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PARTICULATE GLYCOGEN

a correlated electronmicroscopical and biochemical study

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and biochemical study

PROMOTOR: PROF. DR. CH.M.A.KUIJPER

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PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR
IN DE WISKUNDE EN NATUURWETENSCHAPPEN
AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN,
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OP DONDERDAG 16 DECEMBER 1965
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ADRIANUS MARTINUS STADHOUDERS
GEBOREN TE BOXTEL

1965
THOBEN OFFSET NIJMEGEN

Reality is the embodiment of structure;
Structures are the embodiment of properties;
Properties are the embodiment of harmony;
Harmony is the embodiment of congruity.

Written by Ts'ao T'ien-ch'in (Fourth century B.C.)
Translated by Gustav Haloun (1951)

Ter nagedachtenis aan mijn Moeder

Aan mijn Vader

Voor Jeanny en de kinderen

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GENERAL INTRODUCTION

In 1942 Lazarow isolated a submicroscopic particulate component from guinea pig liver cells, which on a dry-weight basis, contained about 93% of glycogen. The term "particulate glycogen" was introduced for these cellular elements. It was thought that the particulates exist as such in the liver cells. Definite proof, however, could not be forthcoming until appropriate high resolution microscopy would show them in a recognizable form in situ, the appropriate technique naturally being electron microscopy. Early attempts of Morgan and Mowry (1951) were rather unsuccessful, principally because at that time the methods of handling biological material for this microscopic technique were still inadequate.

Thanks to the recent refinements in electron microscopic instruments and preparation techniques, cytologists are well equipped for a detailed analysis of the submicroscopic organization of the cell; the resolving power of present-day microscopes (better than 10Å) goes down even to the molecular level and thus offers possibilities of relating morphological and chemical structures.

Nevertheless, Lazarow's proposition has not been definitely confirmed up to now; this is illustrated by the fact that in the literature descriptions of cell-areas containing glycogen differ greatly from one author to the other. Recently Foster (1960) described the identification of glycogen in electron microscopy as occurring through "a process of elimination - the irregular areas and particles of low density which have been identified as this material are clearly dissimilar to any of the recognized cytoplasmic organelles such as mitochondria, microsomes etc."

The deeper ground of this unsatisfactory situation is to be found in the fact that cellular ultrastructure, as revealed by electron microscopy is essentially a "membrane-morphology". Indeed, the membranous components of the cell, in their characteristic arrangements of lamellar, tubular or vesicular structures, clearly express the "many-phasedness" (Ruska, 1961) of the cytoplasm. In the cellular organelles, like mitochondria or the Golgi apparatus, the membrane is mostly arranged in a specific way. The identification of these elements in electron micrographs usually causes little trouble.

The situation is quite different with regard to those cellular constituents that are not built up from membranes. In some cases the histological origin of the object under examination can function as a reliable parameter for identifying certain structures, e.g. myo-

fibrils in muscle cells, but this does not hold for glycogen, which can occur in all types of cells. Yet the ability to recognize glycogen at the subcellular level would be of great potential value for both normal cytohistology and pathology; not only because very small quantities might be detected, but even more so on account of the fact that a precise intracellular localization would become possible (Biava, 1964).

The urgent need for a reliable identification becomes all the more clear if one considers the fact that a particular granular component of livercell plasm is interpreted by several authors (Millonig and Porter, 1960; Biava, 1963, 1964; Revel, 1964) as particulate glycogen; but is regarded by others as representing virus-particles in cases of infective hepatitis (Bearcroft, 1962; Bearcroft and Peachey, 1962).

Still other workers have identified similar particles as aggregated ribosomes or polyribosomes, for example Björkman (1964) while studying pig liver cells and dogliver cells when inoculated with H.c.c. (Hepatitis contagiosa canis) virus, and McGavran and White (1964) who found them in monkey liver cells after infection with yellow-fever virus.

The first part of this thesis will deal with the methodological aspects of a procedure for the selective identification of glycogen in the electron microscope. It will be introduced by a short survey of the molecular structure and the in vivo state of glycogen (Chapter 1). The methods of identification already proposed in literature will be reviewed critically and a partially new method will be introduced, which we found to be suited for differentiating between glycogen and structures containing nucleic acids (Chapter 2). Some quantitative aspects of fixation of glycogen by means of histological procedures in electron microscopy will be studied in Chapter 3, together with some chemical aspects of the "staining" with lead. The morphology of glycogen as it appears in various tissues will be compared with that of chemically well-defined particulate glycogen isolated by centrifugation techniques (Chapter 4). This gives the opportunity to study the effect of chemical extraction procedures on the morphology of particulate glycogen, by correlating sedimentation diagrams with electron microscope pictures of the same material.

Chiefly as a consequence of the difficulties mentioned above with regard to the identification of glycogen at the subcellular level, the literature is rather contradictory in its descriptions of functional relationships between cell-organelles and glycogen metabolism. Thus Carasso (1960) believes that ergastoplasm is involved in glycogenesis. Themann (1963) on the other hand points out that there is an inverse relationship between RNA-content and glycogen-content

in liver cells, and states that in areas rich in ergastoplasm no glycogen is present. Porter and Bruni (1959) in the course of a study on the effect of azo-dyes on rat liver cells, perceived the presence of a membrane system in those areas of the cytoplasm that are rich in glycogen; this system was identified as "smooth endoplasmic reticulum". Changes in the appearance of this structure during periods of fasting and refeeding led them to conclude that the system has a function in glycogen storage and glycogen depletion.

Peters (1962) came to the conclusion that this smooth-surfaced system is only involved in glycogenolytic processes and not in glycogenesis.

Evidence, presented by Luck (1961), suggests that UDP-glucosyl-glycogen synthetase distribution in cell fractions of rat liver can be attributed to the binding to glycogen rather than to the association of this enzyme with membranous elements. Anderson-Cedergren and Muscatello (1963), however, isolated the sarcoplasmic reticulum of frog skeletal muscle cells and found that the synthetase enzyme was bound to the membranes of this reticular system.

The question whether or not the endoplasmic reticulum of the hepatic cell is somehow involved in synthesis and storage, or in degradation and depletion of glycogen has been restudied both by electron microscopical and biochemical analysis, through the perinatal changes in glycogen reserves in the rat as experimental object. Towards the end of fetal life glycogen appears abruptly in the liver and accumulates to at least twice the concentrations found in adults. Immediately after birth glycogen content falls very rapidly, leaving the cells almost depleted within the first 12 hours. Thus this perinatal period offers an optimum for studying ultrastructural aspects of glycogenesis and glycogenolysis separately. This perinatal period seems all the more favourable as an object for study because evidence has been brought forward that bile pigment metabolism i.e. bilirubin conjugation may interfere with glycogen metabolism. For example Halac and Stuart (1960) found that the enzyme glucuronyl-transferase, which transfers the glucuronic acid moiety from uridine-diphosphate glucuronic acid (UDPGA) to bilirubin, was bound to glycogen. Other workers found that this enzyme activity is greatest in the microsomal fraction. Due to the functional incapacity of the fetal liver to clear bilirubin (Grodsky et al, 1958), in the object chosen, no interference in this respect is to be expected.

Similar considerations apply to the so-called "detoxification mechanisms" that take place in the adult liver, but which are still undeveloped in the fetal liver. This seems especially important because the sensitivity to various drugs of the endoplasmic reticulum of liver cells is well-known (Remmer and Merker, 1963; Stei-

ner et al. 1964).

For this reason studies making use of toxic substances for the study of glycogen metabolism are not readily interpretable.

Moreover, extremely long fasting periods,- as used by Millonig and Porter (1960) and Themann (1963) in their experimental arrangements for obtaining glycogen depletion - are at least unphysiological and may ultimately induce pathological changes in the liver cells. Postnatal glycogen depletion is not known to go with such pathological alterations.

Especially the findings mentioned above of Halac and Stuart (1960) have led us to include in our studies an analysis of the jaundiced Gunn rats, which are known to have a deficiency in glucuronyltransferase (Van Leusden, 1963).

The second part of this thesis can be outlined as follows: Chapter 5 summarizes recent knowledge of the biosynthesis of glycogen, and discusses in more detail the evidence that has been brought forward of cytological structures being implicated in glycogen metabolism.

