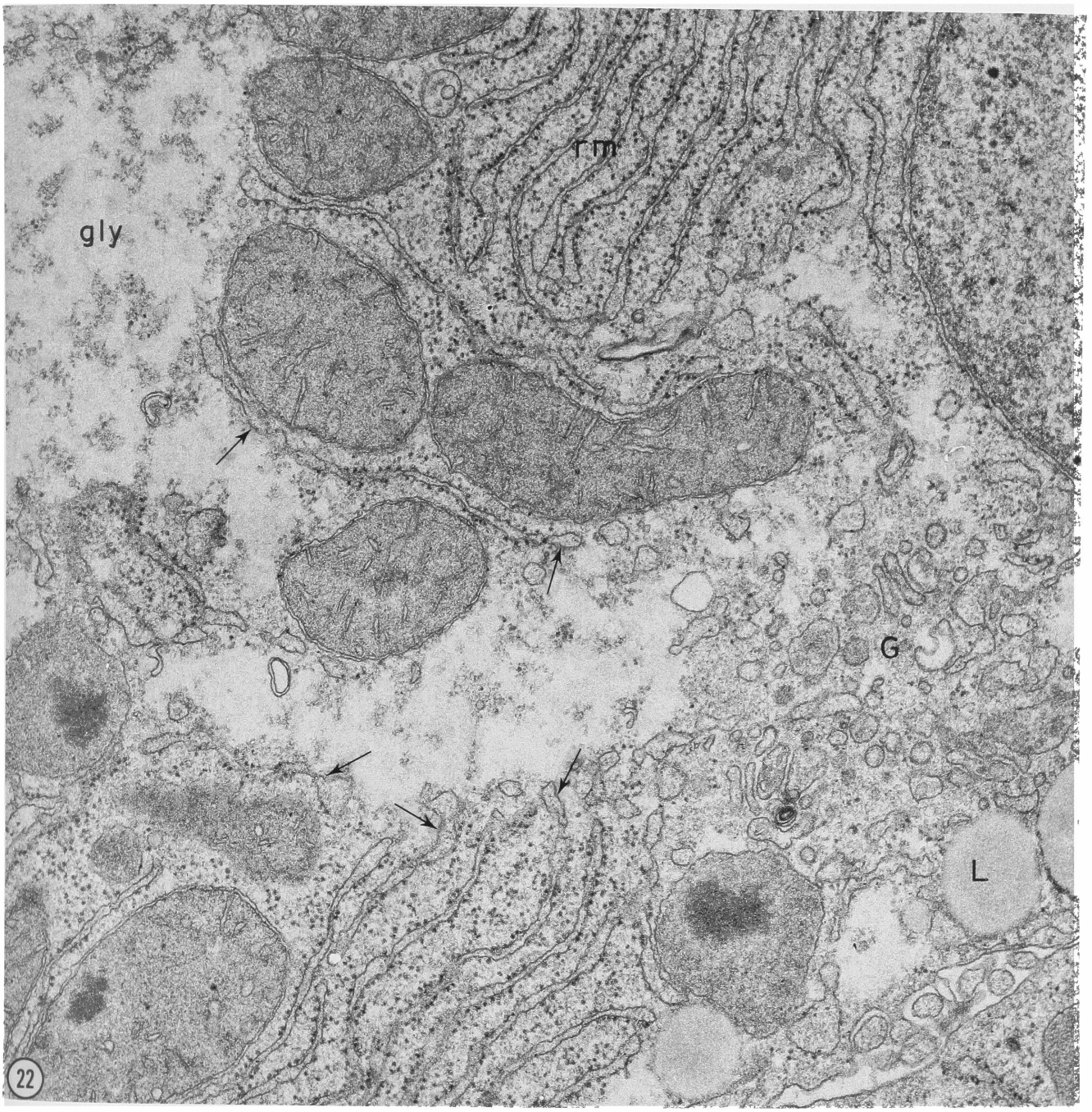

gly
rm
G
L
22

Plate 23. Hepatocyte 24 hours after birth. This field shows a somewhat later stage in the formation of the smooth ER. Rough ER encircles portions of the glycogen (gly) deposits and appears to divide the glycogen regions into smaller units. Areas along the length of the rough ER surrounding the glycogen deposits appear free of ribosomes; smooth-surfaced extensions of it penetrate the edges of the glycogen masses (arrows). At this age there are even fewer attached ribosomes than at 6 hours after birth. At the upper left is a small region in which the development of the smooth ER has proceeded farther, producing inter-connected tubules of smooth ER (sm). This tissue was not incubated for histochemisty.

Magnification: x 23,000.

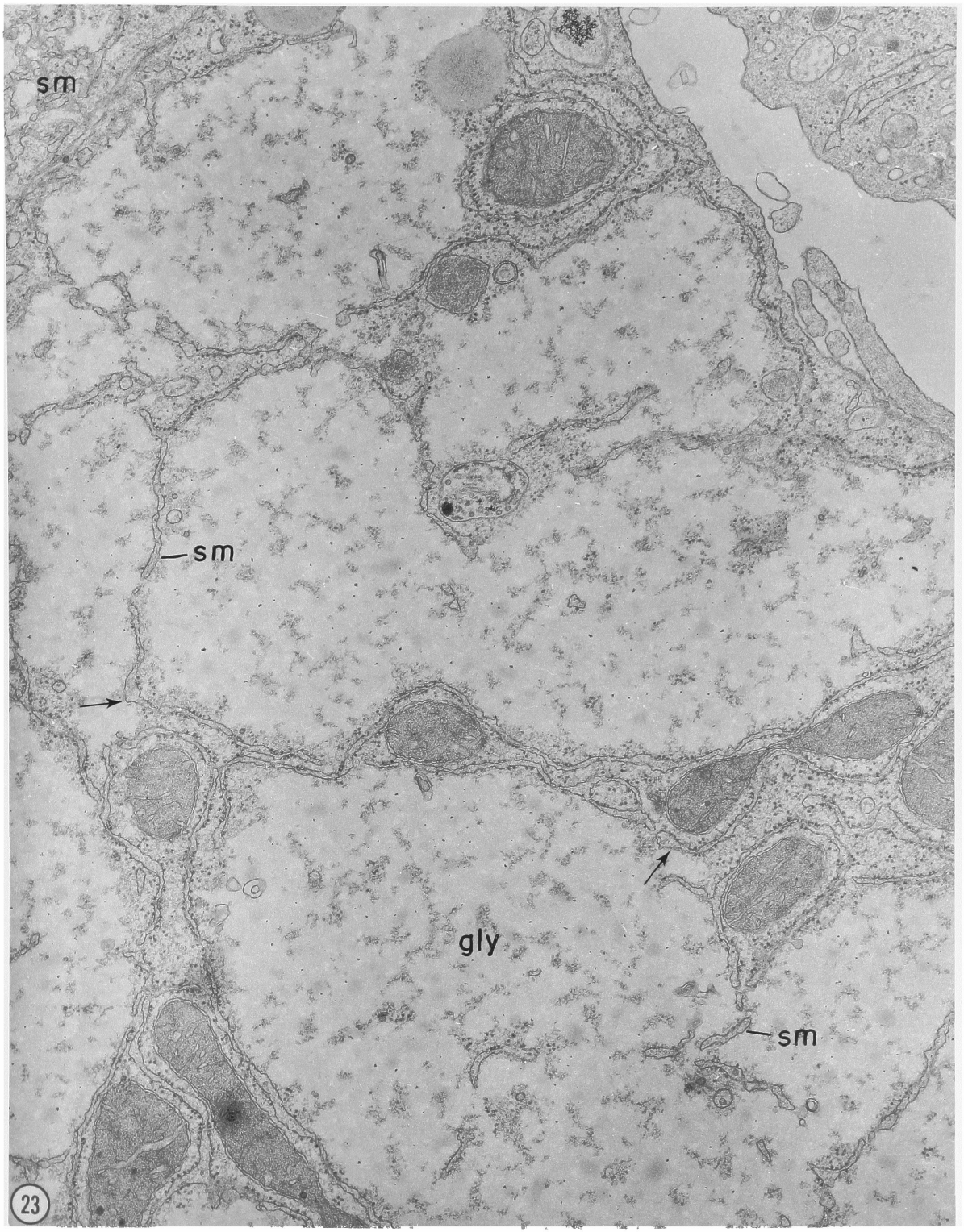

sm
sm
gly
sm
23

Plate 24. Histochemistry in hepatocyte 6 hours after birth. In addition to illustrating the histochemical reaction, this picture shows an area similar to that seen in Plate 23. The ER has completely surrounded regions of glycogen (gly), and membranous profiles are seen penetrating the glycogen regions. Lead phosphate deposit is present along the length of the rough ER cisternae (rm), in the ER surrounding the glycogen deposits and in the membranes within the glycogen regions themselves (arrows). At the lower left is a small area which contains reacted membranes arranged in a tubular network (sm). Comparison with Plate 25 suggests that this is probably newly formed smooth ER. All of these membranes contain reaction product. The edge of the slice is to the left. The tissue was incubated for 30 minutes.

Magnification: x 17,000.

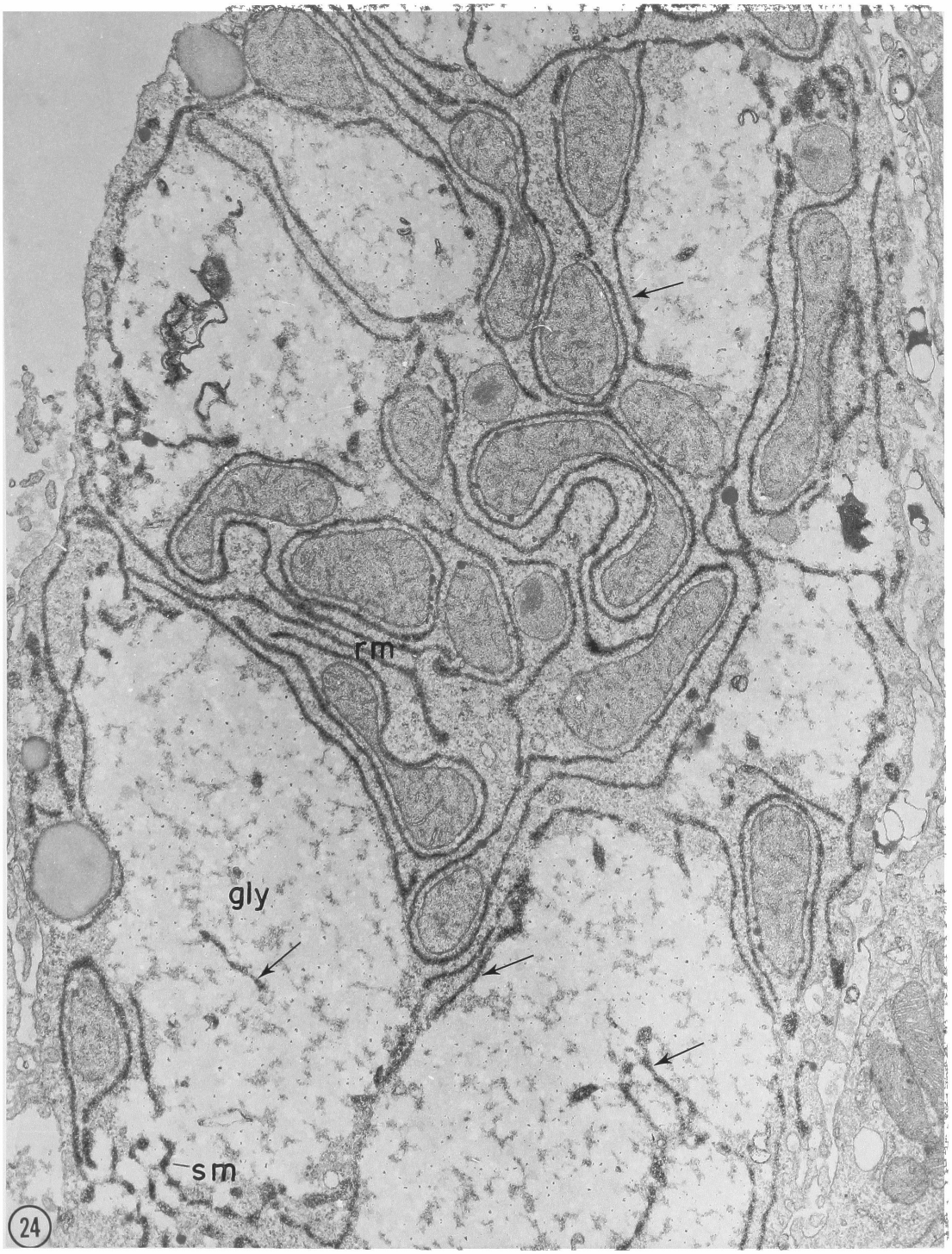

rm
gly
sm
24

Plate 25. Hepatocyte 6 hours after birth. In this cell the smooth ER development is farther advanced than in Plate 23 even though the cell is from a younger animal. Smooth-surfaced ends (arrows) of rough-surfaced cisternae (rm) are seen. In addition, a tubular network of smooth ER (sm) has already developed along the edge of the glycogen region (gly). Two large lipid droplets (L) are present in the glycogen region. The tissue was not incubated in the histochemical medium.

pm - plasma membrane

Magnification: x 35,000.

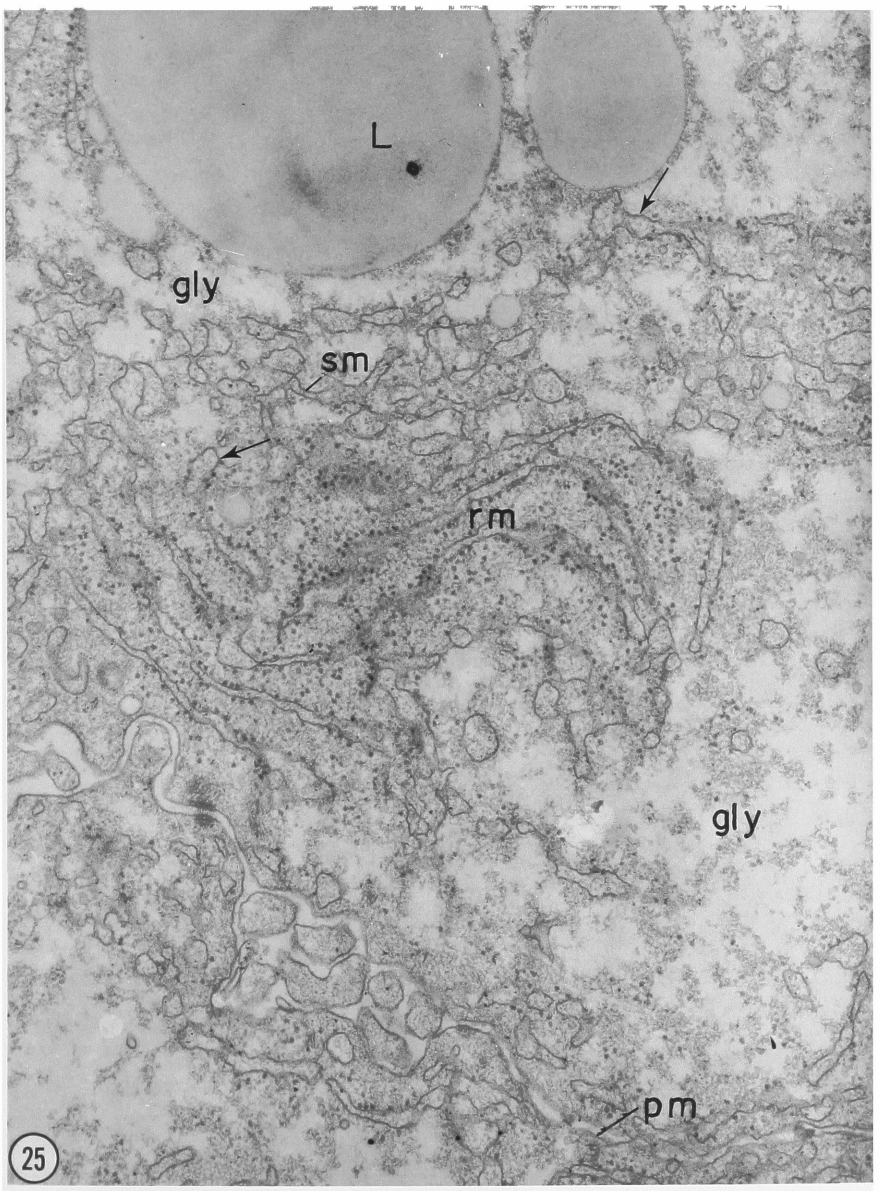
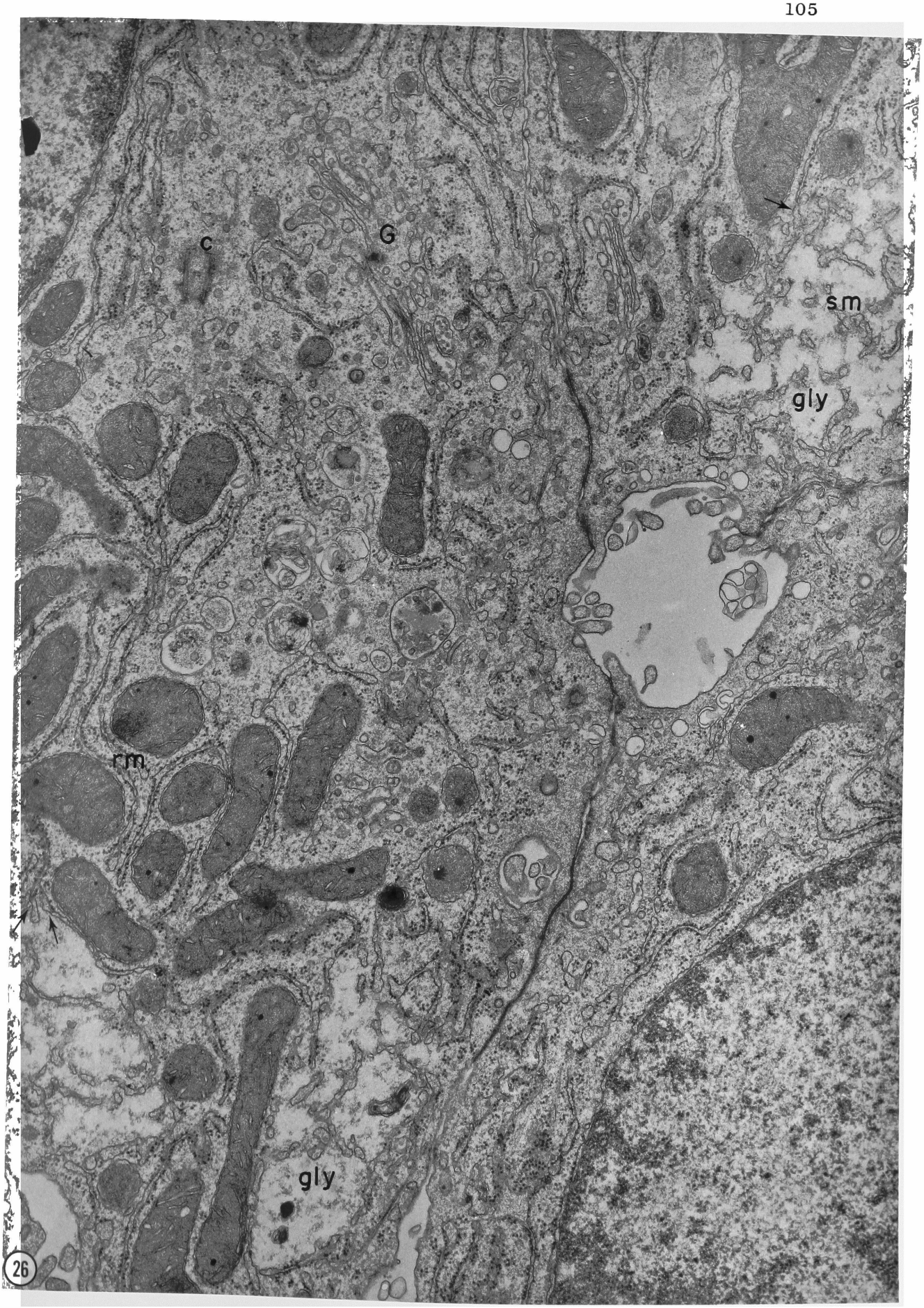

L
gly
sm
rm
gly
pm
25

Plate 26. Hepatocytes 24 hours after birth. The formation of smooth ER in these cells is further advanced than in Plate 24. The glycogen regions (gly) are small and are completely permeated with smooth ER (sm) profiles. The network of smooth-surfaced tubules visible in the glycogen region at the upper right is reminiscent of that seen in adult liver (cf. Plate 4) except that the membranes penetrate into the center of the glycogen deposits. Arrows indicate points of continuity between the rough and smooth ER. Along the length of the rough ER cisternae (rm) there are regions which are free of attached ribosomes. The Golgi complex is large (G) and a centriole (c) is apparent near the nucleus at the upper left.