The data on the beginning of glycogen accumulation in the fetal rat liver differ somewhat in literature; our findings are reported in Chapter 6. In Chapter 7 the pattern of differentiation of the enzymes active in glycogen metabolism is studied. Chapter 8 continues this analysis of hepatocyte development with an investigation of the structural development of the hepatocyte during the perinatal period.

PART I

CHAPTER 1

THE MOLECULAR STRUCTURE OF GLYCOGEN

1-1. INTRODUCTION

Glycogen was isolated for the first time by Claude Bernard (1857) and denominated "matière glucogène animale". It is the most important storage carbohydrate of animal organisms and can be found in almost all types of cells, but is found especially in liver and muscle cells.

Glycogen is a polymer composed exclusively of D-glucose residues, which are interconnected through α-1,4 glucosidic bonds. In the chains thus formed branching points arise through α-1,6 glucosidic linkages.

This basic chemical structure of glycogen is closely related to that of starch, which is the storage carbohydrate of the vegetable kingdom. Starch, however, is composed of a mixture of two polysaccharides, namely amylopectin, which - like glycogen - is a multi-branched polysaccharide, and the essentially linear polysaccharide amylose.

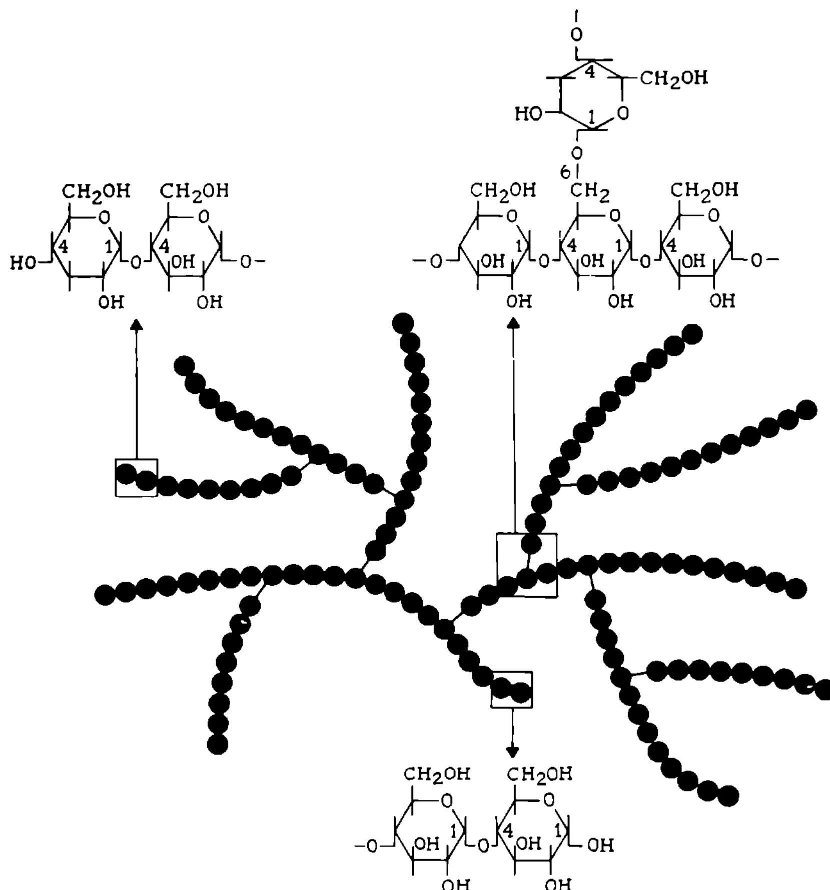
Glycogen differs from the amylopectin component of starch only in the relative degree of branching. The ratio of α-1,4 to α-1,6 bonds in glycogen lies between 12 and 18, whereas this ratio varies in amylopectin from 24 to 30. In the Cori-type IV glycogen storage disease (Andersen's disease or "amylopectinosis") with a suggested defect in amylo-(1,4 $\longrightarrow$ 1,6)-transglucosidase or branching enzyme, the liver and muscles contain an amylopectin-like polysaccharide with relatively long inner and outer chains (Sidbury et al. 1962; Illingworth and Brown 1964).

According to Wolfrom and Thompson (1957) a small percentage of α-1,3 glucosidic linkages whould be present both in glycogen and in amylopectin, but their experimental arguments have been criticized (Manners, 1962; French, 1964).

Glycogen belongs to the largest polymers that occur in nature. The multi-branched macromolecules consist of several thousands of D-glucose chains. Given a molecular weight of about 5×10^6, such a molecule contains about 31.000 glucose units and about 2500 α-1,6 linkages. Three schematic designs for the interpretation of the molecular structure of glycogen have been presented in literature: the "laminated" form, proposed by Haworth et al. (1937); the Stau-

dinger and Husemann (1937) "comb"-form and the "tree"-form as suggested by Meyer (1943).

Most authors now agree that the Meyer depiction of the molecular architecture as a bush-like structure conforms best to reality: see textfig.1.



Textfig.1. Structure of the glycogen molecule according to Meyer (1943), modified. Every circle represents a D-glucose residue. Direct apposition of the circles indicates α-1,4 linkages; the α-1,6 interchain linkages are depicted as short lines. Some segments, i.e. the reducing endgroup, a non-reducing endgroup and a branching point, are enlarged to show chemical structure.

It can easily be seen from this scheme that the regularity of the branching may range from an absolutely regularly branched "ideal" situation to a completely random structure ("haphazard Meyer model", French, 1964). In the following paragraph we will briefly discuss

some of the aspects of glycogen fine structure and the methods to study it.

1-2. BRANCHING PATTERN, AVERAGE CHAIN LENGTH

Besides the total number of glucose residues present in a glycogen molecule and the character of the linkages between the glucose monomers, a third important feature of the molecular structure of a branched polymer like glycogen is the way in which the branching points are distributed along the chains; in other words: the branching pattern (Remarque, 1958).

In glycogen this distribution of branching points is essentially a random one. Yet the molecular architecture of glycogen can be described in terms of the following three parameters:

a. average chain length
b. exterior chain length
c. interior chain length (Kjölberg et al. 1963).

An exterior chain is the part of a linear α-1,4 chain between a terminal glucose residue and the most peripheral branching point on that chain. An interior chain is the part of a chain between two successive branching points.

The average chain length $\overline{CL}$ is defined as the quotient of the number of glucose residues present and the number of non-reducing terminal residues.

Since the molecule is the more highly branched the more the length of the chains decreases, the branching pattern is determined by the mean length of the exterior and interior chains; that is: by the average number of glucose residues in these chains. Degree of branching and average chain length therefore are reciprocal magnitudes. A linear, unbranched α-1,4 glucose-chain consists of one terminal or exterior chain. With the introduction of one branching point, two exterior chains arise. With every following branching point one new exterior chain is formed and one new interior chain. More generally it follows that $E = I + 2$, in which E and I represent the number of exterior and interior chains respectively. In the case of a very large molecule both numbers can be taken to be equal: $E = I$.

The number of exterior chains equals of course the total number of non-reducing terminal glucose-units E.

Let the total number of glucose residues present in a molecule be N (degree of polymerization), then the average chain length is by definition: $\frac{N}{E}$.

When the total number of glucose units in the exterior chains is indicated by M, then the average number of glucose units in these

exterior chains is: $\frac{M}{E}$.

Thus there remain for the interior chains N - M glucose units, from which it follows that the average number per interior chain equals $\frac{N - M}{I}$, or (because I = E) $\frac{N - M}{E}$.

The average number of glucose units per exterior chain plus the average number of glucose units per interior chain is therefore:

$$\frac{M}{E} + \frac{N - M}{E} = \frac{N}{E}\text{, that is: equal to } \overline{CL}.$$

If the peripheral branching point is taken to belong to the exterior chain, as is done by Kjölberg et al. (1963), then the total number of glucose units in the interior chains is N - M - E (because there are as many branching points as there are exterior chains). The average number of glucose units per interior chain is in that case

$$\frac{N - M - E}{I}\text{, or, because } I = E,\ \frac{N - M - E}{E}$$

The sum of the average numbers of glucose-units in the interior and exterior chains is then:

$$\frac{M}{E} + \frac{N - M - E}{E} = \frac{N - E}{E} = \frac{N}{E} - 1$$

The average chain length $\frac{N}{E}$ thus equals the sum of the average number of glucose units per interior chain plus 1.