Magnification: x 14,000.


c
G
sm
gly
rm
gly
26

Histochemistry: 6 hours after birth. By 6 hours after birth the histochemical deposit is very heavy and continuous within the ER cisternae (Plate 27 incubated for 30 minutes). It is still specifically localized to the ER and nuclear envelope and is well distributed throughout all of the ER of the cell. Where mitochondria are ringed by ER the dense deposits are clearly seen to be present in the ER and absent from the mitochondria. The ER is outlined in black against a paler cytoplasm. All of the membranes encircling the glycogen areas are filled with deposit (Plate 24) and the ER elements penetrating the glycogen regions also contain reaction product. By comparison with unincubated tissue (Plate 23) these membranes are identified as being mostly smooth ER. Since the glycogen appears light, the dense deposits in the membranes are easily distinguished.

Histochemistry: 24 hours after birth. At this stage G6Pase activity is at its highest, 2 1/2 times that of the adult. Plate 28 shows cells incubated for 30 minutes in the histochemical medium. The edge of the block is towards the upper left and the deposit begins to be spotty towards the right. Several stages in the proliferation of the smooth ER are seen in this picture. The topmost glycogen area is circumscribed by ER. Right below is an area already penetrated by smooth ER membranes, while the glycogen region in the lower cell is completely permeated with smooth ER. The heavy deposit in the ER surrounding the glycogen areas and the dense deposit in the smooth ER as it pervades the region are illustrated in the upper two glycogen regions (compare with Plate 23 and Plate 26 for localization of the two types of ER). Note that again the elements of the Golgi complex and the plasma membrane are negative. The faint background deposit caused by the non-specific binding of the lead is seen in the lipid droplets and in the Golgi cisternae. Plate 29 shows a higher power view of the deposit in the areas of rough and smooth ER. One region of glycogen surrounded by rough ER is seen and so is the edge of another. The deposit inside the membranes is heavy. An area of proliferated smooth ER also contains heavy deposit.

Plate 27. G6Pase histochemistry in a cell 6 hours after birth. The dense and continuous reaction product is clearly visible in all of the rough ER elements (rm) and in the nuclear envelope (nm). No deposit is seen along the plasma membrane (pm) or over the nucleus (N) or mitochondria (m). Several mitochondria appear encircled by ER elements. The glycogen region (gly) at the left is free of ER profiles. The tissue was incubated for 30 minutes.

Magnification: x 20,000.

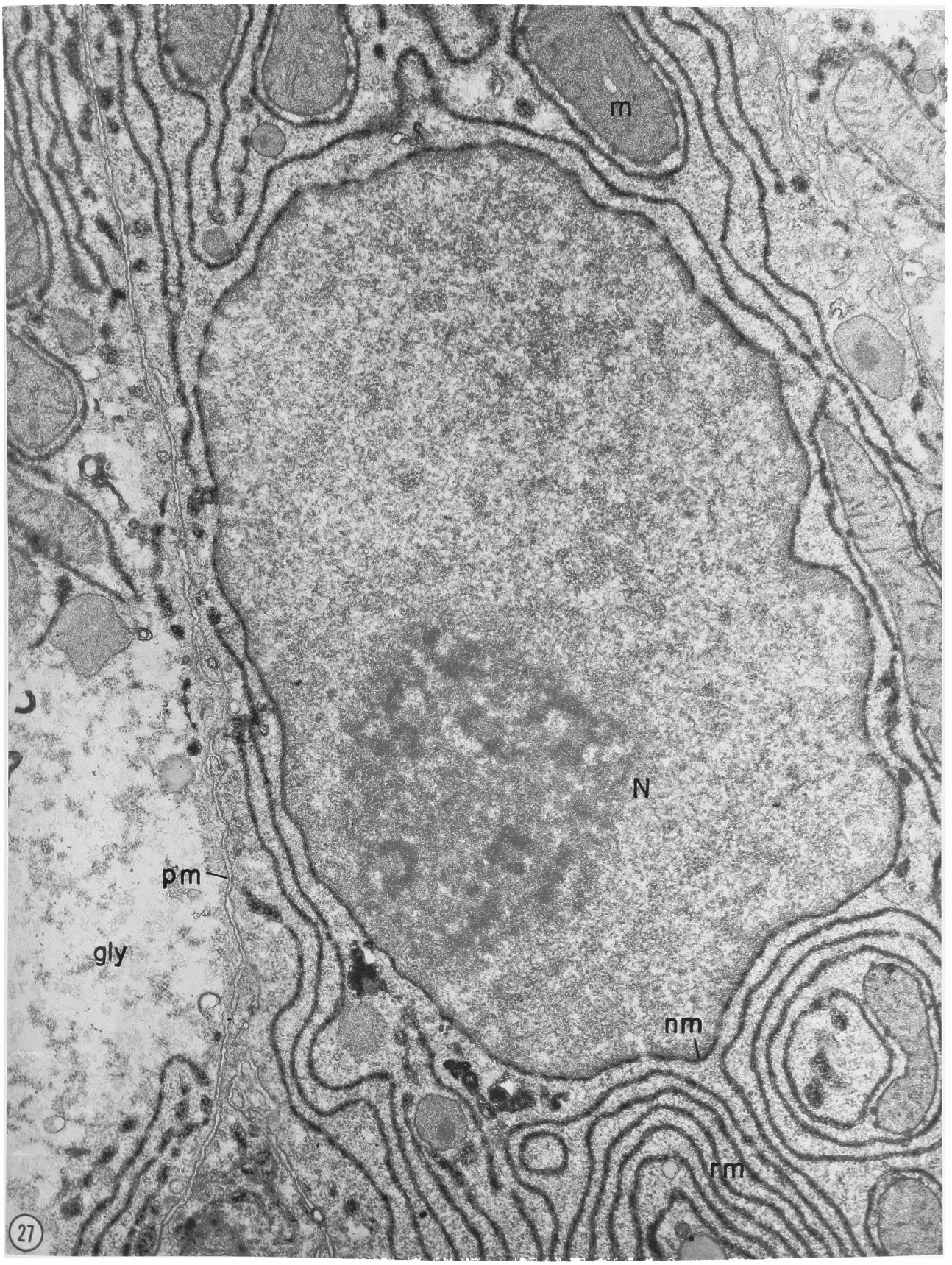

m
N
pm
gly
nm
rm
27

Plate 28. G6Pase histochemistry in hepatocytes 24 hours after birth. This field shows 3 different stages in the formation of the smooth ER. The glycogen region (gly) at the top left is surrounded by ER profiles but does not contain many membranes within it. The glycogen region just below is also surrounded by ER, but in addition, is penetrated by numerous ER profiles. The region of glycogen at the lower right is no longer ringed by the ER but the entire region is permeated with smooth ER (sm) arranged in the typical tubular network. The edge of the tissue slice is towards the upper left and the histochemical reaction is less intense and consistent towards the right of the picture. In the top two glycogen regions all of the ER profiles contain deposit, including those within the glycogen regions (arrows). In the glycogen area in the lower right, which is further into the slice, the reaction is more spotty. The nuclear envelope (nm) contains reaction product although the elements of the Golgi complex (G) and the plasma membrane (pm) do not. Light background contamination can be seen in the lipid droplet (L) and in the Golgi cisternae. The tissue was incubated for 30 minutes.

Magnification: x 18,000.

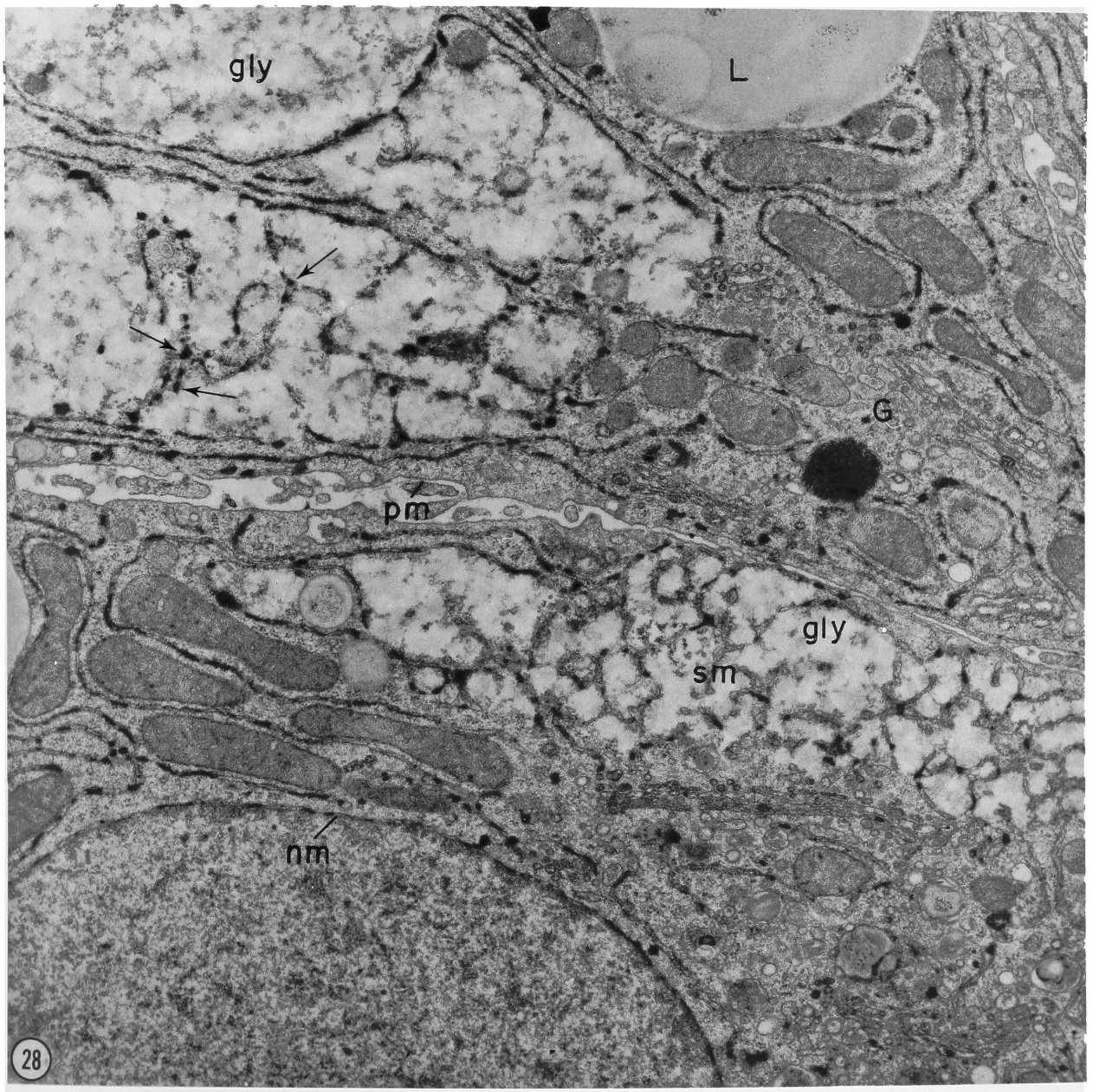
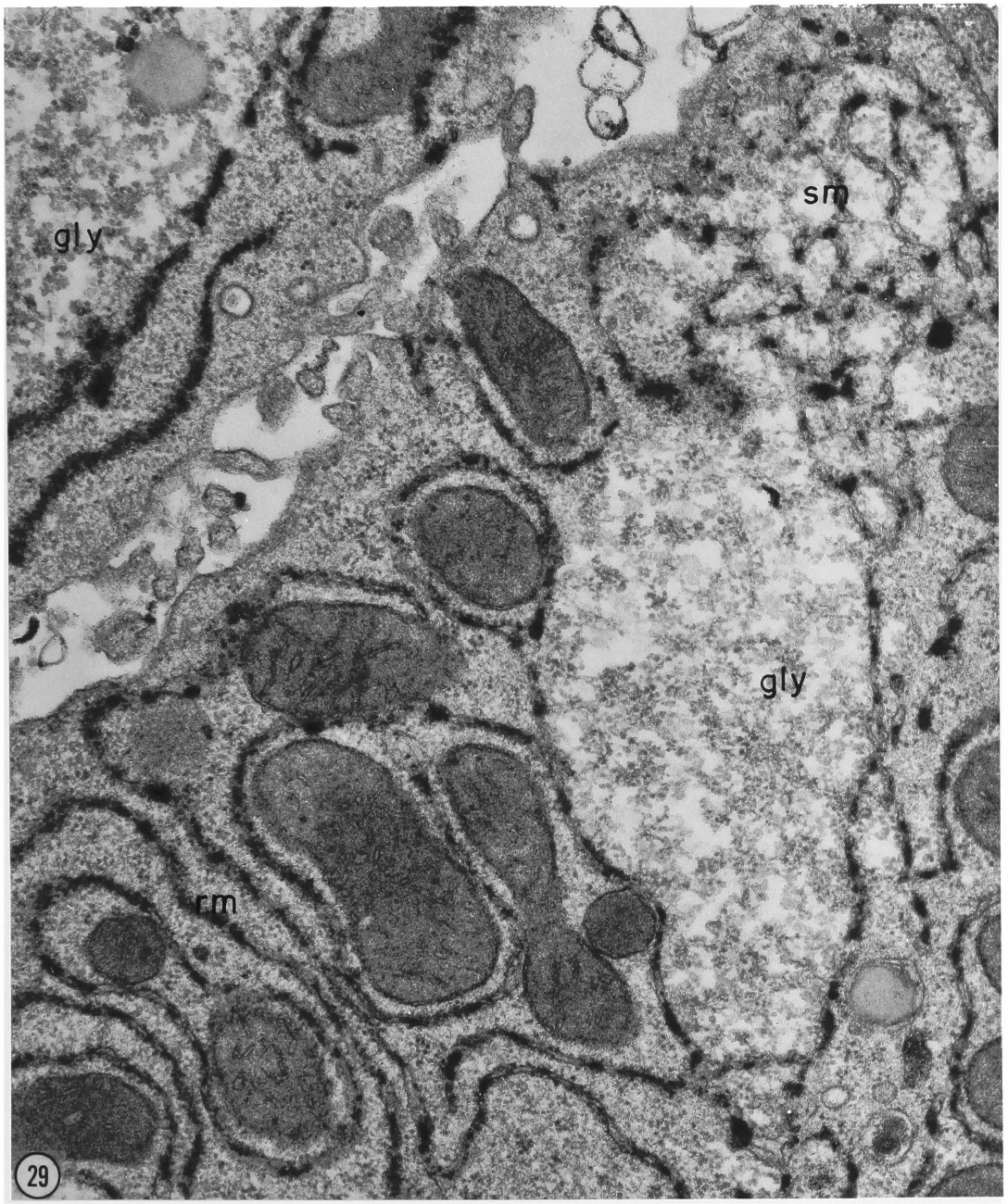

gly
L
G
pm
gly
sm
nm
28

Plate 29. Higher power view of G6Pase histochemistry in a hepatocyte 24 hours after birth. Two regions of glycogen (gly) ringed by ER are seen. In addition, in the upper right, is an area which has been completely permeated with smooth ER (sm). The reaction product is heavily deposited in the rough ER cisternae (rm), in the ER surrounding the glycogen areas, and in the newly formed smooth ER. The tissue was incubated for 30 minutes.

Magnification: x 33,000.


gly
sm
gly
rm
29

Controls

In order to substantiate further the assumption that the activity localized histochemically is actually G6Pase, a number of controls were conducted at all stages. Omission of substrate, prior heating of the tissue for 5 minutes at 80^{o}, substitution of β-glycerophosphate for G6P all produced little or no observable deposit. Plate 30 is a picture of a cell at birth incubated without substrate. No deposit is visible in any of the ER membranes. This indicates that there is very little endogenous substrate in the tissue. The light, punctate, non-specific deposit although present, is not very obvious in this photograph. The electron dense content of the ER cisternae typical of this stage of development is visible, although the Golgi cisternae have a lighter content. The ER membranes are heavily covered with ribosomes which are more visible than in material containing lead deposits but are not as well preserved as in unincubated tissue.

A cell 6 hours after birth, incubated with β-glycerophosphate as substrate instead of G6P, is seen in Plate 31. There is no deposit in any of the ER cisternae. These histochemical findings agree with the biochemical results using the same incubation conditions which showed that no inorganic phosphate was released. Inclusion of oleate during incubation produced variable histochemical results. In some experiments inhibition of activity was complete. In others on ly very slight deposit was visible while at times the oleate itself precipitated non-specifically in the tissue. Inclusion of parahydroximercuribenzoate (PMB) during the incubation reduced the density of deposit while pre-incubation of the tissue with PMB almost completely eliminated the reaction.

Since the histochemical findings parallel the biochemical results with the same inhibitors, they help to confirm that the deposit seen histochemically is indeed caused by the activity of G6Pase.

Plate 30. Hepatocyte at birth incubated for 30 minutes without substrate. This field is from the edge of a slice. There is no lead deposit in any of the rough ER cisternae (rm) or in the nuclear envelope. The electron dense cisternal content typical of cells at this stage is visible in the rough ER, although the content of the Golgi cisternae (G) is of lower electron opacity. The ribosomes are distinguishable, and densely cover the rough ER. The complete lack of lead phosphate deposit is typical of tissue incubated without substrate at all stages of development.

Magnification: x 37,000.