According to the definition, used by Kjölberg et al. (1963), the average chain length equals the statistical average of the lengths of the linear α-1,4 chains. In textfig.1 there are ten α-1,4 chains. Two of them have 1 exterior and 2 interior chains. Four linear chains have 1 exterior and 1 interior chain and there are 4 short linear α-1,4 exterior chains without an interior chain. Taken together, therefore the 10 linear α-1,4 chains are composed of 10 exterior and 8 interior chains. When a new exterior chain is introduced into such a molecule, it necessarily brings about a new interior chain. One might state therefore that n α-1,4 linear chains consist of n exterior and n interior chains (in which n should be taken to denote a high number). Because there are many more short terminal α-1,4 chains than there are long chains, - which can be seen from textfig.1 if one places another branching point on every free terminal chain -, it follows that the average length of the linear α-1,4 chains equals the sum of the average lengths of the exterior and interior chains. In other words

the average length of the linear α-1,4 chains equals the average chain length.

According to Remarque (1958) the average chain-length $\overline{CL}$ is twice the average length of the linear chains. This conclusion, however, is based on an inconsistency in using the symbol K for the total number of chains present. The author uses this symbol to denote, on the one hand, the sum of the number of exterior and interior chains, on the other hand to denote the total number of linear chains present. Remarque's statement is, of course, only true if by "chain" is understood either an interior chain or an exterior chain.

Estimation of the average chain-length (as a measure of the branching pattern of glycogen) amounts to a determination of the number of non-reducing terminal glucose units. For this purpose several methods have been proposed in literature. They can be divided into strictly chemical methods, like the methylation or periodate oxidation methods, and into enzymatic methods.

1. Methylation of glycogen

In such procedures all free hydroxyl-groups of the polysaccharide are converted into methoxy-groups. This is followed by hydrolysis of the product and an analysis of the hydrolysate for the various methylglucosides, particularly for the content of 2, 3, 4, 6-tetra-methylglucose which arises from the terminal glucose residues.
More commonly employed are the

2. Periodate oxidation methods

Through oxidation of the glucopyranose ring, various oxidation products arise, dependent on the position of the OH-groups in the ring-structure (see Remarque, 1958). From the reducing terminal glucose residues as well as from the non-reducing ones formic acid is produced. Since only one reducing group is present in each glycogen molecule, the amount of formic acid that arises from these groups can be neglected when compared with the amount that is produced from the remaining terminal residues. Thus the yield of formic acid is a measure of the number of exterior chains.
Manners and Wright (1961) have pointed out that such estimates require precise conditioning of the experimental procedure. As far as can be gathered from the data published this procedure differs greatly from one author to the other. Therefore those $\overline{CL}$ values for glycogens from different sources, that have been published can hardly be compared with one another.

Manners and Wright found that a first period of rapid formic acid production is followed by a much slower, secundary reaction. Moreover, serious over-

oxidation can occur, dependent on temperature and relative excess of periodate. This also proved to be the case with the procedures developed by Polglase et al. (1952), which were used by Remarque (1958) to establish differences in CL between the two fractions of glycogen (see paragraph 2 of this chapter).

Manners and Wright therefore strongly argue always to combine such periodate oxidation measurements with

3. Enzymatic methods to analyse the molecular structure of glycogen

In the enzymatic procedures, as distinct from the chemical methods, the amount of terminal groups is not measured directly, but deduced from the amount of α-1,6 bonds present.

A depolymerisation of glycogen is obtained through the simultaneous or alternating action of enzymes that attack the α-1,4 and the α-1,6 linkages respectively. In the method developed by Cori and Larner (1951), glycogen is broken down with the aid of the enzyme phosphorylase, which splits the α-1,4 bonds (liberating glucose-1-phosphate), and of the amylo-1,6-glucosidase or "debranching enzyme", which hydrolyses the α-1,6 linkages thus setting free glucose. The yield of glucose therefore is a measure of the percentage of α-1,6 bonds present and thus for the average chain length.

Recently Manners and Wright (1962) developed a method in which only the enzyme α-amylase is used. This enzyme brings about a random hydrolysis of the α-1,4 bonds present in the molecule, but has no effect on the α-1,6 linkages.

The exterior and interior chain lengths can also be determined enzymatically, for instance with the aid of β-amylase. This enzyme causes a stepwise hydrolysis of the α-1,4 linkages only. Moreover, this degradation remains restricted to the exterior chains of the molecule.

The products of this hydrolytic process are maltose and a high molecular β-limit dextrin. In this limit dextrin all the branching points are still present. What is left over of the exterior chains still contains 2 or 3 glucose residues. Given a certain average chain length, the percentage of maltose liberated is a measure of the average exterior chain length.

For a more detailed description of all these methods, the reader is referred to the original literature or to Manners (1957).

When interpreting the results of such estimates of the branching pattern, it should be kept in mind, that the figures obtained always represent mean values. When for example the average interior chain length is stated to be 3 - 4, this does not imply that the chain lengths are uniform. The variation may still be quite considerable. Unfortunately, the methods mentioned above do not give information as to the extent of this variation. As far as we have found out, Wolfrom (1956),

is the only one to have remarked upon it. He interpreted the production of isomaltotriose after acidic fragmentation of glycogen, as indicating that branching points may actually occur in adjacent glucose residues. This would imply that the branching points may occur also much father apart from each other. Whether or not this interpretation is correct, has not been settled up to now. French (1964) believes that the occurrence of isomaltotriose (and nigerose) may arise in part from acid-catalysed intramolecular transfer reactions, "owing to the locally high carbohydrate concentrations inherent in even dilute solutions of branched polysaccharides".

We have treated these analytical procedures to some extent because several investigators have drawn from their results far-reaching conclusions as to structural differences between glycogens of different origin or isolated after various feeding experiments. For example Schlamowitz (1951) found different $\overline{CL}$-values for rabbit liver glycogen isolated after normal feeding and after D-galactose infusion ($\overline{CL}$-values 19 and 23 resp.). Remarque (1958), using the sodium periodate oxidation procedure, found $\overline{CL}$-differences between the lyo-fraction and residual-fraction of rat liver glycogen (see par.1-3).

However, as has been pointed out already, most of the results on glycogen fine structure that have been recorded, are difficult to compare due to considerable variation in experimental techniques. It is therefore of interest to reveal some of the findings, which have recently been obtained by Kjölberg et al. (1963). In this thorough investigation 32 samples of glycogen from 13 different species of animals were examined by means of the following methods of analysis: enzymatic degradation, iodine staining and the concanavalin-reaction.* Part of the samples originated from other laboratories and had been described earlier. The authors found that the majority of the samples had an average chain length of 12-15 glucose residues, whereas the molecular weight values ranged from 5 to 100 x 10^6. They did not find an abnormally low degree of branching of rabbit liver glycogen after D-galactose administration. In this respect their results differ from those of Schlamowitz. It was also found that, as opposed to the findings of Remarque (1958), liver glycogen is chemically not heterogeneous. The lyo- and desmoglycogen fractions, isolated from the livers of rabbits that had as well as of rabbits that had not been fed, showed no differences in $\overline{CL}$. The authors conclude that "the results reflect a remarkable consistency in the relative activity of the chain-forming enzyme-system (presumable UDP-glu-

* The concanavalin reaction, giving a certain "glycogen-value", is based upon measurements of turbidity, produced by the interaction of glycogen with the protein concanavalin A. It appears that these glycogen values are approximately related to the average chain length and the exterior chain length of the glycogen under study (Manners and Wright, 1962).

cosyl glycogen synthetase, see chapter 5) and the branching enzyme during glycogen synthesis".