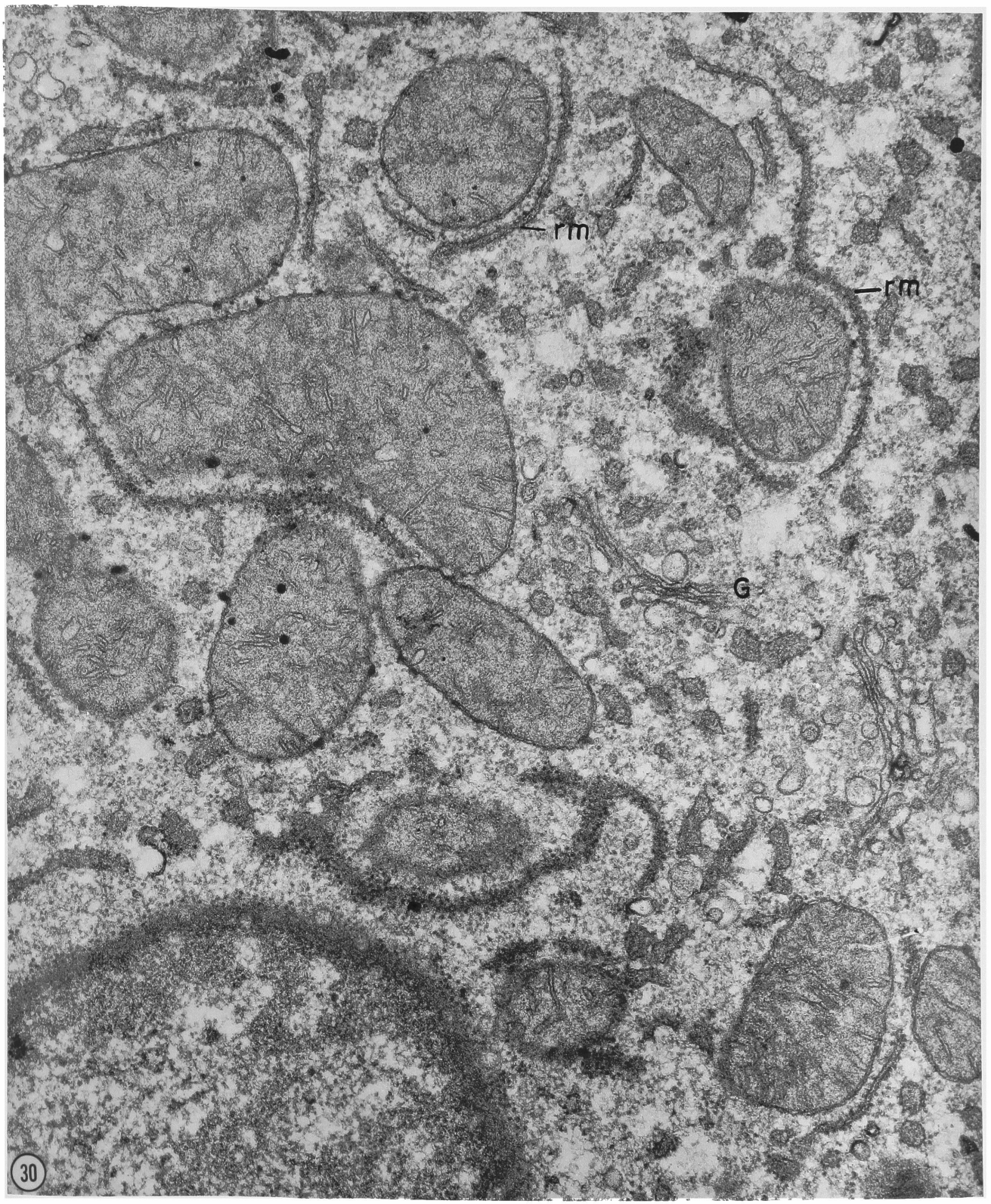
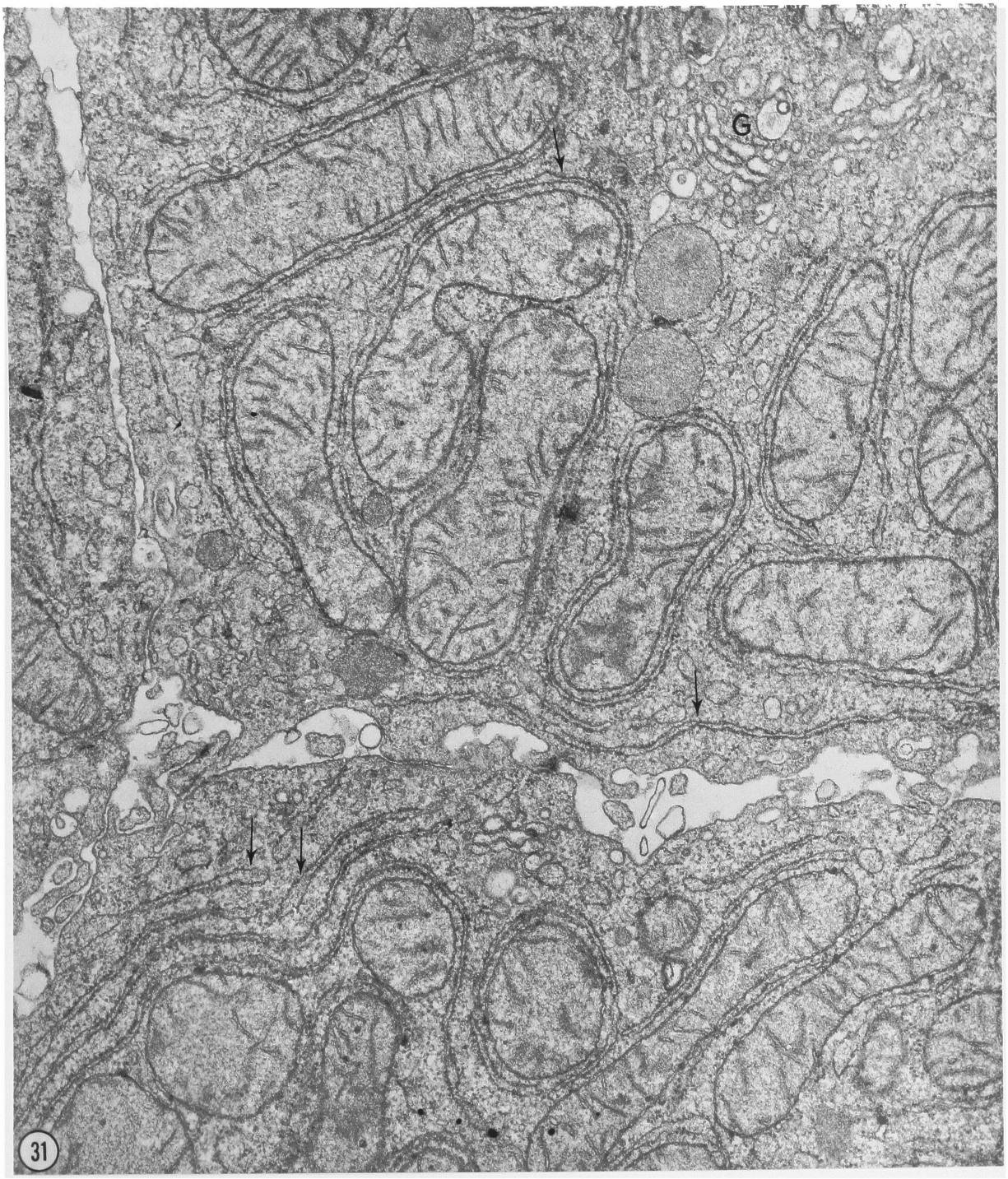

rm
rm
G
30

Plate 31. Hepatocyte 6 hours after birth incubated with β-glycerophosphate instead of G6P. After a 30-minute incubation there is no deposit in any of the rough ER cisternae (arrows). This cell is near the edge of the slice. At no stage in development is β-glycerophosphate utilized as a histochemical substrate.

G - Golgi complex

Magnification: x 23,000.


G
31

Summary Part III

During development, as the biochemical activity of G6Pase increases, the lead phosphate deposit seen in histochemically reacted cells becomes heavier. At all stages, it is localized exclusively in the cisternal space of the ER and the nuclear envelope. No deposit is seen in the elements of the Golgi complex, along the plasma membrane or over other cell organelles. The results of several histochemical controls parallel those found biochemically with the same incubation conditions and help to substantiate that the histochemical deposits are a result of G6Pase activity.

Further, the histochemical results provide information about two levels of development of the enzyme. First, within the population of hepatocytes, individual cells differ in the time during development when they acquire G6Pase. That is, the hepatocyte population differentiates asynchronously with respect to the enzyme. At four days before birth, the majority of the hepatocytes lack G6Pase activity, while at the time of birth, the vast majority of the cells react positively for the enzyme. The biochemical activity of G6Pase detected early in development is a result of the activity in a small number of cells, while the activity measured at birth is due to a contribution from all of the hepatocytes.

Second, within a given cell, all rough ER membranes acquire enzyme activity synchronously. As soon as the enzyme can be detected in a cell, it appears in all of the rough ER elements present in that cell. When smooth ER is formed during the first 24 hours after birth, it too contains G6Pase activity and no smooth ER lacking the enzyme is seen. At no time during differentiation is G6Pase activity restricted to certain parts of the ER. This indicates that there is no regional differentiation within the ER of a cell with respect to this enzyme.

IV. SUB-FRACTIONATION OF ROUGH MICROSOMES

There remains one possible source of serious error in the interpretation of the results of the histochemical experiments presented in Part III. It has been assumed so far that the distribution of reaction product within the ER of a cell represents the actual distribution of sites of enzyme molecules. In other words, the even and uniform appearance of the lead phosphate deposits reflects a widespread and relatively homogeneous distribution of sites within all of the ER of a cell. However, if released phosphate diffuses rapidly within the cisternae of the ER prior to its precipitation by the lead, then this pattern of lead phosphate deposits could also be found even if the enzyme sites were unevenly distributed within the cell. Two possible ways to help in choosing the correct interpretation are available. Both involve converting the continuous ER cisternae into disconnected units to prevent the possible diffusion from occurring. First, it might be possible to cause the ER within the intact cell to fragment into small, unconnected vesicles. The phosphate released by the activity of G6Pase could not diffuse from one vesicle to another and thus the distribution of reaction product in the population of vesicles would reflect the distribution of enzyme sites. Second, isolated microsomes, formed from the ER by homogenization, could be reacted. Again diffusion of phosphate would be precluded. In either case, only those vesicles which contained enzymes sites would acquire lead phosphate deposit after reaction. If only some of the vesicles contain reaction product, the apparently widespread pattern of deposit seen in the intact cells would be a result of diffusion of phosphate from widely-spaced enzyme sites. However, if virtually all of the vesicles react, the interpretation that the histochemical findings result from a uniform distribution of closely-spaced enzyme sites would be confirmed.

Two methods of forming unconnected vesicles within the intact cell were tried: mechanical damage by slicing unfixed liver tissue, and osmotic damage by perfusing the liver with hypotonic medium prior to fixation. In both cases damage to the tissue occurred and although the ER did fragment, the preservation of the tissue was poor and the histochemical results were unsatisfactory.

The second approach, homogenization, was more successful. Since the histochemistry on the tissue slices showed that the lead deposit was found within the cisternae of the ER and not on the outside of the membrane, it was thought that if microsomes were reacted in the histochemical medium, the lead phosphate deposit would be found within the vesicles. Preliminary experiments with total microsomes from liver (and also the work of El-Aaser 1967) indicated that this was indeed the case; the lead deposits were seen within the microsomal vesicles. This observation led to the formulation of a method to separate those vesicles containing enzyme and lead phosphate after incubation, from those vesicles lacking the enzyme. The fractionation scheme designed is shown in Figure 5. The microsomes formed from fragmentation of the ER are reacted in the histochemical medium. Those vesicles containing at least one site of G6Pase activity should acquire lead deposit after reaction while those vesicles lacking enzyme should not. The lead phosphate precipitate increases the density of the G6Pase-containing microsomes and makes possible their separation from the G6Pase-free vesicles by isopynic centrifugation in a 2-step density gradient.

The unreacted vesicles should float above the layer of 2.0 M sucrose, as do normal rough microsomes; however, vesicles containing precipitate should sediment through the 2.0 M sucrose, due to their increased density, and form a pellet in the bottom of the tube. By this means it is possible to determine the proportion of vesicles which react and thus to distinguish between the two alternative explanations for the pattern of lead phosphate deposit seen histochemically.

Isolation of Rough Microsomes

Since at the time during development in which I was interested the cells contain very little smooth ER, the first step in the procedure consisted in obtaining a fraction of rough microsomes. This eliminates any confusion due to the presence of smooth-surfaced vesicles derived from plasma membrane or from elements of the Golgi complex, none of which should react as judged from the histochemistry.

FRACTIONATION SCHEME FOR ROUGH MICROSOMES BASED ON GLUCOSE-6-PHOSPHATASE ACTIVITY

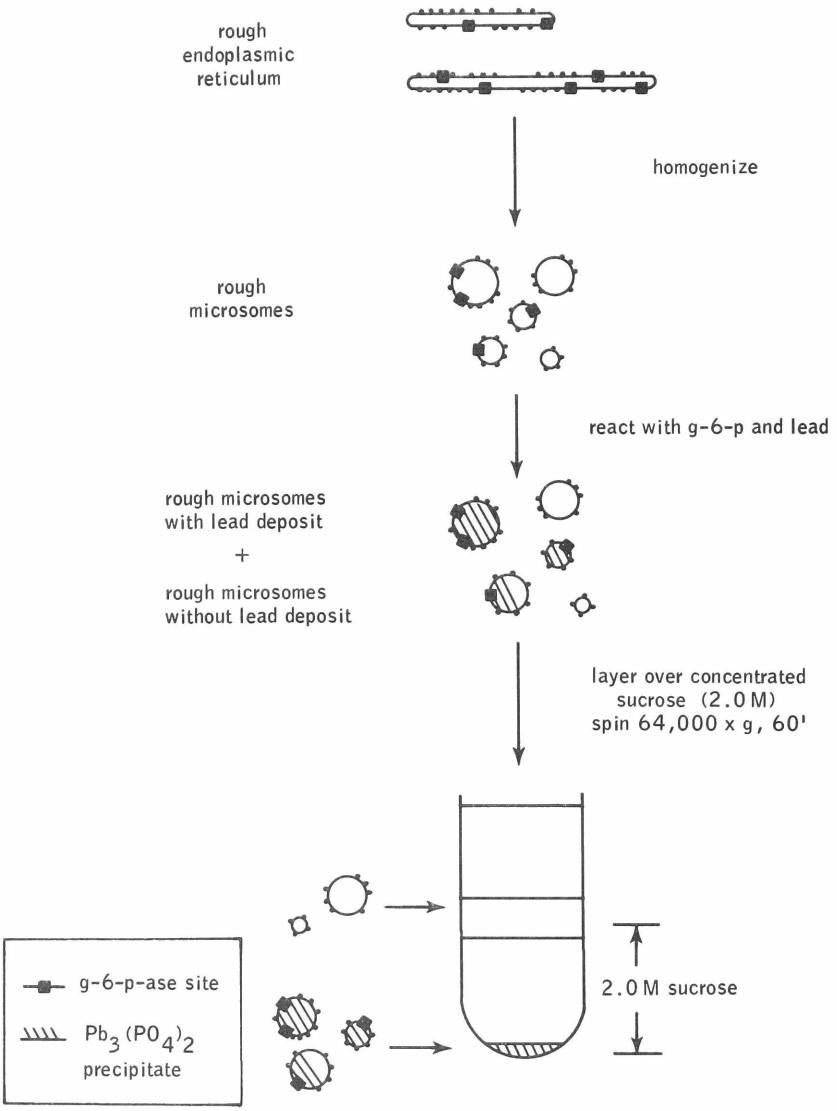


Figure 5. Sub-fractionation scheme for rough microsomes based on G6Pase activity. Normal rough microsomes float above 2.0 M sucrose while microsomes containing lead deposit sediment through the dense sucrose, and pellet.

Procedure

The procedure used for the separation was a modification of that of Rothschild (1963). The changes employed included:

1. the use of 0.88 M sucrose as the suspending medium for the homogenate,

2. the addition of a bottom cushion of 2.0 M sucrose, and

3. the use of the larger Spinco S 30 rotor in order to process larger volumes.

The 0.88 M sucrose allowed a better separation of the rough and smooth microsomes than did the 0.25 M sucrose. The 2.0 M sucrose cushion is denser than the rough microsomes and was used to prevent the rough microsomes from pelleting during the spin. Thus, any aggregation caused by the pelleting could be avoided. Such aggregation could complicate the final subfractionation of the rough microsomes. The rough-smooth separation method of Dallner (1963), which employs cesium ions to speed up the separation, was not used. The cesium binds to the rough microsomes and increases their density, and since a density difference was to be used to subfractionate the rough microsomes, this would confuse the interpretation of the final results.

In the lower tracing in Figure 6 is reproduced the absorbance at 550 mμ of the step gradient in which rough and smooth microsomes were separated. The peak at the lower interface marks the position of the rough microsomes, and the double peak at the higher interface is composed of smooth microsomes, which collect at the interface, and free hemoglobin which remains in the load zone.

The upper tracing shows the pattern from another experiment, in which the gradient was tapped by hand; one milliliter fractions were collected. The peaks of absorbance at 260 mμ or 280 mμ mark the position of the rough and smooth microsome fractions. The rough fraction has both a higher absorbance and a higher 260/280 ratio, due to the presence of ribosomes containing RNA. The hemoglobin is not seen as a separate peak by the ultraviolet absorbance measurement.

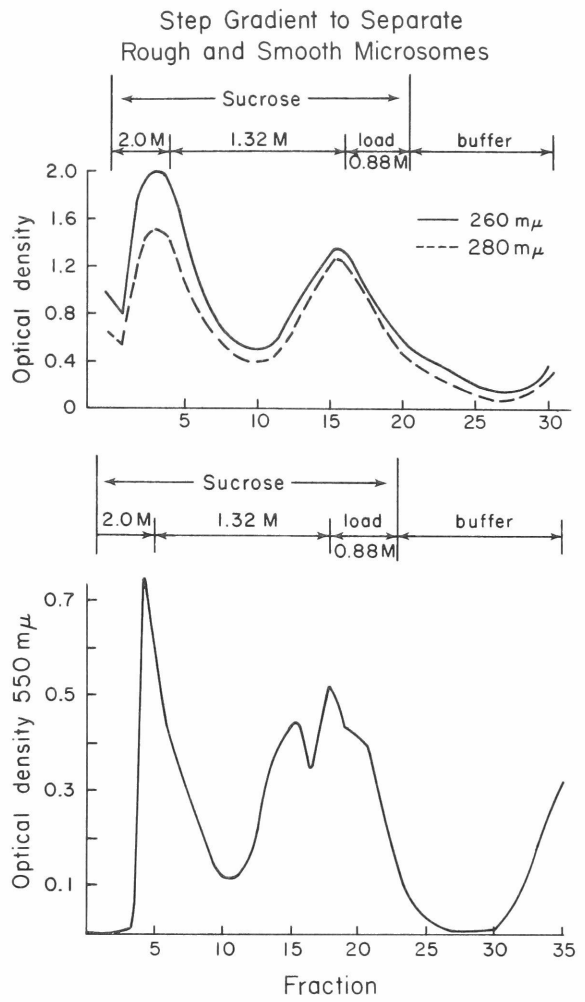


Figure 6. Optical density tracings of step-gradient to separate rough and smooth microsomes.

Top figure: Gradient tapped by hand, 1 ml fractions collected. Peak at interface between 2.0 M and 1.32 M sucrose represents rough microsomes, and peak between 1.32 M sucrose and load zone is smooth microsomes. In this experiment the post-mitochondrial supernatant was not treated with amylase and a pellet of glycogen was found.

Bottom figure: Gradient tapped on fractionator. Peak between 2.0 M and 1.32 M sucrose again represents rough microsomes. The lower part of the double peak at the interface between 1.32 M sucrose and the load zone is smooth microsomes, whereas the upper part is probably hemoglobin. In this experiment the post-mitochondrial supernatant was treated with amylase and no pellet was visible.

Morphology

The morphological purity of the rough microsome fraction is shown in Plate 32. The microsomes were prepared from an animal approximately 2 hours after birth. The majority of the vesicles are intact and are covered with ribosomes on the outer surface. Contaminants include some pieces of plasma membrane, lysosomes, mitochondria and free ribosomes.

Biochemistry of Rough Microsomes

A number of biochemical parameters were tested to ensure that the fraction was composed of typical rough microsomes. These include protein, RNA and phospholipid content, and G6Pase activity. Table 7 compares this fraction with a rough microsome fraction obtained by Dallner (1963). In general the correspondence is good. The RNA/protein rtaio is slightly lower than that found by Dallner. This may be due to the difference in methods used to assay RNA. The rough microsome fraction as isolated contains 50% of the total RNA in the homogenate.[1] The distribution of G6Pase activity in the fractions also suggests that approximately 70% of the rough microsomes are recovered in the final fraction.[2] Since the

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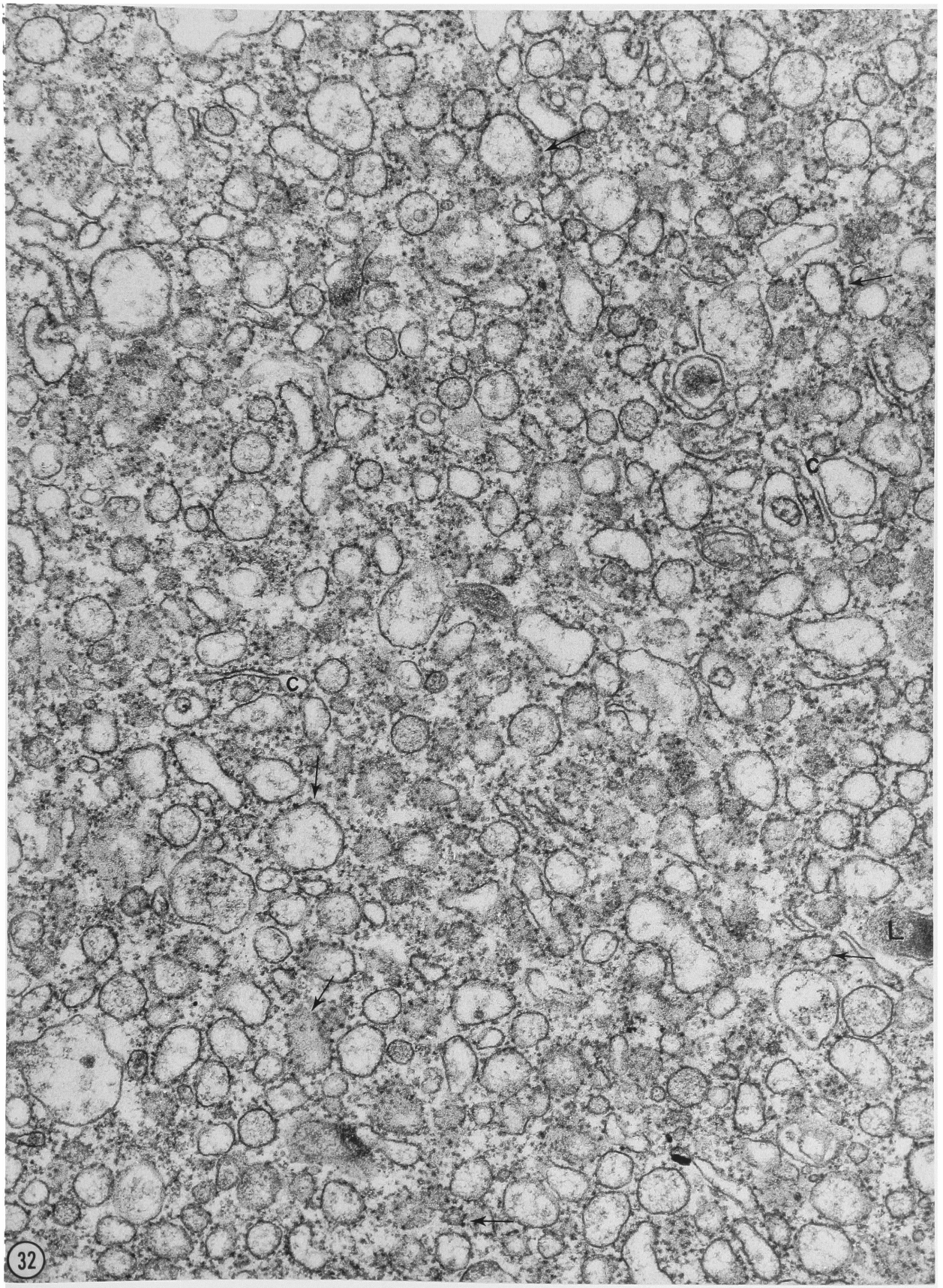
[1] If 15% of the total RNA in the homogenate is unsedimentable, and 5% is nuclear RNA, then a maximum of 80% of the total RNA should be in the post-mitochondrial supernatant. This consists of RNA in both the bound and free ribosomes. Approximately 25% of the ribosomes (20% of total cell RNA) are free (unattached to membranes, Blobel and Potter 1967). If only half of these pellet in the step gradient during the 12-13 hour spin then 70% of the total RNA of the whole homogenate should be found in the rough microsome fraction. The recovery of 50% is then 70% of the maximum calculated value.

[2] The post-mitochondrial supernatant contains approximately 75% of the total G6Pase activity, while the mitochondrial pellet retains 25%. The activity in the mitochondrial pellet is contributed by smooth microsomes sedimenting with the glycogen and rough microsomes which begin to sediment under the centrifugation conditions used. If the contribution from rough and smooth are approximately equal, then about 25% of the rough microsomes are lost to the mitochondrial pellet. This leaves a maximum recovery of 75% of the rough microsomes in the final rough fraction. 90% of the G6Pase activity in the post-mitochondrial supernatant is recovered in the combined rough and smooth microsome fractions; the activity is sometimes evenly distributed between the two fractions although sometimes more is found with the rough microsomes. Thus, approximately 67% of the total rough microsomes are found in the final fraction.

Plate 32. Micrograph of the middle of the pellet showing the composition of the rough microsome fraction prepared from the liver of an animal 1-2 hours old. In this case the post-mitochondrial supernatant was not treated with amylase; the field is similar to microsome fractions obtained after amylase treatment. The pellet consists mostly of intact rough-surfaced vesicles including some flattened cisternal elements (c). A few of the vesicles have moderately electron-dense content. Occasional contaminants of the fraction include mitochondria, plasma membrane fragments and lysosomes (L). Arrows mark areas where ribosomes are clearly visible on the outer surface of the vesicles.

Magnification: x 40,000.


C
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32

TABLE 7

CHEMISTRY OF ROUGH MICROSOME FRACTION FROM ADULT LIVER

Determination	Leskes*	Dallner**
1. G6Pase (μmoles Pi/mg protein/20')	3.44 3.26 3.91	4.03
2. Percent of total G6Pase activity in rough microsomes	41% 34% 51%	---
3. G6Pase (μmoles Pi/20'/gm liver)	86 75	52
4. mg protein/gm liver	25 23	13 19
5. mg RNA/gm liver	3.6 4.3	4.2
6. mg RNA/mg protein	.15 .12	.33
7. Percent of total RNA in rough microsomes	44% 59%	---
8. mg phospholipid/gm liver	4.0	2.2
9. mg phospholipid/mg protein	.20	.16

* Duplicate and triplicate determinations are from different experiments.

** Dallner (1963).

smooth microsome fraction as isolated from the gradient contains a large amount of free hemoglobin, it is not meaningful to compare rough and smooth fractions in terms of specific activities on a protein basis. With regard to total recovery, however, 2-13% of the total RNA of the homogenate is recovered in the smooth fraction compared to 50% in the rough fraction.

Comparisons between the isolated rough fraction and the unpurified homogenate show an increase in G6Pase specific activity of 1.9 to 2.8 fold. The average value for the homogenate was 1.72 μmoles Pi/mg protein/ 20 minutes and that for the rough fraction was 3.73 (average of 4 experiments). The RNA to protein ratio in the rough fraction also increased 1.8 to 3.7 times over that in the homogenate. The rough fraction then, appears to be a typical rough microsome fraction and contains an acceptable proportion of the total rough ER present in the cell.

Reaction and Sub-fractionation of Rough Microsomes

Reaction

The rough microsomes were incubated for 60 minutes in the histochemical medium. As Figure 7 shows, this allows the enzyme reaction to reach maximal rate and then level off. Thus, any vesicle containing enzyme would contain as much lead phosphate as possible. During the incubation the microsomes aggregate. The aggregation is probably due to the presence of excess lead in the incubation medium. Lead, a bivalent cation, may form ionic bonds between negatively-charged groups on two vesicles and thus bind the vesicles together. In addition, microsomes incubated with lead alone become more dense and sediment through the 2.0 M sucrose, which should float unreacted rough microsomes. This increase in density is probably also due to binding of lead ions by the membranes.[1] Thus it was necessary to remove the excess lead in order to reverse the aggregation of the reacted membranes, and to separate reacted from unreacted membranes on the basis of the difference in density due to lead phosphate deposits within the reacted vesicles.

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[1] The non-specific binding of the lead to the membranes may be what produced contrast in the incubated tissue sections which had not been stained (Plate 6).

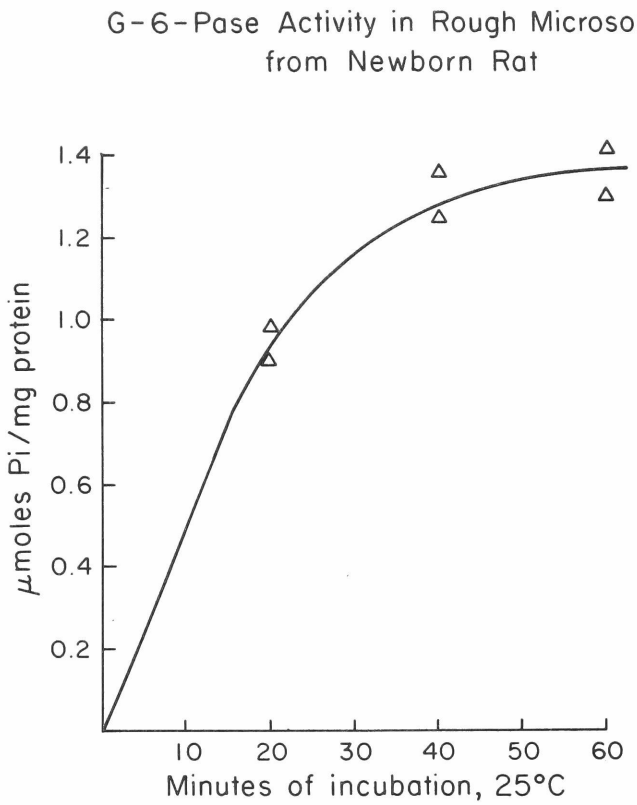


Figure 7. G6Pase activity in rough microsomes from newborn rat. Assay conditions, 1 mM G6P, 2 mM lead nitrate, 50 mM cacodylate buffer, pH 6.6, are same as those used for histochemistry.

Dialysis

In order to remove the free lead ions, the reacted microsomes were dialyzed against 100 μM EDTA. This concentration was more efficient than 40 μ M in reversing the aggregation, but no further difference was observed by increasing the concentration of EDTA to 1000 μM. Two changes of dialysis medium were necessary, since aggregation of the membranes was still visible after the first change. It was also important that the bags spin freely during the dialysis, to prevent the aggregated vesicles from settling to the bottom of the bags. Gentle sonication of the entire dialysis bag further helped reverse the aggregation, possibly by enabling the EDTA to better penetrate the aggregates. Several different pH's and buffer concentrations were tried. Dialysis against water instead of buffer resulted in vesicle aggregation probably due to a lowering of the pH. A pH of 7.4 was more effective than pH 6.6, possibly because at the higher pH the membranes are more highly charged and tend to repel each other; 10 mM cacodylate buffer was more effective than 50 mM. Inclusion of highly charged molecules or high molecular weight polymers such as dextran were not particularly helpful in preventing the aggregation and in addition, some of them interfered with the final assays of phospholipid and protein. Inclusion of high concentrations of salt (0.5 M potassium acetate) in the incubation medium prevented aggregation of the microsomes and did not inhibit the enzyme reaction. However, when this concentration of salt was included during dialysis even the reacted vesicles floated at the interface. Thus, the salt either interfered with the lead phosphate precipitation or caused solubilization of some of the precipitate. When glycogen was present in the rough microsome fraction it tended to bind vesicles together and so the original post-mitochondrial supernatant was treated first with amylase to digest the glycogen.

Test of Aggregation

The final procedure used, which is described in "Materials and Methods", was successful in reversing the aggregation. The degree of aggregation was measured in the following way. Aliquots of rough microsomes from newborn rats were incubated in the histochemical medium

in the presence or absence of substrate. The microsomes incubated with lead and substrate should acquire lead phosphate deposit and become dense while the microsomes incubated with lead only should remain less dense. Newborn tissue was chosen since the histochemistry had shown that at this time most of the cells react and the deposit is fairly heavy. The use of adult tissue would have been an alternative, but the aggregation is more of a problem in the microsomes from foetal and newborn tissue.

The two samples of vesicles were allowed to dialyze separately. After overnight dialysis, equal quantities of the two were combined and dialysis was continued for several hours. This combination should produce a population in which half of the vesicles float and half pellet. The mixing was done after overnight dialysis to ensure that the concentration of G6P in the suspension of reacted microsomes was substantially reduced. In this way membranes originally incubated only with lead would have no chance to react with the substrate. Dialysis of the mixture was continued, to allow the two types of membranes to coaggregate, if they were to aggregate at all. When the vesicles did aggregate, all of the membranes sedimented through the dense sucrose into the pellet. When the aggregation was reversed half of the vesicles remained at the interface above 2.0 M sucrose and the others sedimented into the pellet. Figure 8 shows the results of such an experiment in which the reacted microsomes were processed so as to completely reverse the aggregation. This figure shows the distribution of phospholipid and protein in the gradient. In general, phospholipid is a better marker for the membranes since some of the ribosomes break down during the dialysis against EDTA and the released ribosomal protein remains at the top of the gradient. Therefore the proportion of protein recovered in the sedimenting fractions is decreased. In several experiments RNA was assayed and a high proportion of it was also located at the top of the gradient in the load zone.

The first tracing (Figure 8A) shows the pattern obtained when the "light" microsomes, incubated with lead alone, are spun. All of the vesicles collect at the interface; none are in the pellet. After reaction with both lead and substrate (8B), the vast majority of the microsomes sediment into the pellet; only a small amount of material remains at the interface. When the two types of vesicles are mixed (8C) half of

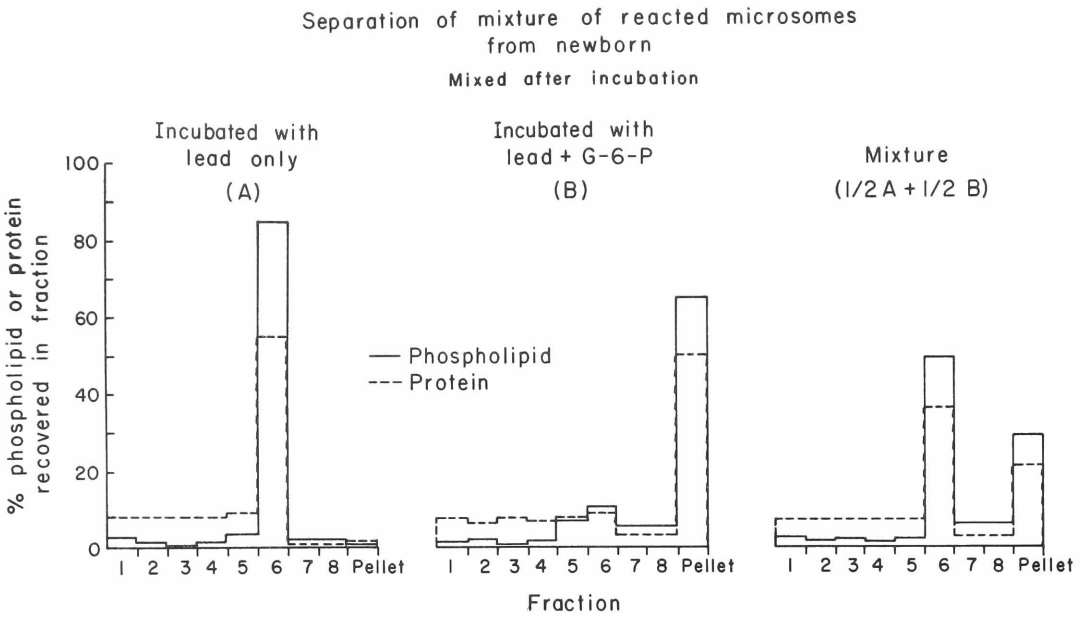


Figure 8. Distribution of phospholipid and protein in gradient separating mixture of reacted microsomes from newborn; mixed after incubation.

An aliquot of rough microsomes was incubated with 1 mM G6P, 2 mM lead nitrate and 50 mM cacodylate buffer, pH 6.6 ("lead + G6P"), while another aliquot was incubated without substrate ("lead only"). The aliquots were dialyzed separately overnight and then equal amounts of the two were combined; dialysis was continued for several hours.

The microsomes were layered over 2.0 M sucrose and were spun at 63,600 x g_{av} for 60 minutes. Four milliliter fractions and the pellet were assayed for phospholipid and protein. The ordinate is the amount of material recovered in the fractions as a percentage of the total recovered on the gradient. Fraction 6 is the interface between the load and the 2.0 M sucrose. All three tracings are from the same experiment. Phospholipid and protein were determined in duplicate on aliquots of fraction 6 and the pellet as well as on aliquots of the microsomal suspension loaded on the gradient.

the membranes float and half are found in the pellet. The results of this experiment and of a similar one using adult microsomes are summarized in Table 8. Comparison between the actual recovery figures and the theoretical values for complete separation of the two types of vesicles (bottom line) indicates a close agreement between the two. Thus the aggregation has been successfully reversed.

Retention of Lead Phosphate

It was possible that loss of lead phosphate deposit from within the reacted vesicles might occur during the sonication and dialysis against EDTA. EDTA binds lead ions very strongly. If free lead ions are produced by the slight dissociation of the lead phosphate, the EDTA could bind to these ions and shift the dissociation equilibrium so that solubilization of the lead phosphate would be increased. To eliminate the possibility that vesicles which float have lost the lead phosphate which they contained after reaction, aliquots of reacted microsomes were tested for the presence of inorganic phosphate before and after dialysis. The tissue was treated with 5% TCA to release the lead phosphate precipitate from the tissue. Table 9 shows that there was no loss of inorganic phosphate during the sonication and dialysis. In fact, in all cases the amount of inorganic phosphate found after dialysis was slightly higher than that present before dialysis. This increase is probably due to the fact that the enzyme reaction is not actually stopped after a 60 minute incubation period. Dialysis at 4^{o} only slowly removes lead and G6P from the microsomal suspension and further reaction probably proceeds slowly for some time.

The finding that there is no loss of lead phosphate is supported by the very small solubility product constant of lead phosphate, $k = 10^{-32}$. There are, therefore, very few free lead ions, formed by the dissociation of the lead phosphate, with which the EDTA could combine. The retention of inorganic phosphate during the procedure confirms the histochemical finding in the intact cell, that all of the phosphate released by the action of G6Pase is released into the cisternae of the ER. It also suggests that the capture efficiency of the released phosphate by the lead is excellent; no inorganic phosphate

TABLE 8

SEPARATION OF MIXTURE OF REACTED ROUGH MICROSOMES

Mixed after Incubation

Incubation Conditions	Percent of Recovered Material			
	at Interface		in Pellet	
	PLP	Protein	PLP	Protein
Birth				
A. Lead only	85	54	1	2
B. Lead + G-6-P	10	9	65	50
C. Mixture (1/2 A + 1/2 B)	49	36	29	22
Calculated (1/2 A + 1/2 B)	48	32	33	26
Adult				
A. Lead only	84	62	1	1
B. Lead + G-6-P	5	6	79	65
C. Mixture (1/2 A + 1/2 B)	55	37	30	32
Calculated (1/2 A + 1/2 B)	45	34	40	33

Total recovery on the gradients was between 74 and 119%. The figures on the chart compare phospholipid and protein found in the fractions to the total recovered on the gradients. The data for the animal at birth are the same as those plotted in Figure 8. The mixture contained equal parts of "lead only" and "lead and G6P".

TABLE 9

RETENTION OF INORGANIC PHOSPHATE DURING DIALYSIS AGAINST EDTA

Experiment	Age	Total μmoles Pi/ml*	
		end of reaction	after dialysis
1	-2 Days	.017	.028
2	Birth	.101	.121
3	Birth	.061	.088
4	Adult	.067	.076

*Values are averages of two determinations.
Essentially no volume change occurs during dialysis;
no change in μg protein/ml.