Thus stated, however, this conclusion can only be partly correct since it leaves out molecular structural aspects of the substrate-enzyme complexes involved. Indeed French (1964) argues that the actual detailed structure of glycogen is primarily a reflection of the steric specificity of the branching enzyme. He gives evidence that the α-1,6 bonds are thermodynamically more stable than the α-1,4 glucosidic linkages and therefore rejects the statement made by Myrbäck and Sillen (1949) that glycogen (with less than 10% α-1,6 bonds) would represent an equilibrium between α-1,4 and α-1,6-linkages. When glycogen is brought to an equilibrium mixture by a yeast branching enzyme, this has no noticeable effect, whereas amylopectin is readily converted into a glycogen-like polysaccharide. This indicates, according to French, that in the case of glycogen a large proportion of the α-1,4 links cannot participate in the branching reaction for steric reasons. Through enzymatically mediated linear elongation, the outer chains have to attain to a critical length before they correspond to the steric specificity of the branching enzyme. Assuming the branching enzyme to act by transferring a part of one chain to another chain, French states that "in all probability the outer chain lengths cover a range between the shortest possible chains that fall within the specificity of the branching enzyme and the longest chains which are just too short to serve as donors in the branching reaction."

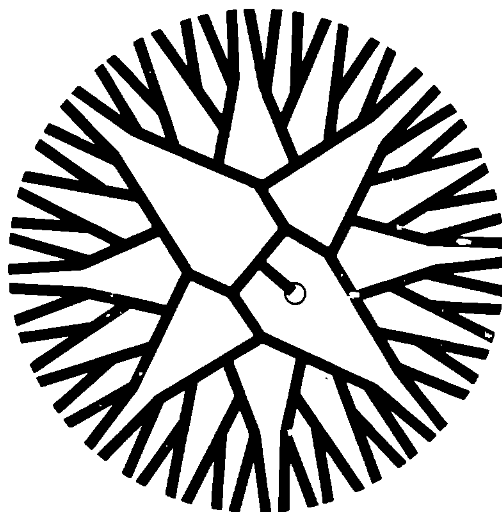
Another point that might be of importance is the likelihood that, when the glycogen molecule is growing, the space available for further branching might be consumed. Indeed it has been suggested that glycogen, with respect to size, must be considered to be a self-limiting structure (Madison and Cori, 1958).

If the Meyer-depiction of the glycogen molecule as a regularly branched, treelike structure is strictly adhered to, it is easy to understand that the periphery of the molecule becomes more and more crowded as the molecule grows in size (textfig.2). When a number of approximations are made, maximum molecular weight and maximum diameter can be calculated. We need not go into detail to summarize the conclusions drawn from such calculations.

Pollard (1958) stated that the limit molecular size was somewhere between 130 Å (Mol. Wt. 8 x 10^6) and 200 Å (Mol. Wt. 10^9). Stetten (1961) calculated that the largest number of completed tiers (cf. fig.2) possible in such a regularly branched spherical molecule ($\overline{ICL}$ =4; $\overline{ECL}$ = 8) is 17, with a molecular weight maximum of 138 x 10^6.

French (1964) arrived at a molecule radius r = 180 Å and a molecular weight of 20 x 10^6. This implies that much larger molecules

must have a number of interiorly terminating chains. Due to this sterical protection such chains might be very resistant to enzymic action.



Textfig.2. Idealized Meyer-model of a glycogen molecule, having 5 successive tiers of glucosyl residues. The crowding at the periphery is apparent. 'O' indicates the free reducing endgroup. After French (1964).

Of course such calculations can hardly take account of the fact that the glycogen molecule is highly hydrated (approximately 2 g of water per gram of polysaccharide) whereas they also do not account for the fact that a number of space-consuming enzyme-proteins may be firmly attached to the molecules (see page 25).

Moreover, from a physiological point of view, it may prove to be more sensible to consider glycogen as a biological unit rather than an organic chemical unit.

The question arises to what extent electron microscopy may be helpful in clarifying the glycogen fine structure. The answer must be rather disappointing. Even if there would be no instrumentally conditioned restriction to resolve such tiny structures as the individual chains or the branched twigs, there would still remain the difficulty of adequate preparation of the structure. This would require a flattening down of the individual chains on the supporting film, without overlapping, for study by means of shadowcasting or negative staining techniques, but this does not seem achievable. As matters stand at present, electron microscopy is, at best, capable

of dealing with what one may call in analogy to protein-molecular configuration, the "quarternary structure" of glycogen: the way glycogen particles are possibly built up from sub-units.

1-3. STATE OF GLYCOGEN IN THE LIVING CELL

There exists an extensive literature on the problem raised by the state of glycogen in the living tissues. Especially the problem of the easily extractable, so-called free or lyo-glycogen against the cold-acid-nonextractable, residual or desmo-glycogen has been the subject of numerous studies since Pflüger's (1903) original approach to this puzzle.

According to a great many authors different solvents extract different amounts of glycogen from tissue. For example water or dilute trichloroacetic acid (TCA) extract only a part of the glycogen present, whereas hot concentrated alkali removes all of it. The difference in the solubilities of the two forms of tissue glycogen has been attributed to many factors. For example, the suggestion has been made that residual glycogen is protein-bound, which might explain the insolubility in TCA. The older literature on this subject has been thoroughly reviewed by Remarque (1958) and is not further dealt with here. His own studies are essentially a verification and a further elaboration of the findings of Bloom et al. (1951). The conservative character of the residual fraction could be confirmed under the experimental conditions which Remarque used. It was found that this fraction tends to maintain a nearly constant level, except when the amount of "free" glycogen falls below approximately 0,5%. The readily extractable portion on the contrary shows wide quantitative variations. Recently, Brodskaya (1964) reported that both in liver and in brain, the bound fraction increases somewhat with age.

In his attempts to characterize the two fractions of glycogen as regards structure and properties, Remarque found that the residual glycogen was less soluble in water, gave more opalescent solutions and showed a different absorption spectrum with iodine. From researches made by means of the Polglase periodate oxidation procedure (criticized above), Remarque quoted the average chain length of the residual glycogen as being 30% shorter on an average than the $\overline{CL}$ of the TCA-extractable fraction. From sedimentation experiments he concluded that the residual glycogen was more polydisperse and contained considerably lighter molecules than did the TCA-glycogen.

Remarque also pointed out again the importance of the temperature with regard to the yield of the TCA-extraction. About 40% of the amount of residual glycogen determined at 0 - 3°C passed into the TCA-portion when the extraction was carried out at 37°C. Obviously, the

choice of a particular temperature is arbitrary, as is in fact also the choice of TCA as the extraction fluid. It follows from the studies by Carroll et al. (1956), Hanson et al. (1960) and Roe et al. (1961) that the same holds true for the degree of homogenization of the tissue. Carroll and co-workers found that repeated homogenization with 5% TCA increased the recovery of glycogen from liver and muscle. As they could show, moreover, that some of the material, extracted by KOH and measured by anthrone, was dialyzable and appeared to decompose after prolonged digestion in KOH, the reality of the two fractions was seriously questioned. The suggestion was made that the distinction between "free" and "fixed" glycogen is simply the result of inadequate homogenization. (Koritz and Munck, (1960) could not confirm the decomposing-effects on the residual fraction of prolonged hot-KOH-digestion).

Hanson and co-authors (1960) also found different extractabilities of the glycogens depending on the agent employed (hot and cold TCA; hot and cold water) but they stated that if the homogenization of the tissue is not thorough enough or if the amount of solvent used is insufficient relative to the tissue, the results might be distorted.

Even more serious objections were advanced by Roe et al. (1961). They showed that after homogenizing the tissue at 45.000 rpm in the presence of glassbeads, the bound fraction was completely set free in TCA. They therefore concluded that a distinction between free and bound glycogen, based upon non-extractability with cold TCA, is no longer tenable and, in addition, that glycogen is not bound in the tissue in the sense of being held by a bond that is not broken by cold TCA.

As a matter of fact such a treatment is very drastic. It is quite conceivable that large proteins or protein-glycogen complexes will be destroyed (Kits van Heijningen, 1963). This will certainly be the case with mitochondria or sarcosomes, so that by such vigourous dispersion-methods possibly "physically" detained glycogen, too, is released through rupture of cellular organelles. Roe and his co-workers themselves do not deny that there exists in the tissues some glycogen that is protein-bound. This is already to be supposed from the generally accepted enzyme-action hypothesis.

It is well-known that glycogen readily associates with the enzymes involved in its metabolism. The Michaelis constant for the glycogen rabbit-muscle phosphorylase association is, according to Cori, Cori and Green (1943), 1.3 mM (expressed in glucose units).