Rough microsomes were incubated in the histochemical medium. The microsomal suspension was dialyzed against 100 μM EDTA, 10 mM cacodylate buffer, pH 7.3-7.4, for approximately 18 hours, and was gently sonicated during the dialysis. Aliquots were removed before and after dialysis, the tissue was precipitated with TCA, and the inorganic phosphate in the suspension was measured.

is left unprecipitated, or it would escape during dialysis. Note that the amount of inorganic phosphate produced during the enzyme reaction in the -2 day animal is less than that produced in the newborn animal. This is in direct agreement with the biochemistry done on the homogenate and with the histochemistry in the intact cell.

Subfractionation of Rough Microsomes

Rough microsomes isolated from animals at several stages of development were reacted with glucose-6-phosphate and lead, and treated to reverse the aggregation as described above. They were then sedimented on a step gradient to determine the proportion of vesicles which contained reaction product. For all stages, controls were incubated with lead but without substrate to check that the excess lead had been removed. In addition, mixing experiments such as that just described were performed to ensure that the aggregation had been successfully reversed.

Figure 9 shows the results of these experiments. The first tracing, of reacted microsomes from an animal three days before birth, indicates that almost no rough microsomal vesicles have become dense and therefore very few contain the enzyme. The actual amount of material found in the pellet at this stage of development varies somewhat (see Fig. 10), since it is impossible to determine the exact age of the animals. The histochemistry on intact cells from an animal at this stage showed that only a small number of hepatocytes contain G6Pase. The specific activity of the enzyme is approximately 1/10 that of the adult.

Two days before birth a larger number of hepatocytes contain lead phosphate deposit, although they are still in the minority. At this stage (second tracing), a small proportion of the reacted rough microsomes is found in the pellet.

By the time of birth almost all of the cells react and have histochemical deposit in all of their ER. The enzyme activity is approximately equal to that in the adult. The vast majority of the microsomes at this stage (third tracing) are found in the pellet, indicating that all of the vesicles have reacted and contain enzyme.

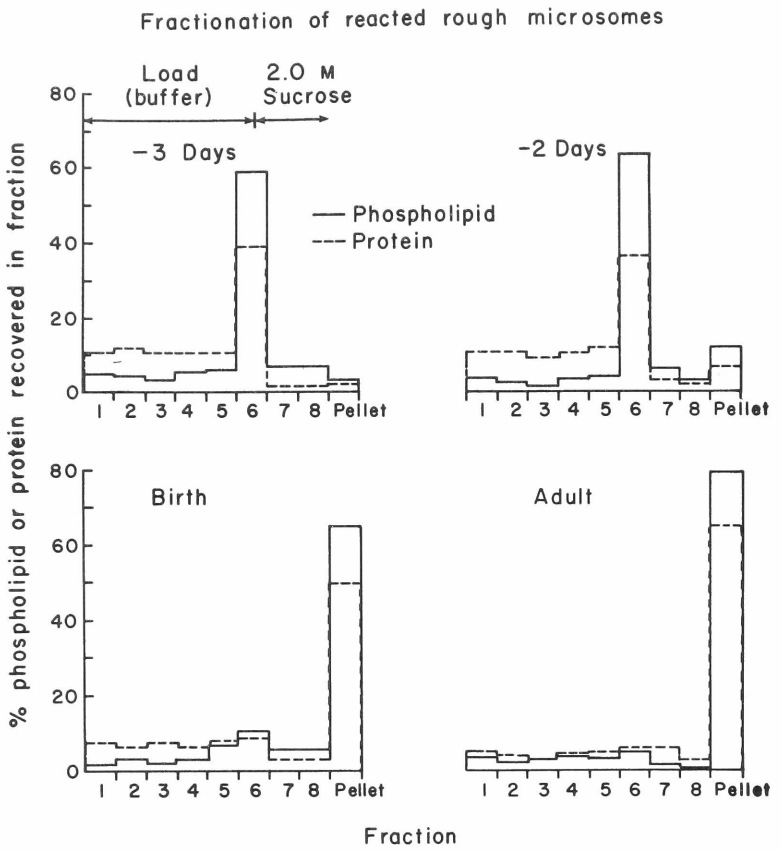


Figure 9. Fractionation of reacted rough microsomes from animals at 4 stages of development; distribution of phospholipid and protein.

At each stage rough microsomes were incubated with 1 mM G6P, 2 mM lead nitrate and 50 mM cacodylate buffer, pH 6.6, for 60 minutes at 25°. The suspensions were dialyzed overnight against 100 μM EDTA, 10 mM cacodylate buffer, pH 7.3-7.4, and were gently sonicated. The suspensions were loaded above 2.0 M sucrose and were spun for 60 minutes at 63,600 x g_{av}. Four milliliter fractions and the pellets were assayed for protein and phospholipid. Determinations on fraction 6 and the pellet in each gradient were done in duplicate. Ordinate compares protein and phospholipid in the fractions to that recovered on the total gradient. In each experiment microsomes incubated without substrate remained in fraction 6.

The histochemistry on the adult liver shows a pattern similar to that at birth. All of the cells react and all of the ER in each cell contains lead phosphate deposit. In the fractionation (fourth tracing) all of the vesicles become dense after reaction. The distribution of material at the interface and in the pellets of these four gradients is summarized in Table 10.

Table 11 compares the specific activity of G6Pase in the isolated rough microsomes with the amount of material (as phospholipid) recovered in the pellets. Column four relates the specific activities of microsomes from fetuses or newborn animals to that of the adult and the last column normalizes the phospholipid recovered in the pellet to that recovered in the pellet from the adult. Comparison of these two columns indicates that in general the activity of the enzyme in the microsomes agrees very well with the amount of heavy lead phosphate-containing vesicles found after reacting the microsomes.

Thus, the results of fractionating the reacted rough microsomes are consistent with the hypothesis that G6Pase molecules are well distributed in closely-spaced sites throughout the ER of the hepatocyte. There is no need to postulate diffusion of released phosphate within the ER of cells reacted histochemically, to explain the uniform and widespread distribution of lead phosphate deposit. This distribution results from, and is a reflection of, a uniform and widespread distribution of enzyme sites.

However, a further control experiment more rigorously to exclude aggregation of the reacted microsomes could be done with the above results in hand. It was conceivable that the aggregation of vesicles which takes place during the reaction is not completely reversed by the sonication and dialysis, although no <u>further</u> aggregation takes place under these circumstances as was shown by the experiment in which reacted and unreacted vesicles were mixed. If there were irreversible aggregation, some vesicles which did not contain deposit may have been bound to heavy vesicles, leading to a spuriously high proportion of the microsomes pelleting through the 2.0 M sucrose.

TABLE 10

FRACTIONATION OF REACTED ROUGH MICROSOMES
AT FOUR STAGES OF DEVELOPMENT

Age	Percent of Recovered Material			
	at Interface		in Pellet	
	PLP	Protein	PLP	Protein
-3 Days	59	39	3	3
-2 Days	63	36	12	7
Birth	10	9	65	50
Adult	5	6	79	65

These are the same data that were presented in Figure 9. All determinations were done in duplicate. Total recovery figures for the gradients were between 74 and 119%.

TABLE 11

CORRELATION OF G-6-Pase ACTIVITY IN ROUGH MICROSOMES WITH PERCENT PHOSPHOLIPID RECOVERED IN REACTED PELLET

Exp.	Age	Specific Activity		Percent of total PLP recovered in pellet	PLP recovered in pellet as percent of adult value
		μmoles Pi/ mg prot/20' 37°C*	percent of adult		
1	-3 days	.10	3	3	4
2	-3 days	.20	6	12	15
3	-2 days	.61	19	12	15
4	Birth	2.94	90	65	82
5	Birth	3.24	99	73	92
6	Adult	3.26	100	79	100

* Values are average of two determinations.

This Table includes data from Tables 8, 10, and 12. The specific biochemical activity was determined on an aliquot of the same fraction used for incubation with G6P and lead ions. Assay conditions for biochemical specific activity: 30 mM G6P, 30 mM cacodylate buffer, pH 6.6. Assay conditions for incubation with G6P and lead: 1 mM G6P, 2 mM lead nitrate, 50 mM cacodylate buffer, pH 6.6.

To rule out this possibility, isolated rough microsomes from animals at two different stages of development, -3 days and birth, were mixed before reaction in the histochemical medium. When reacted alone, vesicles from -3 day animals all remain light, and vesicles from newborn animals all become dense. Equal quantities of vesicles from the two stages were mixed and reacted, and the mixture was dialyzed and analyzed on a step gradient. Equivalent amounts of each of the types of microsomes were also reacted separately and analyzed. The results are shown in Figure 10.

The first tracing (10A) shows the pattern obtained using rough microsomes from an animal three days before birth. The majority of the membranes float and a small fraction pellets. The second tracing (10B) from the animal at birth, is similar to that shown before (Figures 8 and 9); all of the membranes pellet. The last tracing (10C) shows the distribution of material in the mixture. The results are just what is expected in the absence of aggregation; half of the membranes float and half pellet through the heavy sucrose. These results are summarized in Table 12. The phospholipid in the two fractions accounts for 70-80% of the total phospholipid recovered in the gradients. However, these two fractions do not account for as high a percentage of the protein. This is probably due to the presence at the top of the gradient of protein released from the ribosomes. The bottom line shows the recoveries expected in the mixture if aggregation does not occur. The actual values obtained are very close to these theoretical recoveries. It seems quite clear then, that there is no aggregation present at the time that the material is sedimented on the step gradient. Therefore, in the cases of the newborn and the adult, all of the rough microsomes sediment through the heavy sucrose because they contain lead phosphate. And to go back one step, they contain the precipitate because every vesicle contains at least one site of G6Pase.

G6Pase Activity of Fractions

The elegant corollary to this fractionation would be the demonstration that the vesicles which remain at the interface lack G6Pase activity while those that pellet contain it. This determination was

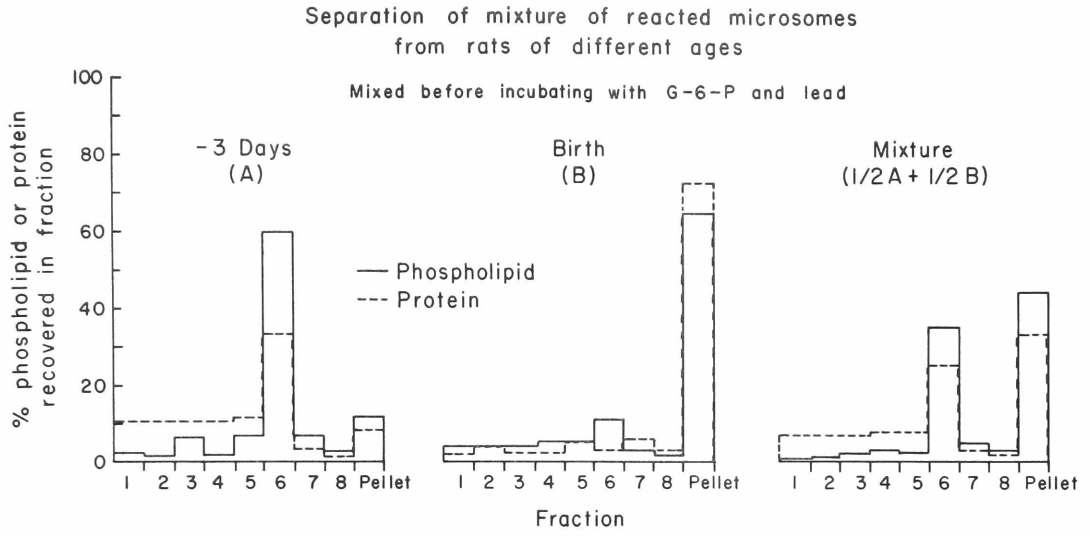


Figure 10. Distribution of phospholipid and protein on gradient separating mixture of reacted rough microsomes from -3 day and newborn rats.

Equal aliquots of rough microsomes prepared from rats of the two ages were mixed before incubating with G6P and lead. The suspension was dialyzed overnight against 100 μM EDTA, 10 mM cacodylate buffer, pH 7.3-7.4, and was gently sonicated. The microsomal suspension was layered over 2.0 M sucrose and was spun for 60 minutes at 63,600 x g_{av}. Four milliliter fractions and the pellets were assayed for phospholipid and protein. All three gradients are from the same experiment. Fraction 6 is the interface between the load zone and the dense sucrose.

TABLE 12

SEPARATION OF MIXTURE OF REACTED MICROSOMES FROM -3 DAY AND NEWBORN RATS——MIXED BEFORE INCUBATING WITH G-6-P AND LEAD

Age	Percent of Recovered Material			
	at Interface		in Pellet	
	PLP	Protein	PLP	Protein
A -3 days	60	33	12	9
B Birth	3	11	73	65
C Mixture (1/2 A + 1/2 B)	35	25	44	33
Calculated 1/2 A + 1/2 B	32	22	43	37

These data were presented in Figure 10. Determinations were made in duplicate. Total recoveries on the gradients were between 79 and 115%.

attempted several times but unfortunately without any success. G6Pase is a rather labile enzyme. Table 13 shows that if the isolated rough microsomes are kept at 4° C overnight, the time required to dialyze the reacted microsomes, they lose 85% of their original G6Pase activity. The rest of the activity is lost if the microsomes are dialyzed against EDTA. Preincubation of the microsomes with Mg^{++} before assay does not increase their G6Pase activity. Possibly the enzyme could be stabilized by inclusion of substrate during the entire procedure, but this was not attempted.

Morphology of Fractions

In all of the fractionation experiments, the material at the interface and in the pellet was examined by electron microscopy. Plate 33 shows material from the interface of a gradient loaded with a mixture of adult microsomes: microsomes reacted with both lead and substrate, and microsomes incubated with lead alone. The material was pelleted and processed for electron microscopy.

Plate 33A shows a portion of the top of the pellet. Many of the vesicles are intact and have ribosomes on their outer surfaces. There are some broken vesicles and some membrane fragments. Plate 33B shows a similar field from the bottom of the pellet. The vesicles are tightly packed and many are cut tangentially. Most of the vesicles are again intact and only occasionally is a reacted vesicle seen at the bottom of the pellet.

Plate 34 shows the material which pellets in the step gradient. Most of the vesicles have electron dense lead phosphate deposit inside of them. Some of the vesicles are completely filled with deposit. In others, which are not completely filled, the deposit remains closely applied to the inner surface of the vesicle. This suggests that the inorganic phosphate does not diffuse far before it is precipitated by lead. In addition, it attests to the insolubility of the lead phosphate precipitate since it does not travel away from the membrane. No deposit is seen on the outer surface of any vesicle. Sometimes an occasional mitochondrion, some membrane fragments and some vesicles that appear to lack deposit are seen contaminating the fraction.

TABLE 13

LOSS OF GLUCOSE-6-PHOSPHATASE ACTIVITY DURING DIALYSIS

(Rough Microsomes from Newborn Animal)

Fraction	Specific Activity μmoles Pi/mg Protein/ 20', 37°*
Rough microsomes - assayed day isolated	2.62
Rough microsomes - assayed 2nd day, stored 0° **	.40
Rough microsomes - assayed 2nd day, dialyzed against 100 μM EDTA, 0.01 M cacodylate buffer, pH 7.4 **	0

* Average of two determinations

** Preincubated with 5 mM Mg^{++} before assay

All three determinations were done on aliquots of the same rough microsome fraction. The aliquot which was dialyzed was kept in the 4° C room during dialysis. None of the aliquots was incubated with lead or G6P.

Plate 33. Interface fraction from a step-gradient containing a mixture of rough microsomes incubated either with lead and G6P or with lead but no substrate. Microsomes derived from adult rat liver. These are the vesicles which remained above 2.0 M sucrose.

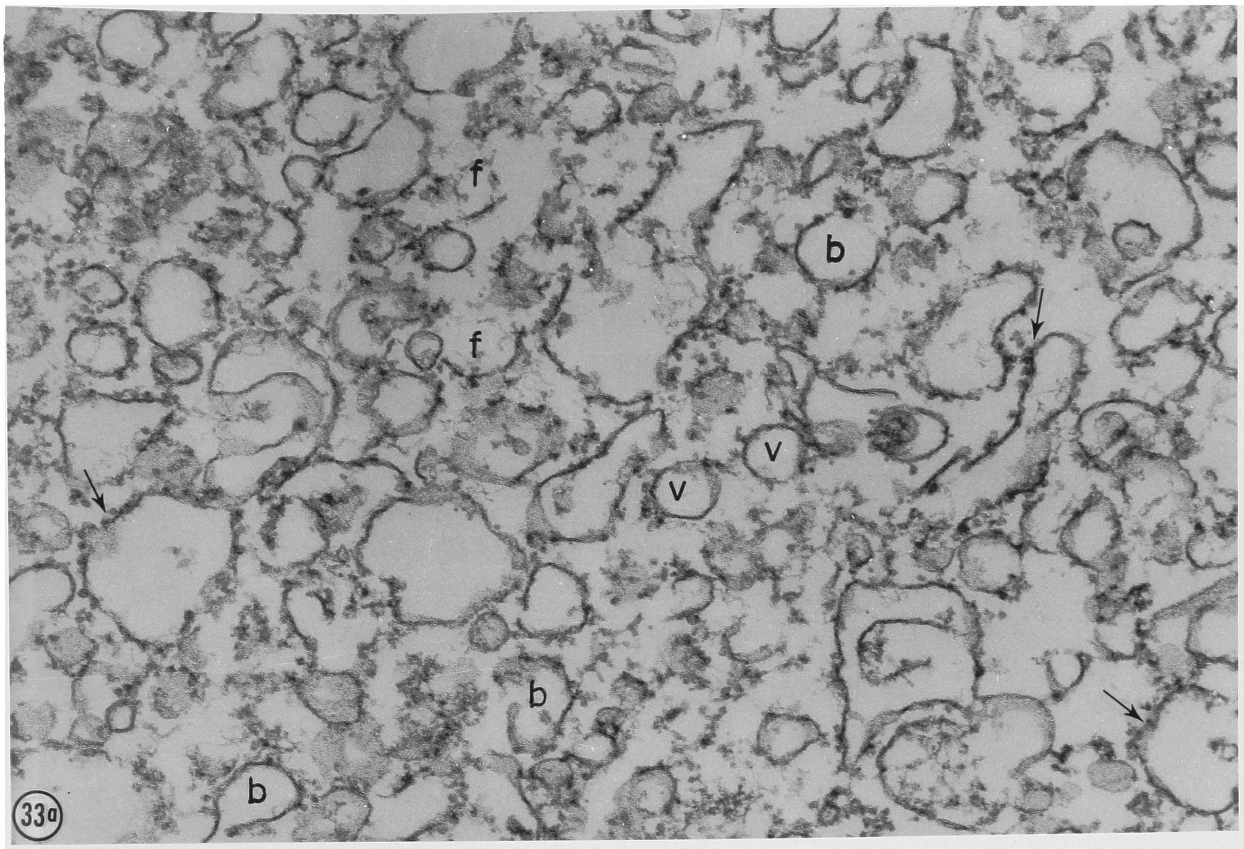
Plate 33A. Top of the pellet prepared by re-sedimenting this fraction. The microsomes do not contain lead phosphate deposit. The pellet includes intact vesicles (v), broken vesicles (b) and membrane fragments (f). Ribosomes are visible on the outer surfaces of the vesicles (arrows).

Magnification: x 57,000.

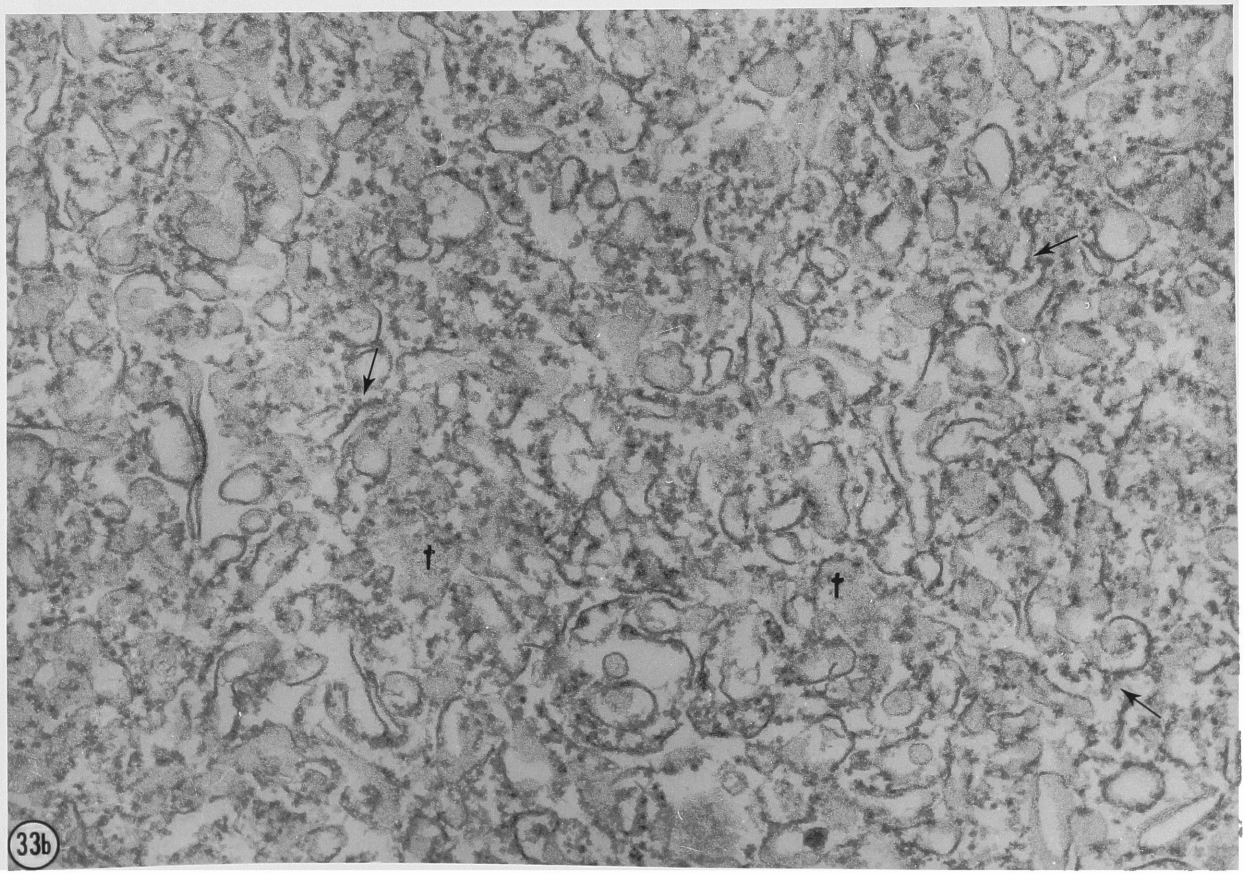
Plate 33B. Bottom of the pellet. The vesicles are more tightly packed and in this field many are cut tangentially (t). Only occasionally are vesicles containing lead phosphate encountered (none present in this picture).

arrows - ribosomes attached to the membranes

Magnification: x 75,000.



33a



33b

Plate 34. Pellet fraction from a step-gradient containing a mixture of rough microsomes incubated either with lead and G6P or with lead but no substrate. Microsomes derived from adult liver. These are the microsomes which sedimented through 2.0 M sucrose. The pellet consists mostly of vesicles containing within them electron-opaque lead phosphate deposit. Some of the microsomes are completely filled with deposits (f) while others are partially filled (p). In addition some empty microsomes are seen (e). In the partially filled vesicles the deposit often remains closely applied to and completely covers the inner surface of the membranes. Other partially filled vesicles contain clumps of deposit which does not completely line them (arrow). This field is near the top of the pellet and is representative of its entire depth.

Magnification: x 105,000.

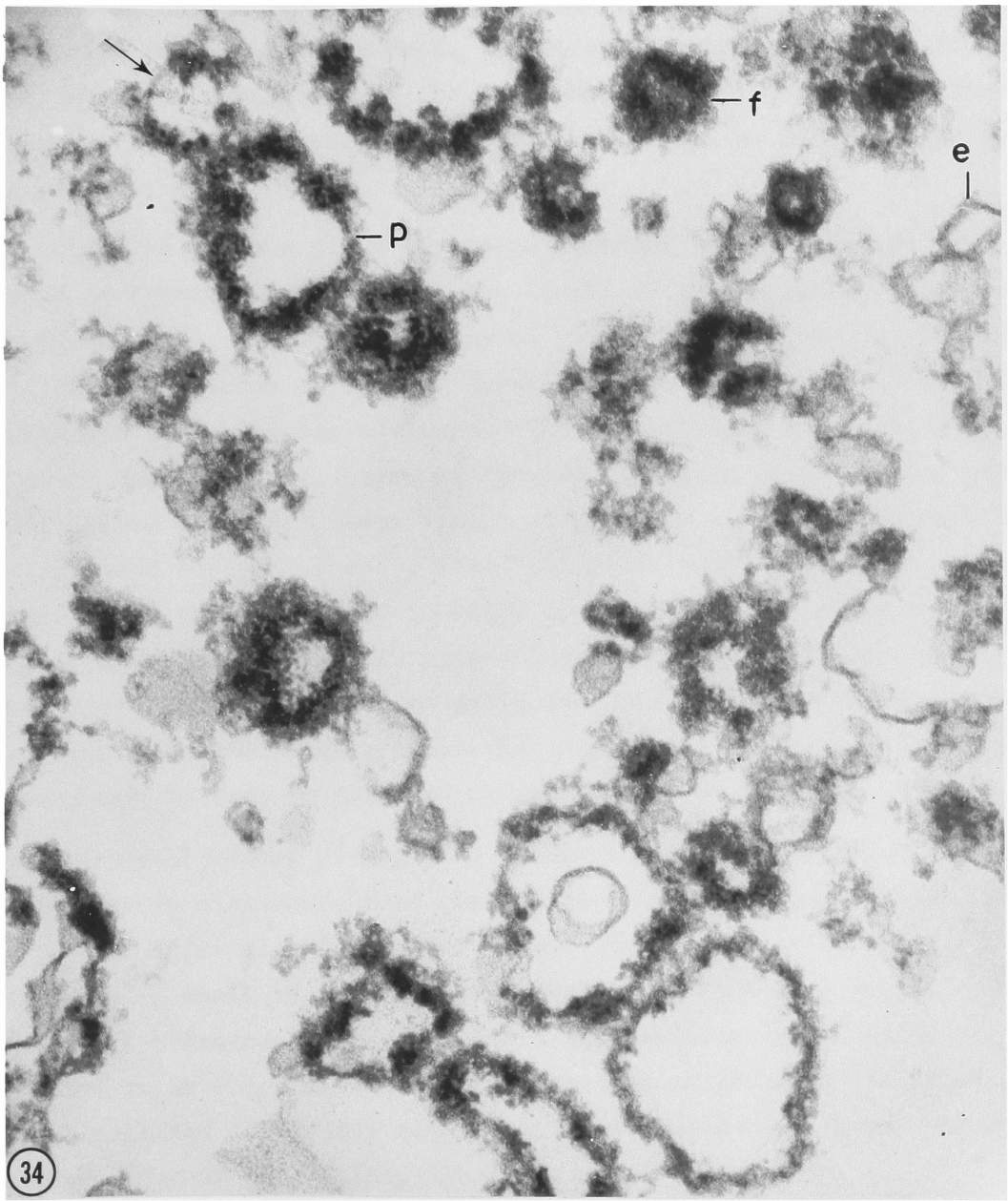

f
e
p
34

Plate 35 illustrates the material found in the pellet of a step gradient loaded with reacted microsomes isolated from an animal immediately after birth. The material looks much like that found in the pellet of reacted microsomes from the adult. Most of the vesicles contain lead deposit. The insert shows an area in which the membrane surrounding the lead deposit is more clearly seen. Considering the treatment -- dialysis against EDTA and sonication -- the membranes are well preserved. They are mostly intact and have ribosomes or the remains of ribosomes on their outer surfaces.

Plate 36 shows a piece of plasma membrane found in the pellet. Although no deposit is seen along the length of the membrane, a finding which agrees with the histochemistry on the intact cell, small vesicles joined to the membrane do react. These are probably ER fragments which are attached to the plasma membrane. This observation can be used to illustrate another point. Some of the vesicles seen in the pellets lack deposit; often these are large vesicles. If only a part of the vesicle contained lead deposit, then it would be possible for a section to cut through a part of the vesicle in which the deposit was not visible. Such a thing could happen with this plasma membrane if the section passed through an area where the small vesicles were not present. It would then be assumed that the material was in the pellet as a contaminant although in fact it pelleted because of the precipitate.

The small amount of material found at the interface in the gradients of reacted microsomes from the newborn animal look much like that found at the interface of the mixing experiment. The total amount of material is so small that sometimes it was not possible to pellet the material for microscopic observations. The vesicles are probably derived from a few cells which still do not react. In cases where the material could be pelleted (Plate 37) some reacting vesicles were found contaminating the bottom of the pellet.

Plate 38 shows the interface and pellet fractions from an experiment in which microsomes from a -3 day and a newborn animal were reacted together. Again the material at the interface is composed of vesicles lacking lead deposit while the pellet contains lead-filled vesicles.

Plate 35. Pellet from a step-gradient containing reacted rough microsomes from an animal at birth. In general the fraction looks much like that in Plate 34. The majority of the vesicles encountered in the pellet contain electron-opaque lead phosphate deposit, which partially (p) or completely (f) fills them. Some vesicles which appear free of deposit are also seen (e). The reaction product is always found within the lumen of the microsomes.

Magnification: x 75,000.

Insert: In this field the membranes of the microsomes are clearly visible surrounding the lead phosphate deposits.

r - ribosomes attached to the vesicles

Magnification: x 148,000.

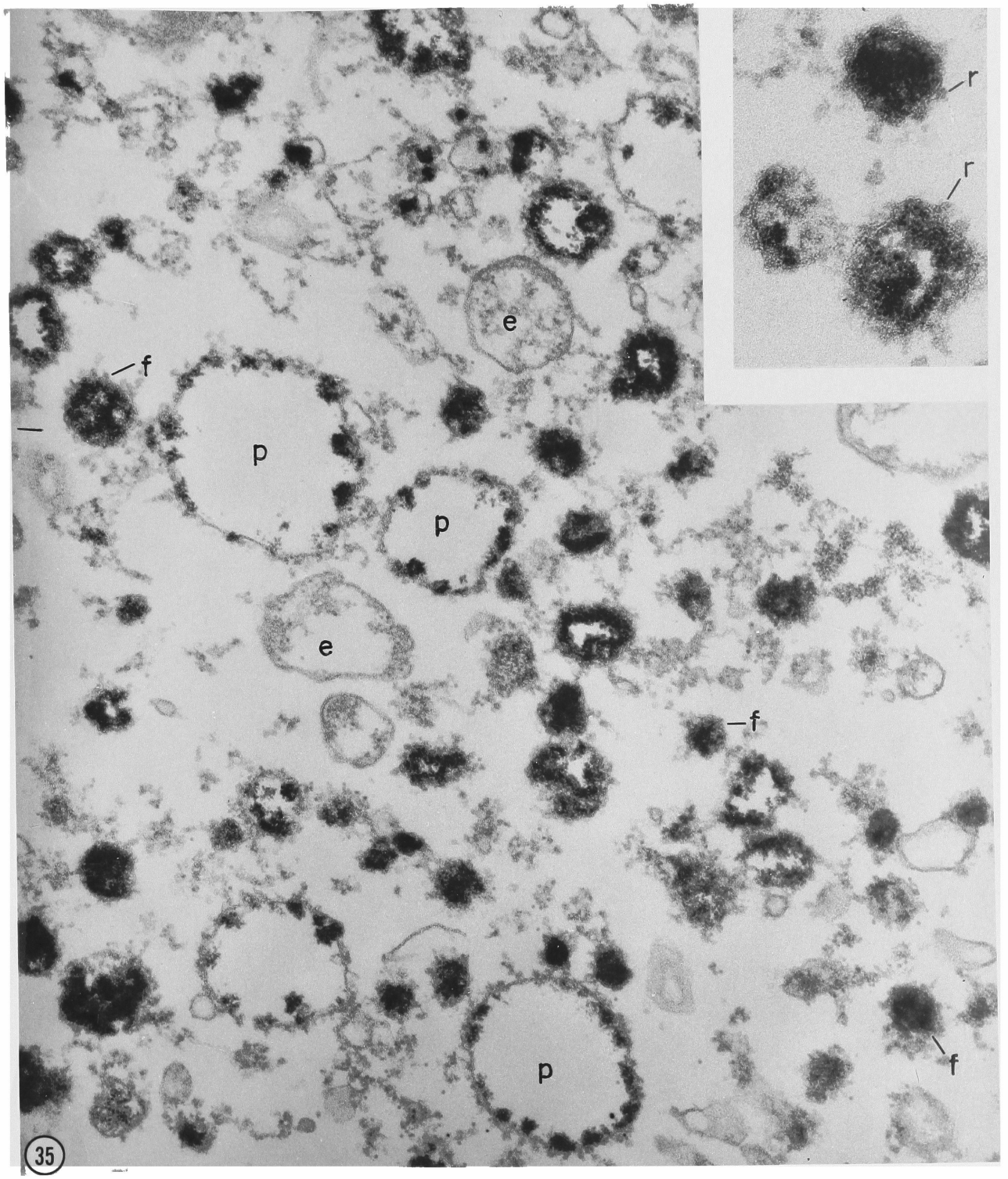

r
r
e
f
p
p
e
f
f
p
35

Plate 36. Plasma membrane found in pellet from step-gradient of reacted rough microsomes from an animal at birth. There is no lead deposit along the length of the membrane although deposit is seen in small vesicles adjoining the membrane (v). Some of the vesicles seem to be covered with ribosomes and may be rough ER attached to the plasma membrane. The arrow marks a point of obvious continuity between a vesicle and the plasma membrane. There is some fuzzy material lining the inside of the plasma membrane. Bottom of pellet.

Magnification: x 66,000.

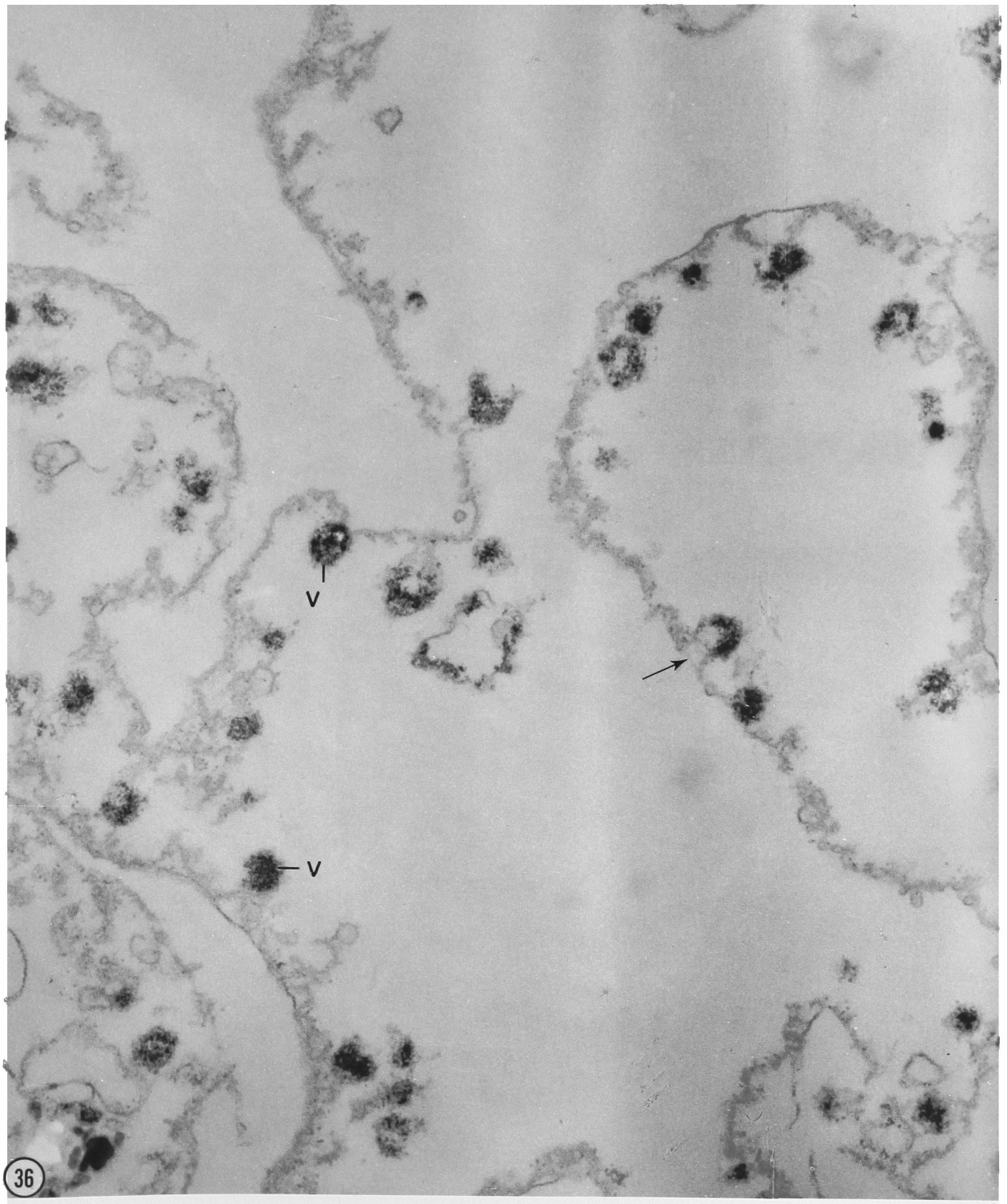

V
V
36

Plate 37. Interface fraction from step-gradient of reacted rough microsomes from an animal at birth (pellet shown in Plate 35). At this stage the amount of material found in this fraction is very small. A number of the vesicles are broken, some are smooth-surfaced, and numerous ribosomes appear scattered in the interstices. This field is at the bottom of the pellet prepared by re-sedimenting the interface fraction, and some vesicles containing lead deposit are present (r).

v - intact vesicles

f - membrane fragment

Magnification: x 47,000.