Recently Selinger and Schramm (1963) demonstrated that muscle phosphorylase a as well as b interact with soluble glycogen to form an insoluble enzyme-substrate complex. The glycogen-phosphorylase association in liver has been shown by Sutherland and Wosilait (1956) seriously to hinder the purification of the enzyme. High concentrations of glycogen were present in the subcellular fraction containing the enzyme UDP-glucosyl glycogen synthetase

(Leloir and Goldemberg 1960). Luck (1961) could show that the activity of this enzyme in his preparation was parallel to its glycogen content.

Non-enzymatic association of glycogen with proteins can also occur, e.g. with serum globulin, myosin, concanavalin A etc.

From the above it follows that one may agree with Stetten and Stetten (1960) that the subdivision of cellular glycogen in two fractions, based upon the extractability or non-extractability by cold TCA is arbitrary. Indeed the commonly employed method of differential solvent extraction does not fulfil the requirements of an experimentally well defined approach to the problem of the condition of glycogen in the living cell.

However, to describe these methods of analysis as "totally inadequate to supply a final answer" is in our opinion, to take a rather sombre view of things. A great many workers agree as to their experience that a variety of nutrional, endocrinological or other experimental conditions (for a review: see Stetten and Stetten, 1960) induce shifts in the TCA-extractable portions, but leave the residual fraction unattacked, at least initially. It is the present writer's opinion that all this evidence already justifies the conclusion that tissue-glycogen is metabolically not homogeneous. The real significance of the problem lies in the question whether or not the "residual" glycogen is physiologically distinct from the "free" glycogen. Albeit that the residual fraction does not go through abrupt quantitative changes, this does not mean that this fraction is metabolically inert. On the contrary, it has been claimed that residual glycogen is metabolically the more active of the two, as was deduced among other things from tracer experiments.

After intraperitoneal injection of a single dosis of glucose-^{14}C, in short-term experiments from 20 min. up to 3 hours, Stetten, Katzen and Stetten (1958) invariably found a higher specific activity in the residual fractions derived from liver and skeletal muscle of the rat. Brodskaya (1963), studying the rate of glycogen metabolism in the brain and liver of rats of different ages, also found that the specific activity of the bound glycogen always considerably exceeds the specific activity of the free glycogen. In these experiments the radioactive glucose was administered 1 hr before analysis of the fractions. She concludes that the results prove "beyond any doubt that there is a difference in the metabolic activity of the two fractions, namely, that the lability of the bound fraction is higher".

Singh and Venkitasubramanian (1963) also believe that the residual glycogen is metabolically more active than is the TCA-extractable glycogen. They could show synthesis of both free and fixed glycogen from the non-carbohydrate source glycine-1-^{14}C in guinea pig liver. Three hours after one single intraperitoneal injection of

glycine-1-^{14}C the specific activity of residual glycogen was approximately nine times as great as that of the free glycogen portion.

Results which appeared difficult to reduce to the same denominator were gained by Kits van Heijningen (1963) in in vitro experiments with rat diaphragms. After incubation periods of no more than 1 and 2 min the fixed glycogen fraction had a higher total as well as a higher specific activity, but after 7 min or more there was no longer a difference between the labelling of the two fractions. From then on the specific activity of both fractions increased at about the same rate. When insulin was added to the incubation-medium, causing an increase in free glycogen content, the specific activity of both fractions increased but now with a much more pronounced effect in the free portion. When the influence of insulin was combined with that of adrenalin, it appeared that the fixed fraction increased in content but showed a decrease in specific activity, "presumably due to a dilution of the fixed portion with inactive glucose from an unidentified source".

After ample discussion especially of the value of tracer studies in dealing with the metabolism of such a complex molecule like glycogen, the author concludes that her findings both with and without insulin addition to the incubation-medium are compatible with the view that fixed glycogen is the precursor of free glycogen. Indeed such a supposition can explain the change in relative specific activity with time. But the combined insulin-adrenalin effects are apparently incompatible with this hypothesis.

As a matter of fact completely opposite findings were reported by Figueroa and Pfeifer (1962). After in vitro incubation of slices of rabbit livers with radioactive glucose, the TCA-extractable fraction showed a greater incorporation of radioactivity than did the residual glycogen. A 2 to 3 times higher specific activity of the free fraction relative to the fixed fraction was already established as soon as 10 min. after incubation. The specific activity-time curve was a straight line for both fractions up to 1 hr of incubation. However, experiments of very short duration as in the study of Kits van Heijningen were not performed.

The authors suggest as a possible explanation for the discrepancy between their results and those of Stetten et al. (1958) that their experiments were conducted in vitro but they do not argue this statement.

Nevertheless, this aspect may be of importance, as will be illustrated.

The same authors (Figueroa et al. 1962) obtained glucose-^{14}C incorporation into glycogen when working with a crude liverhomogenate. Addition of yeast hexokinase increased the formation of $^{14}CO_2$ from radioactive glucose but did not modify the incorporation into

glycogen. These findings were taken to indicate that phosphorylation of glucose is not necessarily a step towards glycogen synthesis. Similarly Nigam and Friedland (1964), using pigeon-liver homogenates, showed that the percentage of conversion of glucose to glycogen is unaffected by the glucose concentration, whereas addition of inactive G-1-P, G-6-P or UDPG showed no decrease of glycogen-labelling as might be expected from isotopic dilution. Apparently under such in vitro conditions, the UDPG-linked pathway, believed to be the in vivo pathway to glycogen synthesis, is circumvented.

Indeed, Hers et al. (1964) made it very probable that the incorporation under these circumstances is due to the activity of the enzyme amylo-1,6-glucosidase, which under normal conditions is active in the glycogen catabolic pathway. Of course, the findings gained from a crude homogenate cannot be extrapolated to those obtained by the use of liver slices. Nevertheless it may illustrate the point that in vitro findings should be carefully regarded before a comparison with in vivo findings are made.

This holds true especially when glycogen metabolism is studied since in these objects glycogen synthesis requires mitochondrial ATP generation (which is easily disturbed in vitro) and is balanced by hormonal factors (also easily upset in vitro).

The results, obtained in vitro from liver or muscle-tissue need not be comparable with each other either.

It is well known (Sols, 1964) that the livercells are freely permeable to glucose, whereas in muscle there is a insulin-governed control of transport.

Another point to be taken into account is that the muscle cells are more highly compartmentalized than the liver cells, which may be of importance in view of the suggestion made by Lourau and Meyer 1958 that the residual glycogen is located inside the cell in a site less accessible to exogeneous glucose.

The problem of the metabolic heterogeneity of cellular glycogen has also been approached from a somewhat different angle.

Stetten and Stetten (1958) showed that the rate of incorporation of glucose-^{14}C into glycogen depends on the molecular weight of the glycogen. When liver glycogen was studied, an inverse correlation between molecular weight and incorporated activity was found; that is: the glycogen molecules of greatest radioactivity were found in the fractions having the lowest average molecular weight.

On the other hand, when muscle glycogen was analysed, the fractions of larger molecular size had incorporated the largest amount of radioactive glucose. The results were not influenced by the type of solvent used for the glycogen extraction (either TCA or KOH).

Colucci et al. (1964), too, found a differential incorporation of ^{14}C-labelled glucose into the high and low molecular weight glycogen, isolated from the parasitic tape worm *Hymenolepis* by means of a cold water extraction.

When the initial glycogen levels were low, through starvation of the host, the lower molecular weight fraction showed higher specific activity (Orell et al. 1964) which suggests that this fraction is synthesized at a more rapid rate. These differences in the specific activities of the two mol. wt. fractions were also reflected in differential labelling of the outer chains and in the phosphorylase limit dextrins of either fraction. It was therefore concluded that the higher molecular weight fraction could not have been formed artificially during the extraction-procedure, but must have been present in the living cells. This suggestion of the metabolic heterogeneity of cellular glycogen as expressing itself in molecules of different sizes, may prove to be a valuable one. Nevertheless, in this case too, additional studies (Colucci et al. 1965) revealed facts that are difficult to correlate with the statements mentioned above. When the mol. wt. fractions were analyzed for radioactivity while only a moderate or no preceding glycogen depletion was induced, the exposure to ^{14}C-glucose resulted in a greater specific activity of the high molecular weight fraction.

It is evident from the information quoted above, that no definite conclusions about the *in vivo* state of glycogen (or glycogens) can be reached at the moment. The results of analysis of the glycogen fractions with regard to their importance as physiological distinct entities, (as obtained after differential extraction of glycogen with TCA and KOH), appear to be contradictory. Since in all probability the methods used to isolate glycogen change its natural state, the need for direct *in situ* visualization is apparent. The next chapters will be devoted to such attempts.

CHAPTER 2

SELECTIVE STAINING OF GLYCOGEN IN ELECTRON MICROSCOPY

2-1. INTRODUCTION

Before we are going to describe in this chapter the possibilities of a selective staining of glycogen in electron microscopic specimens, it seems useful to state briefly some principles that govern contrast-formation in this type of microscope. It will appear that, strictly speaking, the use of the word "staining" in electron microscopic histology is not correct; in fact one should use the term "contrasting". In the literature, however, one is apt to employ the misnomer since the effect of the specimen-treatment is analogous to the results of staining a specimen for light microscopy. Both in light and electron microscopy the details of the object are visible as contrast-variations, present in the final image. In both microscope types this contrast is, of course, determined by the nature of the beam-specimen interactions. However, the way in which contrast arises and the mechanisms that determine contrast variations are distinctly different in either of these microscopic systems.

In the light microscope the differential absorption of light by the details in the object results in visible differences in the various parts of the image. Speaking of light as an undulatory movement one might say that the various details in the object decrease the amplitude of the incident lightwaves. These differences in amplitude are perceived by the eye as bright-dark differences and thus as contrast differences. Untreated light microscopic specimens contain few such "amplitude details". Therefore the sections are stained; that is: one effectuates a colour-contrast between the various object details. The differential absorption of light by the object then results - mainly on the principle of complementary colours - in amplitude changes between light of different wave lengths; and the eye percieves different colours. In the electron microscope, the part of the incident beam of electrons that is absorbed by the specimen - in other words, the number of electrons that "stick" in the specimen - is so small that this does not contribute significantly to image-contrast.

The main factors that influence this contrast in the electron microscope are: scattering of electrons in the object, phase-phenomena and

diffraction-effects. For the relative importance of each of these factors under different circumstances of specimen characteristics or instrumental conditions (like: homogeneity of the object, size of the object details in relation to resolution, crystalline conditions of object-parts, acceleration-voltage, optimum focus, etc.) the reader is referred to the studies by Hall, 1953; Reimer, 1959 and Magnan, 1961. Only the contrast that arises due to differential scattering of electrons in the object will briefly be discussed here. Under the impact of the electron beam upon the specimen, part of the electrons is transmitted without being changed, but the others are scattered due to the forces of attraction and repulsion that are exerted by the electrostatic charges of the atomic nuclei and electrons in the specimen. This scattering is either elastic, in which case the energy remains unchanged, or inelastic and accompanied by loss of energy.

The angle of deflection of the incident electrons, when passing an atomic nucleus, depends among other things on the charge of this nucleus, i.e. on the atomic number Z.

Those electrons that are scattered at a particular object-detail by more than the angle defined by the effective aperture of the objective lens, are trapped by the objective diaphragm and thus cannot contribute to the brightening of the relevant image-points on the screen. The result is variation of intensity at the image-plane.

The dependency of electron microscopic contrast on the atomic number of the object-components is a much more complicated problem than might be deduced from the above simple construction. In some theoretical (Lenz, 1954) and experimental studies (Hall, 1955; Reimer, 1959) it is stated that the contrast only depends on the mass-thickness (product of physical density and thickness), the acceleration voltage and the aperture angle and is essentially independent of the atomic number.

Indeed, Cosslett (1958), referring to the study by Hall (1955), points out that the rise in atomic weight A through the periodic system offsets the rise in "scattering cross section" of a particular scattering nucleus with increasing atomic number, so that the value of the scattering coefficient varies little with atomic number.

Since, according to the same author, it is the weight-for-weight uptake of "stain" that counts in enhancing contrast, it is not essential that the "stains" used in electron microscopy should contain a heavy element. Nevertheless, as there is a twenty-fold variation in physical density over the periodic table, so the preferential use of heavy elements for increasing the mass-density of particular structural elements in the electron microscopic specimen can easily be understood.

This also explains the widespread application of osmiumtetroxyde as the fixative chosen in electron microscopy. Besides the outstanding qualities as a fixative (i.e. a stabilization of the lipids and a precipi-

tation of the proteins), osmium also causes an appreciable increase in mass of the specimen (osmium is the densest element known) and thus an enhancement of the contrast in the electron microscopic specimen.

As a matter of course, the fluorescent screen or the photographic emulsion "translates" *the variations in contrast in the final image* into gradations from black to white. This limits, to a certain extent, the range of possibilities of staining tissue components differentially. If we define with Watson and Aldridge (1964) a specific staining procedure as the one in which only a single chemical grouping or tissue component is rendered visible, then all the other tissue constituents will remain invisible. In that case, however, a study of the cellular topographical interrelationships will be impossible since the other cellular elements are not depicted. Under such conditions the light microscopist can use a "counterstain"; i.e. he causes the other components to take up a different colour. Counterstaining in electron microscopy would mean that the other cell- or tissue-components are also made visible; this being possible only in the same grey-black gradations allowed by the image-translating system. The method to be preferred will therefore be a selective staining procedure, which will emphasize a particular component without preventing the other cellular elements from gaining some contrast, too.

In this chapter, the various methods proposed in the literature as being suitable for the staining of glycogen, will be critically examined.

2-2. EXPERIMENTAL PROCEDURE

2-2-1. Animals

The tissues used for these studies were: liver of adult guinea-pig; rabbit and adult and fetal rats; glycogen body of the chick; malpighian tubules and midgut of insects (Periplaneta americana). Some human liver biopsies were also included in these studies.

2-2-2. Fixation, Embedding

The tissues were fixed in OsO_4 - or glutaraldehyde solutions. Osmiumfixation was carried out with the use of veronal acetate (Palade, 1952) or phosphate buffer solutions; the latter were used because Millonig (1961) has claimed that this buffer has advantages for OsO_4 - solutions with respect to the preservation of glycogen. Palade's fixative has an osmolar concentration (the molar concentration, calculated from the total osmotic effect of all molecule - or ion-species present) of about 0,20 and already Palade noticed that his fixation-fluid was distinctly hypotonic when compared to blood-plasm.

The literature is contradictory as regards the importance of adapting the tonicity of the fixation-fluid for optimum preservation of the tissue. In our experience the quality of fixation of liver- and muscle-tissue (judged by the standards of the electron microscopic literature for a good fixation result) is not improved by adjustment of the tonicity of the fixation-fluid, for example by adding sucrose (0,045 gr/ml fixative, Caulfield, 1957).

It has recently been established that the addition of sucrose, or of dextran and sucrose, to collidine-buffered osmium-fixatives, causes a considerably greater extraction of proteins when compared with fixation-fluids from which these saccharides are omitted (Wood and Luft, 1965).

An obvious effect on the fixation result of adapting the tonicity of the fixatives employed was encountered when the malpighian tubular epithelium was processed. This well agrees with the observation, made by Wigglesworth (1953), that this system is especially sensitive to osmotic variations.

The same applies to the epithelium of the kidney proximal tubules (Pease 1955[a,b]).

The following reports served as a basis for preparing a theoretically ideal fixative.

1. Barsa (1954): insect tissue is highly tolerant of variations in the Na/K ratio, but sensitive to variations in the K/Ca ratio.

2. Ludwig et al. (1957): the hemolymph of Periplaneta americana is isotonic to a 0,224 M. NaCl-solution. In a Periplaneta-Ringer the Na/K ratio may vary between 3 and 30; the K/Ca ratio between 0,9 and 3,5.

3. van Asperen and van Esch (1956): the hemolymph of P. americana contains 360 $\pm$ 59 mg Na, 30 $\pm$ 5 mg K, 17 $\pm$ 1,9 mg Ca and 512 $\pm$ 40 mg Cl per 100 ml.