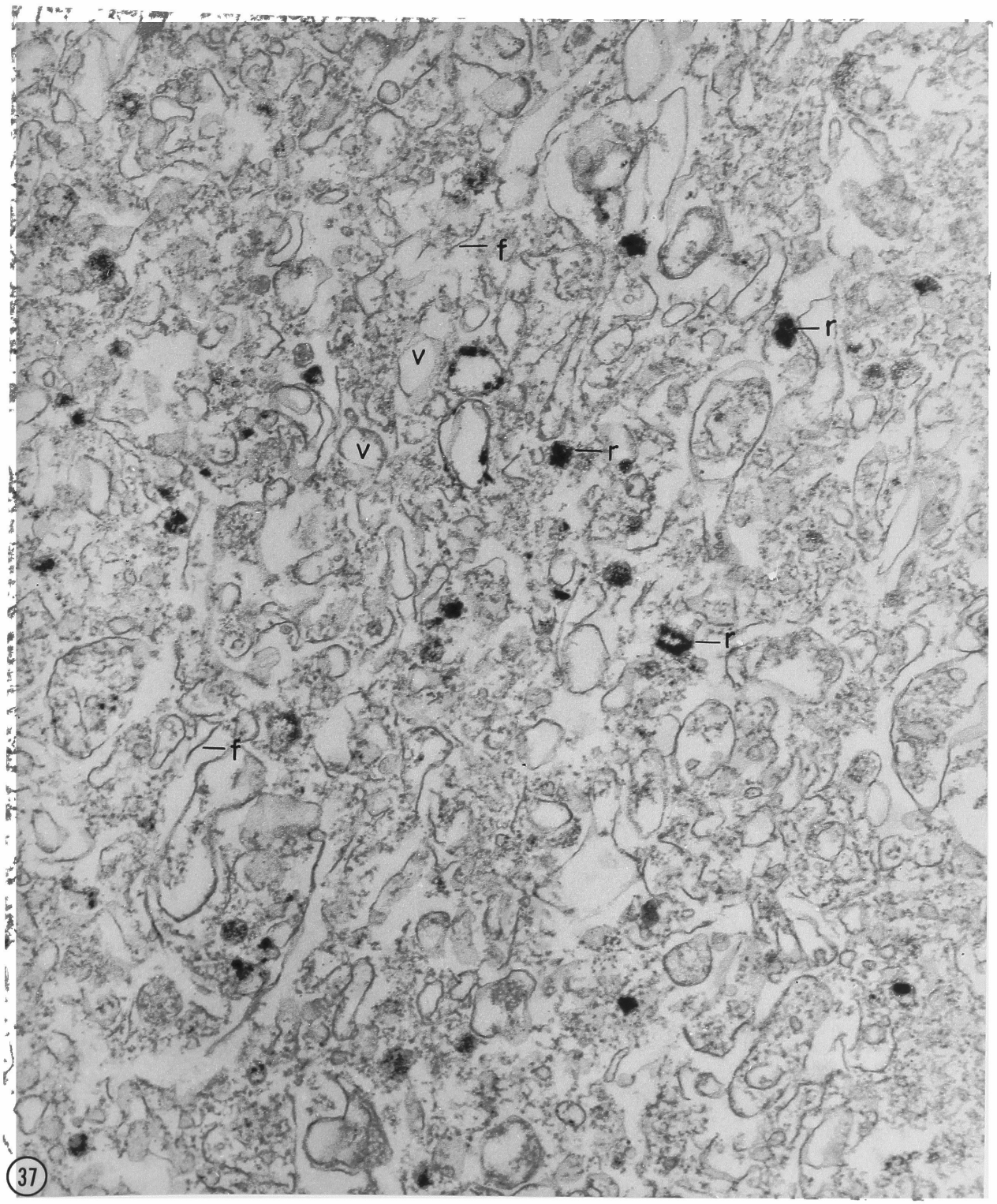

f
v
v
r
r
r
f
37

Plate 38. Interface and pellet fractions from a step-gradient containing a mixture of reacted rough microsomes from a -3 day and a newborn animal.

Plate 38A. Interface fraction: vesicles which floated above 2.0 M sucrose. The fraction consists of vesicles lacking lead deposit. In addition to the unbroken (v) and broken (b) vesicles and membrane fragments, there are free ribosomes in the fraction. This picture is taken at the top of the pellet prepared from the peak fraction.

r - ribosomes attached to vesicles.

Magnification: x 55,000.

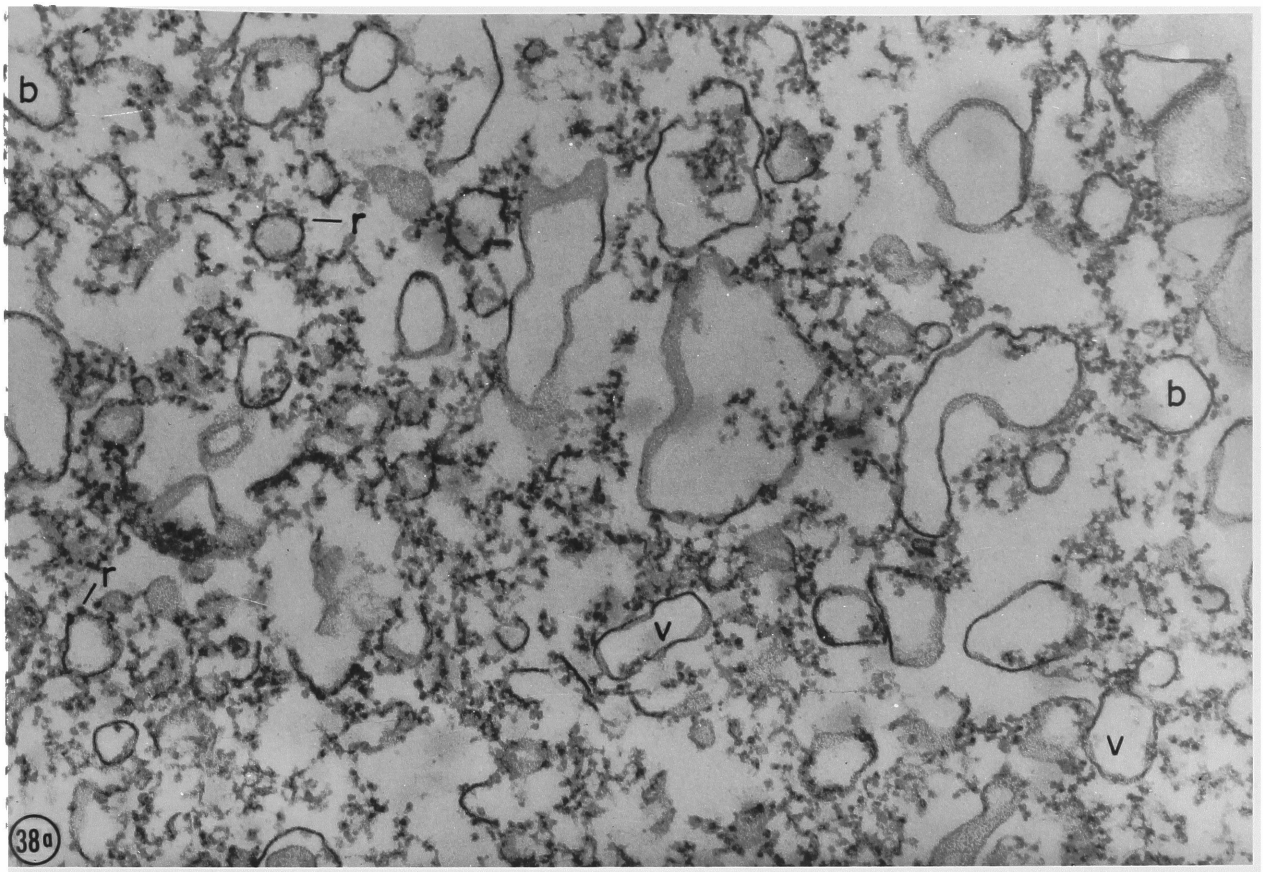
Plate 38B. Pellet: microsomes which sedimented through 2.0 M sucrose. The pellet consists of microsomes containing lead phosphate reaction product. In this field several empty vesicles are also seen. The location of the deposit within the vesicles and the unit membrane (arrows) bounding them are clearly seen in the insert. Some attached ribosomes can also be seen. Picture taken in the middle of the pellet.

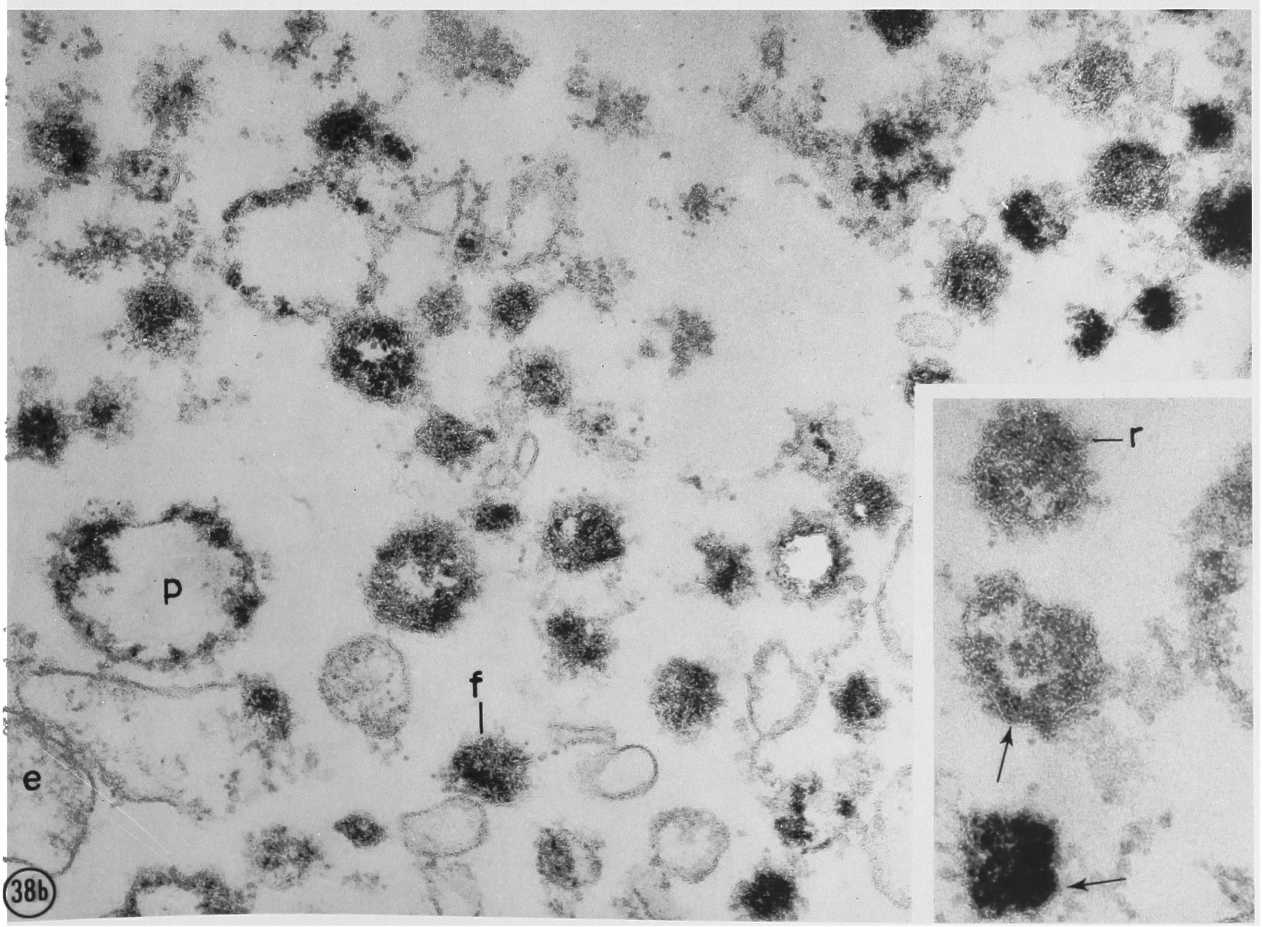
f - vesicles completely filled with deposit

p - vesicles partially filled with deposit

e - empty vesicles.

Magnification: x 75,000. Insert: x 120,000.


b
r
r
b
v
v
38a


p
f
e
r
38b

These fractions chosen for illustration are representative of all the fractions examined. The material that pellets through the heavy sucrose always consists of reacted vesicles while the material which floats is composed of unreacted microsomes. The microscopy thus substantiates that the method can be used to separate two types of rough microsomes: those that contain G6Pase and those that do not.

Correlation with Histochemistry

The results of the cell fractionation study confirm and expand the histochemical findings. At all stages, the proportion of rough microsomes which can be rendered dense by the presence of lead phosphate precipitate correlates well with the biochemical activity of the enzyme and with the amount and distribution of the histochemical deposit in the intact tissue. Further, in the adult and in the newborn, where all of the microsomes react, we can say with confidence that the distribution of deposits seen in situ reflects the actual distribution of enzyme sites. The enzyme molecules are widely distributed in closely-spaced sites throughout the ER of the hepatocyte. There is no need to postulate a diffusion of released phosphate within the ER cisternae to explain the histochemical pattern. The results further suggest that there is no differentiation within the rough ER at a level of resolution defined by the size of the microsomal vesicles analyzed: this point will be expanded in the discussion.

It is not possible to interpret the histochemical results from the animals before birth with such certainty. In those instances in which only some of the vesicles become dense after reaction, one cannot conclude that all of the rough ER from a particular cell contributes enzyme-carrying vesicles to the total microsomal population. However, it is likely that at these stages, too, the uniform distribution of reaction product results from the even distribution of enzyme sites within the membranes.

Summary Part IV

A method for the sub-fractionation of rough microsomes was developed based on the retention of lead phosphate within fresh microsomes incubated *in vitro* for G6Pase. The reaction product increases the density of the G6Pase-containing microsomes and thereby makes possible their separation from G6Pase-free vesicles by isopycnic centrifugation in a 2-step density gradient. The method permits the virtually complete reversal of the aggregation which occurs during the incubation. The procedure was applied to rough microsomes isolated from rats at various stages during hepatocyte differentiation and the results obtained agree with those given by the histochemical tests *in situ*. Before birth, when only some of the hepatocytes react, only a commensurate proportion of the microsomal fraction can be rendered heavy by the G6Pase reaction. At the time of birth and in the adult, all of the microsomes acquire lead deposit and become dense after reaction. The results further suggest that the pattern of distribution of lead phosphate deposit in the ER of cells in tissue slices can be explained by the actual distribution of enzyme molecules. Enzyme molecules are uniformly distributed, in closely-spaced sites throughout the rough ER of the hepatocyte. There is no qualitative differentiation of the rough ER with respect to G6Pase at this level of resolution.

DISCUSSION

Pitfalls in Histochemistry

Conditions of Reaction

Careful workers have realized that histochemical localization of enzymes has many pitfalls (Gomori 1950a, 1950b, Barrnett and Palade 1958, Holt 1959). The controls conducted during this study were an attempt to avoid some of these problems.

The conditions used for the histochemistry allowed optimal visualization of enzyme activity. The concentration of trapping agent (lead ions) is high enough to ensure that all of the inorganic phosphate released can be precipitated immediately. If this were not the case, non-specific deposition of reaction product might be found due to the diffusion of released phosphate before its precipitation (see Reid et al. 1964). None of the conditions or reagents inhibits the enzyme and there is no non-specific breakdown of substrate during the reaction to complicate the interpretation (Moses and Rosenthal 1967).

Fixation

One of the most important problems faced by histochemists is enzyme inactivation during tissue fixation. The requirements of good tissue fixation and retention of enzyme activity seem mutually exclusive (Barrnett and Palade 1958). Good fixation involves stabilization of molecular configurations while retention of activity implies that the enzyme can interact with its substrate, possibly by changing configuration. Under suitable conditions, however, a compromise can be reached. It is necessary that quantitative determinations of the enzyme remaining in fixed tissue be made. If only a fraction of the original activity is left, the deposit cannot be considered representative of the localization of the enzyme in the unfixed tissue. Since different methods of fixation may selectively inactivate different populations of enzyme molecules, strikingly different results could be obtained from two different methods of fixation (see Goldfischer et al. 1964).

The technique of short fixation by perfusion employed in this study allowed retention of 70-80% of the original enzyme activity. It is, of course, difficult to show conclusively that the 20-30% of the activity

lost was not derived preferentially from one site or configuation of the enzyme. Substrate was included during fixation in an attempt to trap enzyme molecules in their active configuration. It was hoped that more of the enzyme molecules would be in this configuration in the presence of substrate (see Beaufay et al. 1954 for a discussion of the ability of substrate to protect G6Pase against inactivation). But this precaution did not increase the recovery of activity.

Metal Salt Precipitation

The histochemical method first developed by Gomori (1939, 1941) and Takamatsu (1939), employing metal salt precipitation, has been widely used. For a number of enzymes it is the only reliable method available. There are several problems concerning this method. One of these problems is the interpretation of the location of the deposits. How closely does this location correspond to the point of release of the product and to the site of the enzyme molecule itself? How far does the free phosphate diffuse before it is trapped by the lead ions, and how efficient is the precipitation?

The results obtained by incubating isolated rough microsomes in the histochemical medium, followed by extensive dialysis and sonication, indicate that the precipitation of phosphate ions is extremely efficient. All released phosphate ions are trapped by lead and remain precipitated during the entire procedure. Although the sub-fractionation of rough microsomes was undertaken to determine whether diffusion of released inorganic phosphate takes place during the histochemical reaction, the results do not directly answer that question. It has not been conclusively demonstrated that the uniform distribution of the deposits is a direct reflection of the distribution of sites of enzyme activity; the results do not rule out diffusion. Diffusion of the phosphate may, in fact, take place. However, these results do tell us that each microsome has at least one site of G6Pase activity, and that G6Pase molecules are uniformly distributed along the ER membranes. Thus, the distribution of histochemical deposits may result from the release and rapid precipitation of phosphate at these sites.

A further observation lends support to this interpretation. When reacted vesicles are examined in the electron microscope, the precipitate is seen attached to the inside of the membranes in those cases where it does not fill the whole vesicle. Similar findings have been reported for the reaction product of other phosphatases (Tice and Engel 1966, in reacted sarcoplasmic reticulum vesicles, and Goldfischer *et al*. 1964, Tice and Barrnett 1962, in histochemistry *in situ*). This suggests that the phosphate does not diffuse far from its release point before it is trapped. In addition, the observation shows that the lead phosphate precipitate is insoluble and that it does not diffuse away from the membrane.

Position of the Enzyme in the Membrane

In vivo glucose-6-phosphate is available in the cytoplasmic matrix of the cell, where it is formed from glycogen. In the incubated fresh microsomes *in vitro*, the substrate is outside the vesicle. Whether G6P can penetrate through the ER membrane is unknown. If the membrane is impermeable to the substrate, then there must be a binding site for the substrate exposed to the cytoplasmic matrix. If the substrate can pass through the membrane then the enzyme need not be located on one or the other side.

In both the intact cell (fixed) and the isolated microsomes (unfixed) the lead phosphate deposit was always found on the cisternal side of the membrane. In fact, the experiment with the microsomes showed that the recovery of released phosphate within the cisternae was quantitative. Thus there is a directional release of the phosphate which is split from G6P. A possible advantage of this directionality might be the removal of inorganic phosphate from the cytoplasm much as the mitochondria sequester calcium ions.

There is no evidence concerning the site of glucose release. Since it is possible that the membranes are at least semi-permeable to glucose, this site might be difficult to determine: and if the membranes are freely permeable to glucose, then the point of release is irrelevant. Although ER membranes are semi-permeable to sucrose (Share and Hansrote

1960, Palade and Siekevitz 1956a, Tedeschi et al. 1963) there are no data concerning their permeability to glucose. Cahill et al. (1958) report the plasma membrane to be freely permeable to glucose although Dawkins (1963) disagrees. If the ER membranes are impermeable or only partially permeable to glucose, then a release of glucose into the cavity of the ER might be a means of transporting the glucose out of the cell through these intracellular channels. Such an intracisternal transport of glucose is discussed by Siekevitz (1958). Nordlie and Soodsma (1966, also Soodsma et al. 1967), studying kidney G6Pase, hypothesize that the enzyme is involved in facilitated transport across the kidney tubule epithelial cell in diabetic animals. They suggest that G6Pase transphosphorylates glucose as it enters the cell from the tubule lumen, and then dephosphorylates the G6P on the side of the cell near the blood stream. They do not discuss this theory with reference to the physical location of the enzyme itself (which is in the endoplasmic reticulum).

Sub-fractionation of Rough Microsomes

The technique of using a histochemical reaction product to modify the density of microsomes has not been previously reported. Since the lead phosphate deposit has such a high density, even a small amount of precipitate is sufficient to increase the density of a vesicle enough so it can be easily separated from an empty vesicle on the basis of this increased density.

The observed non-specific binding of lead to the membranes increased the density of all vesicles indiscriminately. To separate reacted from unreacted vesicles by density differences, it was necessary that the free lead ions be removed. The procedure developed for doing this, dialysis against EDTA (a chelating agent), does not disturb the lead phosphate precipitate itself and so the enzymatically formed phosphate remains insoluble inside the vesicles.