The following stock-solutions were prepared:

STOCK-SOLUTION A (buffer)	
Veronal - Na	14,7 g
Na - acetate	9,7 g
Aqua dest. to	500 ml

STOCK-SOLUTION B (salt mixture)	
NaCl	40 g
KCl	2 g
$CaCl_2$	1 g
Aqua dest. to	500 ml

The fixation fluid contained:

sol. A	10 ml
sol. B	3,9 ml
0,1 N HCl	7 ml
Aqua dest. to	50 ml
OsO_4 (cryst.)	1 g

The result is a fixative with a 2% OsO_4-concentration, an osmolarity of 0,448, a pH between 7,75 and 7,85, that has an optimum K/Ca ratio, in accordance with the above data. Indeed, the results obtained with this fixative may be qualified as, on the whole, good but it is our impression that not only the adapted osmolarity, but also the relatively high pH-values are of importance.

Another possibility is that the adjustment of tonicity by addition of the various salts is beneficial partly because of specific ion effects (Wood and Luft, 1965) that are not related to the osmotic activity itself. Indeed, the principle of osmotic activity presupposes the presence of a semi-permeable membrane. It is, however, very likely that the cell membrane, which at present is regarded by most authors as the seat of the relative impermeability, - an assumption not shared by others (cf.: Stadhouders, 1963) - immediately looses its specific properties upon exposure to osmium.

Since it has been reported by Palade (1954) and Fawcett and Porter (1954) that tissues which are especially rich in water, may show coarse flocculation when fixed in media that are only slightly alcaline (pH 7,2), embryonic tissues were processed with fixatives adjusted to pH 8,0 - 8,2.

After fixation, the tissues were dehydrated and embedded in accordance with standard procedures in Epon 812 or Araldite. During dehydration insect tissue was vacuum treated.for some time in order to expell the air from the tracheoles adhering to the tissue. After the filling of gelatine capsules they were placed for one night at 35°C and afterwards for 24 hours at 65°C for polymerization.

2-2-3. Microtomy, Microscopy

Sections were cut with glass knives on a LKB-Ultrotome and collected on copper grids that were either covered with carbon reinforced formvar or collodion films or they were used uncovered. After staining, the sections were studied with a Philips EM 100^B electron microscope, operated at an accelerating voltage of 60 kV and an emission current of approximately 25 μ A. Magnifications on the film varied from 2000 to 23.000 x. The instrument was not fitted out with an equipment for measuring the correct exposure-time of the photographic material. Therefore, an exposure-meter of our own design was built in, which registered the intensity of the electron beam on the fluorescent screen.

The mechanical, hand-operated, shutter had also to be replaced.*

The whole system consists of (1) a shutter of the diaphragm shutter type, containing three non-ferrous lamellae. This shutter is built in the microscope tube behind the projector lens; it replaces the μ-mark indicator, which is of little use when the sunken screen is used. The thickness of the shutterhouse, likewise constructed from non-ferrous metals, is equal to that of the μ-mark-device. (2). The fluorescent screen, under the fluorescent layer on the glass coated with a thin conducting chrome layer, is built-in in such a way that it is isolated from mass. The intensity of the electron beam on the screen is determined by measuring the voltage across a resistor which connects the screen to earth by means of (3) an electronic voltmeter. The shutter is operated electro-magnetically by means of (4) an electronic time-switch for 1/2, 1, 2 and 4 sec. This time-switch is controlled by means of (5) a pair of micro-switches that are fitted to the handle of the mechanical shutter present in the standard outfit of the instrument. The arrangement is such that the newly introduced diaphragm shutter closes before the camera is moved into, resp. out of, the beam. In a later stage of this study use was also made of a Philips EM 200 electron microscope. This instrument is fitted out with a double condenser-lens system and an electro-magnetically controlled stigmator for both the illuminating system and the objective lens. Astigmatism can easily be reduced to less than 0,1 μ. This microscope was also operated at 60 kV and an emission current of circa 25 μA., use being made of the double condenser with an illumination-spot between 10 and 15 μ. Magnifications on the film varied from 3.600 to 46.000 x. Pictures were made on Kodak Fine Grain Positive Film type P 246 or on Perutz diapositive plates. Development of film and plates in resp. Kodak D 163 (19°C; 4 min, dilution 1 to 2) or Orbitol (19°C, 4 min, dilution 1 to 10).

2-3. IN SITU MORPHOLOGY OF GLYCOGEN; early descriptions

The first attempts to characterize the *in situ* structure of glycogen with the aid of the electron microscope, were made by Morgan and Mowry (1951). Alcohol-formalin fixed human liver, after previous embedding in paraffin, was re-embedded in methacrylate. Thin sections were cleared again from this embedding-material and thereupon shadowed with palladium. It is evident that these studies must now be regarded as having only historical value. Fawcett (1955), using

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* This important improvement of the instrument, permitting exact exposure of the photographic material, was developed and constructed by Mr.H.G.Kraan in collaboration with the instrument work-shop (chief: Mr. K.Peters).

more refined histological procedures, described glycogen areas in liver-cells as being "diffusely mottled" and as having, at high magnification, a "cloudy amorphous appearance" without clear-cut ultrastructure. Luft (1956), introducing permanganate as a fixative, indicated that certain granules in the livercells, presumed to be glycogen, were preserved better after $KMnO_4$-fixation than after OsO_4-fixation. The granules took the form of cytoplasmic rosettes, composed of small 100-150 Å globular elements.

Bondareff (1957) applied the freeze-drying technique to guineapig liver and stained in toto with Periodic-Acid-Schiff. He discriminated between three orders of particles. Particles of the first order, about 130 Å in diam., were thought to represent the central (denser) part of the glycogen molecule. These granules, appearing in a less dense background, were aggregated to particles of the second order (600-1500 Å). These were thought to correspond to the particles isolated by Lazarow (1942) by means of differential centrifugation. Aggregation of the particles of the second order to levels of the third order was believed to correspond to the granularity that is often discernable in the light microscope after fixation with fluid fixatives. Though the physical density of glycogen through the uptake of leucofuchsin was apparently sufficiently enhanced to account for adequate contrast in the electron microscope, the pictures that illustrate this study are hardly convincing. In fact, no other ultrastructural elements or details are presented. Porter and Bruni (1959) defined "glycogen-units" in OsO_4-fixed, methacrylate embedded liver-tissue as:

"a unit of structure..... circular in outline and about 100 mμ in diameter. It is
"limited by a border of greater density than the center, a fact which accounts for
"its being visualized at all. This limiting border varies in distinctness and is
"sometimes as sharp as one would expect a membrane to be. Outside this border
"the density usually grades off within 50-75 mμ to that of the surrounding matrix
"of the cytoplasm.... The halo of density, presumed to represent glycogen with
"membrane remnants, may be lopsided or of uneven density."

Anticipating the problem of the ultrastructural involvements of glycogen metabolism, to be discussed in detail in the second part of this thesis, it seems worthwhile noticing that this interpretation of "membrane remnants" as parts of the glycogen units might have brought the authors to hypothesize about a functional relationship between smooth membranes and glycogen turn-over (cf. chapter 5).

As a matter of fact, structural elements that quite conformed to the description given above for liver glycogen, were encountered in a tissue as different as insect malpighian tubular epithelium, provided the tubules were embedded in methacrylate (see fig.2). A number of particles, 120 mμ in diam., with a fairly electron-transparent centre and a border of uneven density lie scattered in the cytoplasm.

A distinction between these structures and small endoplasmic reticulum or Golgi vesicles is indeed difficult to make. It is therefore conceivable that the authors looked upon the membrane-like structures as being elements or derivatives from the agranular ER.

Shortly afterwards, Porter in collaboration with Millonig (1960) described the glycogen particles in liver tissue as follows: "structural elements, resembling dense rosettes or clusters, about "100 mμ Ø ". In this instance, Epon-sections subjected to a treatment with lead-hydroxyde, were studied. It was believed at the time that the use of another type of buffer (phosphate instead of veronal-acetate) was responsible for the different aspects found. It is now understood that the use of a different embedding-medium, in combination with an effective lead-staining, are the chief causes of these differences. Fig.3 illustrates the point. This micrograph represents parts of two malpighian tubular cells, embedded in Epon and stained with lead-citrate according to Reynolds (cf. chapter 3). The distinction between the star-like glycogen particles and the vesicular ER-elements is evident.