A drawback to the procedure is the inability to recover G6Pase activity after dialysis. Part of the reason for the loss of activity is the length of time necessary to completely dialyze away the excess lead ions. Even unreacted microsomes kept in sucrose at 0° C for the

same length of time lose much of their activity. Exactly why the remainder of G6Pase activity is lost during the dialysis is not clear. Rough microsomes which were not incubated with lead but which were merely carried through the dialysis procedure lost all G6Pase activity by the end of the dialysis, suggesting the inactivation occurs during the dialysis itself. Possibly EDTA dialysis removes a tightly bound divalent cation necessary for activity, but reincubation of the fractions with Mg^{++} before assay did not reactivate the enzyme. If there is such a removal of cations it may permanently change the configuration of the enzyme. There is no evidence that EDTA has such an effect on the enzyme and the loss of activity may be due to another problem altogether. Beaufay *et al*. (1954) reported losing G6Pase activity during dialysis, but they believed the inactivation to be due merely to a low pH since adding bicarbonate prevented it. In this case, the pH was controlled (and maintained at pH 7.3-7.4) so that a low pH could not be the cause of the inactivation.

A possible modification of the technique to overcome this loss of enzyme activity is the introduction of sephadex chromatography instead of dialysis to remove the excess lead ions from the microsomal suspension. The procedure could be shortened considerably and the exposure to EDTA could be eliminated.

Several other determinations could be made on the sub-fractions of rough microsomes. First, if enzyme activity can be recovered, the G6Pase activity in the dense fraction might be compared to the activity in the light microsomes to further substantiate that the method separates vesicles containing G6Pase from those lacking the enzyme. Second, the microsomes in the two fractions could be tested for other enzyme activities to see if the cells which contain G6Pase also develop other microsomal enzymes early. Third, the concentrations of newly formed membrane constituents (e.g., protein and phospholipid) in the two fractions could be determined to see if the cells containing G6Pase are also preferentially synthesizing or turning over membrane components.

Further Applications of the Microsomal Sub-fractionation Technique

Although so far the sub-fractionation technique has been applied to only one question, many other uses can be envisioned. Considering only G6Pase first, the reaction might be useful as a tool to separate smooth microsomes from Golgi-derived vesicles. The Golgi-derived vesicles should not contain enzyme and should remain light after reaction while the smooth microsomes become dense. Also, the question approached by Orrenius and Ericsson (1966) might be soluble; after phenobarbital induction, are smooth ER membranes formed which lack G6Pase but contain NADPH-cytochrome c reductase? In addition, the basic technique is applicable to any enzyme reaction (all phosphatases) in which the product can be trapped as a heavy metal precipitate, and the technique could be useful as a mechanism for separating various cellular components (e.g., Golgi-derived vesicles from other cell organelles based on the presence in the former of thiamine-pyrophosphatase).

Heterogeneity Within the Endoplasmic Reticulum

Rough ER

The *in situ* histochemistry demonstrated that as soon as a cell acquires G6Pase activity, the enzyme is distributed throughout all of the ER present in that cell. The cell fractionation experiments confirmed this widespread distribution of enzyme; all rough microsomes isolated from adult or newborn rat liver contained at least one site of G6Pase activity.

The fractionation of the ER as measured by the size of the microsomal vesicles sets the limit to the resolution of the procedure. If the vesicles form by pinching off from the ER and thus contain membrane from both sides of the cisternae, then the enzyme sites along a membrane (one side of a cisterna) are, on the average, no further apart than half the circumference of the smallest microsomes. The average half-circumference value for the entire microsomal population,

determined by measuring the vesicles in micrographs, is 180 mμ[1]. Thus, two molecules of G6Pase are no further apart than 180 mμ.

The resolution of the technique can be increased by decreasing the size of the vesicles. Rough calculations estimate the average number of G6Pase molecules per vesicle to be in the range of 4-36.[2] Since Dallman et al. (1969) report being able to reduce the size of microsomal vesicles to 1/100 their original volume by sonication, it should be possible to produce a heterogeneous population of small vesicles, some of which contain enzyme and some of which do not. Thus, the actual distances between sites of G6Pase molecules in the ER might be available to direct measurement and the differentiation with the ER, which must exist at the molecular level, could be observed.

The finding of a uniform distribution of G6Pase in all of the rough ER microsomes is in contradiction to the work of Dallner and coworkers (Dallner et al. 1968). They found an uneven distribution of G6Pase in the population of adult rat liver rough microsomes which were sedimented on a continuous sucrose gradient. The specific activity of G6Pase was greater in the slowly-sedimenting microsomes than in the faster-moving ones.

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[1] This method of measuring would tend to underestimate sizes since only if a vesicle is cut through the center is the actual diameter measured. However, the thickness of the sections (approximately 60 mμ) is of the same order of magnitude as the diameter of the vesicles. The size of the vesicles seen in the sections should be reasonably good estimates of their actual diameters. In addition, for this calculation we are interested in the smallest vesicles in the population, so the figure of 180 mμ can be used as an approximation.

[2] Assume G6Pase is a flat plate 5 mμ on a side. This is in the range of myoglobin (Kendrew 1961) and RNAase (Kartha et al. 1967). The surface area is then 25 $m\mu^2$. If the G6Pase accounts for 0.1-1.0% of the total protein (cytochrome b_5 is approximately 0.8%, Omura et al. 1967), then one molecule of G6Pase would be found per 2500-25,000 $m\mu^2$ of protein. If the protein is evenly distributed on the two sides of a phospholipid bilayer, then one molecule of G6Pase will exist per 1250-12,500 $m\mu^2$ of surface protein. Calculating from the average size vesicle measured, with a half-circumference of 180 $m\mu^2$ and a diameter of 60 μm, the surface area ($4\pi r^2$) is 43,200 $m\mu^2$. There would be 4-36 molecules of G6Pase per vesicle.

The slower-sedimenting vesicles must be either smaller, or less dense, or both, than the faster-sedimenting ones. It is difficult to understand what relation size or density of a vesicle would have to its enzyme content. At any rate, application of a sub-fractionation scheme such as the present one, based on a factor directly related to the enzyme content, ought to give a more straightforward result.

In the same group of experiments, Dallner *et al.* found that the distribution of NADH- and NADPH-cytochrome c reductases followed that of G6Pase while cytochrome b_5 was found uniformly distributed throughout the gradient. The increase in activity of several newly-formed enzymes in the newborn rat was greatest in the slowest sedimenting fraction, as was the phenobarbital-induced increase of NADPH-cytochrome c reductase activity and the starvation-stimulated increase in G6Pase activity. The authors interpret their findings as suggestive of a high degree of heterogeneity within the rough ER although it is surprising that cytochrome b_5 and NADH-cytochrome c reductase, proteins which are involved in a common electron transport chain, show different patterns of sedimentation.

Such groups of enzymes involved in common functional pathways (such as the cytochrome b_5 and NADH-cytochrome c reductase mentioned above) are known to exist in the ER. It is highly probable that these proteins are located close to one another within the membrane. This supports the idea that the membrane is a mosaic of functionally distinct units. Drug stimulation of some enzymes and not others (Ernster and Orrenius 1965) further supports this concept.

The present work on G6Pase does not necessarily disagree with this idea. Resolution of the heterogeneity may only be obtained if the ER is fragmented into smaller vesicles than those obtained by simple homogenization. Alternatively, G6Pase, an enzyme which is unrelated to other membrane-bound ER enzymes, may have a pattern of distribution in the membrane radically different from that of multi-enzyme units. The technical problem is how to separate enzymatically distinct units in order to observe the heterogeneity. Application of techniques similar to the one presented in this thesis, employing reaction products to differentiate microsomes, appears to hold considerable promise.

Smooth ER

The in situ histochemistry shows the enzyme product to be present in much of the smooth ER. From the histochemistry alone, it is difficult to say whether all of the smooth ER contains G6Pase (see Plates 5, 6, 28, and 29). There have been histochemical reports suggesting that G6Pase activity exists in all smooth ER (Orrenius and Ericsson 1966) and biochemical work (Dallner 1963) localizing the enzyme to only some of the smooth ER. Dallner and co-workers have performed experiments similar to those just discussed concerning the rough ER, to examine differentiation within the smooth ER (Dallner 1963, Glaumann and Dallner 1968a, 1968b). They used magnesium to separate two fractions of smooth ER. Magnesium ions aggregate some of the smooth microsomal vesicles but not others. Both fractions are derived from the ER although they have different enzyme compositions. The increase in smooth membrane seen after phenobarbital treatment seems to be limited exclusively to those membranes which aggregate with magnesium. Fragments of plasma membrane and/or Golgi-derived vesicles may, however, account for one of these fractions.

Glaumann and Dallner (1968b; see also Dallner and Ernster 1968) have sub-fractionated smooth ER on a continuous sucrose gradient in the presence of cesium ions. They found that NADH-cytochrome c reductase, NADPH-cytochrome c reductase and nucleoside diphosphatase have high specific activities in the vesicles found near the top of the gradient while G6Pase is highest in the faster-sedimenting microsomes. Adenosine monophosphatase, Mg^{++} activated adenosine triphosphatase and cholesterol content are highest in the pellet, suggesting that the pellet contains plasma membranes.

There is evidence accumulating that protein and phospholipid are assembled into membrane in the rough ER which is then converted into smooth ER. Dallner et al. (1966b) studying the newborn rat liver found newly formed constitutive enzymes first in the rough and then later in the smooth microsome fraction. Omura, Siekevitz and Palade (unpublished experiments) followed the in vivo incorporation of radioactive label into purified membrane proteins and showed first an increase in specific

radioactivity in the enzymes isolated from the rough microsomes and later an increase in the specific radioactivity of the enzyme isolated from the smooth microsomes. Ernster and Orrenius (1965) found that the specific enzymatic activity of the newly formed enzymes induced by phenobarbital, increase first in the rough and then later in the smooth ER. Jakobssen and Dallner (1968) followed the increase in G6Pase activity after starvation and in alloxan-induced diabetes and found that the activity increased first in the rough and then later in the smooth ER.

If this is actually the mechanism of smooth ER formation, then all of the smooth membranes, especially those in the newborn rat liver, might be expected to have G6Pase, since all of the rough membranes contain the enzyme. The first smooth membranes to form right after birth do have G6Pase histochemically. Application of a sub-fractionation technique to the smooth microsomes, similar to that applied to the rough microsomes, would add considerable dimension to our knowledge.

Models for Membrane Assembly

The results of this study can also be used to construct models of membrane assembly. At the very early times examined, when only a few cells show a light reaction, those cells that react contain enzyme in all of their rough ER. In other words, as soon as the enzyme activity appears, it is visible in all of the ER membranes. At the later time points too, the enzyme is found in all of the membranes. At no time are cells seen with two types of ER: membranes that contain the enzyme and those that do not.

Two possible ways in which membranes can be assembled are diagrammed in Figure 11. The first mechanism (A) proposes that there are a small number of growing points where new membrane is being formed. That is, all of the components in these fragments are newly synthesized or at least newly assembled. If enzyme is inserted only into these new membrane areas, the pattern illustrated, with well-delimited areas of only some cisternae reacting, would be the pattern seen after the reaction for G6Pase. Fractionation of the membranes would produce a heterogeneous population of microsomes, some containing G6Pase and some lacking

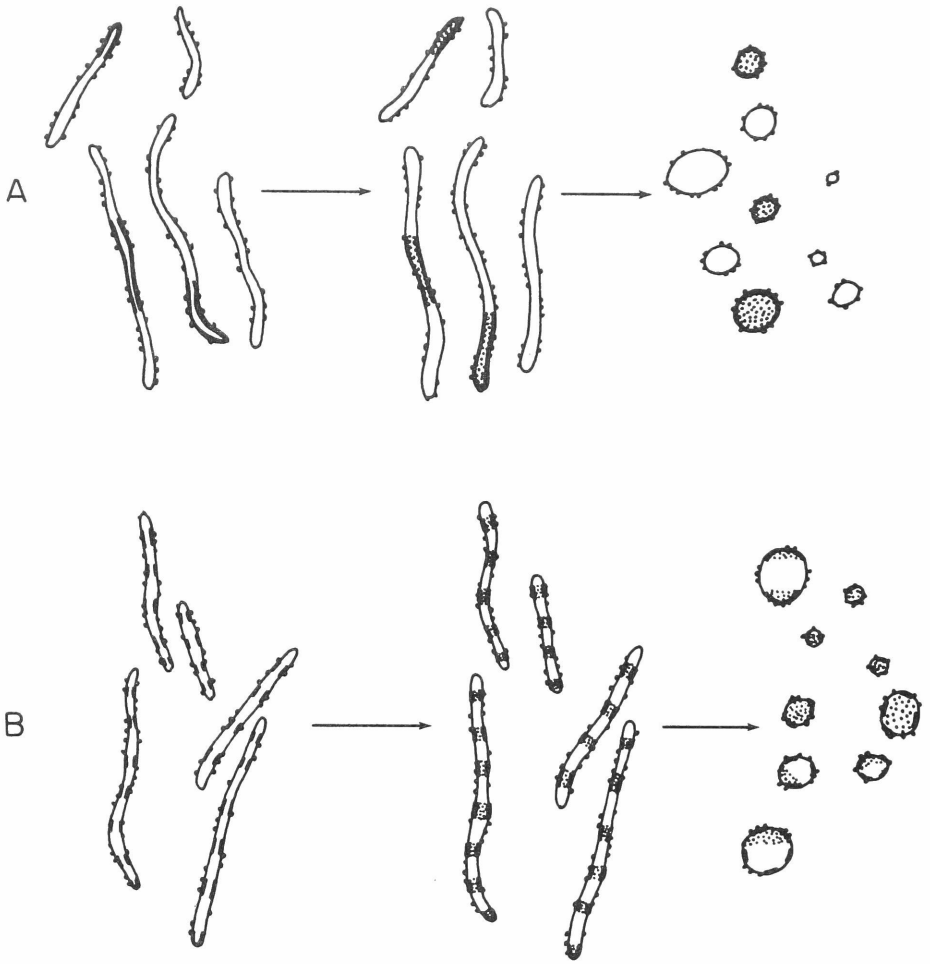

Figure 11. Two models for membrane assembly.

A. Dense line represents regions of newly synthesized membrane containing newly synthesized G6Pase.

B. Dense line represents regions of newly synthesized membrane containing newly synthesized G6Pase, or regions of old membrane containing newly inserted G6Pase molecules.

In both models the stippling represents lead phosphate deposit. The first figure in each model illustrates the composition of the membrane, the second is the pattern of lead phosphate deposit seen after histochemical incubation, and the third describes the population of microsomes after incubation with G6P and lead.

the enzyme. This type of pattern is not seen either by histochemistry or by cell fractionation.

The only way to reconcile the observed histochemical distribution and this model of assembly is to assume that the membrane constituents are turning over very rapidly and that old membrane, lacking the enzyme, is very quickly replaced by new membrane, containing the enzyme. Although there is no data for the turnover of membrane constituents in the foetus, Omura et al. (1967) have studied this turnover in the adult rat liver. They found that the half life of the protein components of the ER membranes is on the order of 3-4 days. This means it would take this long for half of the old membranes to be totally replaced by new ones. If the rate of turnover in the foetus were similar, it would not be sufficiently rapid to reconcile the histochemical findings with this mechanism of assembly. With such a rate of turnover we should be able easily to find cells in which part of the ER, the "new" part, contained enzyme, while the "old" part did not. Such cells are not found, and so unless the turnover in the foetus is much more rapid than that in the adult, this model of membrane assembly cannot adequately explain the findings.

The second pattern (B) can be explained by two mechanisms. One of these assumes that there are a large number of closely-spaced sites where new membrane formation is taking place. If the enzyme is inserted only into these areas, then deposit will be seen along the length of each and every cisterna. The fractionation will produce vesicles which all contain enzyme as long as the microsomes formed are larger than the spaces between the neighboring enzyme sites.

This pattern of lead deposit may also be explained in another way. There may be no areas of completely new membrane at all; that is, no areas where all of the components of the membrane are newly synthesized or newly assembled. Growth and change in the membrane may take place by the insertion of membrane constituents into an already formed sub-structure. The histochemistry would then present a picture of deposit distributed along the whole length of the cisternae and the fractionation

would again produce a homogeneous population of microsomes all containing the enzyme.

The results obtained are those shown in B. The two explanations of the pattern are not easy to distinguish experimentally. The first explanation would predict an increase in the actual amount of membrane in the cell and the second would not. So far it has not been possible to distinguish any difference between the amount of membrane in cells containing enzyme activity and the amount of membrane in cells lacking the enzyme.

The type of membrane formation suggested by either of the two explanations for B implies that any moderate length of membrane is always a mosaic of new and old components. This is the conclusion which appears to be favored by data accumulating from many studies of membranes. Dallner et al. (1966a, 1966b) found that the patterns of development of various ER enzymes during hepatocyte differentiation are very different. Enzymes, even those involved in the same biochemical pathway, appear at different times and increase at different rates. The enzyme composition of the ER is thus continuously changing during development. These authors interpreted their findings as suggestive of a multi-step process of membrane assembly in which membrane components are inserted into a pre-existing structure independently of one another.

Omura et al. (1967) have shown that the phospholipid and protein constituents of the ER have different half lives and are thus turning over independently of each other. Even among the proteins, there is variation; cytochrome b_5 turns over more slowly than NADPH-cytochrome c reductase. In the phospholipids too, it appears that the glycerol backbone turns over faster than the fatty acid portion of the molecule.

Ernster and co-workers (Ernster and Orrenius 1965, Orrenius and Ernster 1964) have studied the effect of phenobarbital on ER enzymes. The drug induces specific increases in NADPH-cytochrome c reductase, cytochrome P450 and oxidative demethylation. This is the pathway which metabolizes phenobarbital. Other enzyme activities actually decrease in specific activity. A proliferation of smooth ER membranes is seen after the phenobarbital treatment but it appears from the limited data

available (Orrenius and Ericsson 1966) that no areas of the membranes contain exclusively these induced enzymes. Since the rate of synthesis of the induced enzymes is increased and the rate of synthesis of the other ER enzymes is unchanged (Jick and Shuster 1966, Kuriyama et al. 1969) the newly-formed membranes probably contain an increased proportion of NADPH-cytochrome c reductase and cytochrome P450. When the drug treatment is discontinued the newly-formed smooth ER remains for several days after the enzyme activity has returned to normal. This suggests that the membrane components are being turned over independently. Kuriyama et al. (1969), studying enzyme activities after a single injection of phenobarbital, found that the increase in the amount of cytochrome P450 lagged behind that of NADPH-cytochrome c reductase although the two are involved in a common pathway.

All of these studies support the same type of model of membrane assembly that is suggested by the work presented in this thesis. Membranes seem to be assembled through a molecule-by-molecule insertion or replacement into a pre-existing structural framework. Exactly how this insertion is accomplished is unclear. A correlate to the theory is the existence of recognition sites which determine the final assembly; the enzyme components of a single pathway are probably located together in the membrane. Possibly the lipid, as well as the protein, plays a role in this recognition, for there are known differences in lipid composition of various membranes (Fleischer and Rouser 1965, inter alios). In addition, studies by Duttera et al. (1968) and Ganoza (1964) have shown that phospholipids can affect the substrate specificity of G6Pase. Other work (Fleischer et al. 1962, Jones and Wakil 1967) have associated certain lipids with enzymes, while Widnell and Unkeless (1968) have found a specific lipid (sphingomyelin) associated with a single membrane protein (5'-nucleotidase).

The model of membrane assembly outlined above does not exclude the existence of functionally distinct units within membranes. Molecules need not be inserted at random into the pre-existing framework. The model does rule out the formation of large regions of membrane where all of the constituents -- lipids, enzymatic proteins and possibly "structural proteins" -- are assembled concurrently.

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