With the intention of finding clear-cut criteria for the EM-morphological identification of glycogen, Revel and co-workers (1960) studied a number of tissues from a variety of species. Glycogen areas in unstained sections of rat liver showed a variable appearance, little affinity for osmium and only a vague indication of a particulate character of the glycogen. In the frog liver, however, individual particles were discernable, as was also the case in the sarcoplasm of the frog muscle. In the frog nephron glycogen had a variable appearance, whereas in the glycogen-body of the chick closely packed granules with low electron density were present.

In each of the tissues studied, lead-staining resulted in an increased contrast of the glycogen areas, revealing 150-400 Å particles. Sometimes these particles could be observed to have a finely stippled aspect (see fig.10), which was not regarded as representing an orderly arrangement of sub-units, but was believed to be due to partial sublimation of the lead during the electron bombardment.

With regard to the lack of uniformity in the descriptions presented, two possibilities must be considered.

One possibility has already been indicated, namely that glycogen is very sensitive to modifications in the materials and methods used during the histological processing of the tissue; we will discuss this point at some greater length in section 5 of this chapter.

What may also be a possibility is that the scale of manifestations of glycogen reflects actual ultrastructural differences between glycogen of different sources. In either case, however, the reliability

of the methods used to differentiate glycogen from other cellular elements is of prime importance. In the following section we will discuss the results of studies that were carried out with the use of a number of methods already proposed in the literature.

2-4. METHODS PROPOSED FOR THE IDENTIFICATION OF GLYCOGEN AT THE SUBCELLULAR LEVEL

A number of investigators has tackled the problem of the identification of glycogen at the subcellular level by comparing adjacent thick (1 μ) sections for light microscopy and thin sections (300-500 Å) for electron microscopy. The thick sections are subjected to a light microscopic-histochemical method for the visualization of glycogen; in preference to the Periodic-Acid-Schiff reaction (PAS). The glycogen-positive areas are compared with the corresponding areas in the electron microscope section. By excluding the structurally well-defined cell-organelles, one arrives, "as the result of a process of elimination" (Foster, 1960), at the subcellular localization of glycogen. Special mention deserve the carefully performed studies by Karrer and Cox (1960[a], 1960[b]), concerning the part the Golgi apparatus plays in the storage of glycogen in the embryonic liver. These studies, however, also demonstrate one of the limitations inherent in this method, which becomes all the more difficult to employ the more the dimensions of the glycogen depositions approach the limits of resolution of the light microscope. Thus, in their first study, Karrer and Cox suggested that the Golgi complex might play a role in the formation of glycogen within the embryonic liver-cells, since it was observed that the Golgi complex showed PAS-positivity. Subsequent study, however, revealed that the Golgi apparatus did not show a positive PAS-reaction. Another disadvantage is the circumstantiality of the method. Moreover, the diastase control, necessary for differentiating glycogen from other PAS-positive materials, gives uncertain and in many cases even negative results when OsO_4-fixed and Epon-embedded tissues are studied. Nevertheless, the method relies on a firm knowledge of the validity of the PAS-reaction and appears to be very helpful in bridging the gap between light- and electron microscopy. The results obtainable by these methods are illustrated in the figures 4, 5 and 6. Fig.4 represents a PAS-stained 1 μ section of foetal rat liver (19 day embryo), fixed with osmium and embedded in Epon. The adjacent thin section is pictured in fig.5 at low and in fig.6 at higher electron microscopic magnification. In order to facilitate orientation, the corresponding areas are outlined in black. For further explanation see the legends to these figures.

Other authors have tried to adapt light microscopic methods for

direct use at the subcellular level. The en bloc staining with PAS of frozen-dried tissue, as applied by Bondareff (1957) has already been mentioned above.

Themann (1960, 1963) worked out the Best carmine procedure for staining en bloc the electron microscopic specimen (for technical data: see original reports). Liver tissue, when treated in this way, revealed 100-200 mμ electron-dense particles that were composed of smaller sub-units 20-30 mμ. When the blocs were pretreated with diastase, no such structures were encountered. An interesting point, revealed by this technique, was the fact that small 40-80 Å particles were invariably found in apposition to the membranes of the mitochondria in heart and skeletal muscle and kidney tissue. These particles too, disappeared upon treatment with diastase. According to Themann, the high Ca- and Al-content of carmine is responsible for the electron density of the glycogen structures.

Although the Best carmine method for the identification of glycogen in the light microscope is, as Revel (1964) characterized it, a "time honored procedure", the validity of Themann's adaptation of this method for electron microscopy remains to be established.

We were specially interested in reproducing the observation of glycogen deposits along the mitochondrial membrane, since this location of part of the glycogen as suggested by Themann, if true, might provide a clue to the problem of the fixed glycogen as against the free. Other techniques for the demonstration of glycogen in electron microscope specimens fail to show such a location. We have therefore made a number of attempts to employ the Themann method, but with rather disappointing results throughout. In every instance a very poor tissue preservation was encountered. When using muscle tissue, we did not succeed in obtaining such a result as he described. With liver tissue, only in one case a result was obtained that was reminiscent of a positive reaction. Our attempts were somewhat more successful when malpighian tubular epithelium was tested. Fig.7 represents the basal part of a tubular epithelial cell. In the right hand top corner a number of glycogen particles show carmine positivity. Only a vague indication of the mitochondrial profiles is discernable, whereas the basal cell-membrane invaginations stand out rather distinctly, due to a fine granular and very probably unspecific deposition of electron-dense material on the membranes. Careful examination of fig.3 of Themann's monograph (1963) shows that in the kidney tissue, too, some carmine deposits are present on the cell membranes as well. Indeed, as Revel (1964) has pointed out, it is quite conceivable that small unspecific binding sites, not detected by light microscopy, might contain sufficient material to give a false positive reaction in the more detailed electronmicroscopic image. Because

of the results that could be obtained with the carmine-method, it is not astonishing that Themann in his monograph gives only a couple of illustrations from sections that have been processed by his own method.

The Gomori periodic acid-silvermethenamine reaction, revealing essentially the PAS positive components, was adapted for electron microscopy by Movat (1961). His report is illustrated with a picture of normal human liver, in which the glycogen areas are clearly demonstrated. We have been able to confirm this result, but - as has already been mentioned by Movat - the method has the disadvantage of granularity, which is predominantly present not only on the phospholipid-membranes of the various cell organelles, but also to some extent in the ground cytoplasm. That is why the method seems less suitable for high resolution electron microscopy.

Methods that do not find their origin in classical lightmicroscopic procedures are: the permanganate staining according to Drochmans (1960); the periodic acid-thiosemicarbazide-osmium method after Hanker et al. (1964) and finally the methods that make use of the preferential affinity of glycogen for lead in strongly alkaline solutions, as first noted by Watson (1958). Both the first and the last method cannot be regarded as specific but in view of the remarks, made earlier in this chapter, concerning the preferential use of selective staining procedures, they should be considered at this place. Following the observation by Luft (1956) that permanganate can take the place of osmium in fixation for electron microscopy, Drochmans (1960) introduced the kaliumpermanganate staining of thin sections as a method for demonstrating glycogen. In general, the staining of sections is preferable to the en bloc staining procedures, since it allows other sections of the same tissue bloc to be studied unstained as a control for the degree of selective contrast-enhancement in the stained sections.

We were readily successful in showing the preferential affinity of glycogen for permanganate, when methacrylate embedded material was studied (cf. fig.8), but it appeared far less easy to obtain tolerable results with Epon embedding. This might be explained by a recently published discovery on the incompatibility of permanganate with the methyl nadic anhydride (MNA), which is used as a hardener for the Epon resin in embedding. The failure to show any gain in contrast over background after staining with permanganate in sections of MNA-cured Epon, is according to Reedy (1965) due to the fact that MNA, even in the cured resin, retains its capacity to react with permanganate. Other disadvantages of the permanganate stain are the readiness with which manganese precipitates are formed and

the generalized destructive effect on tissue detail, if the periods of staining are not carefully controlled.

Though we have not been able to test the method that has recently been introduced by Hanker et al. (1964), it must be mentioned here, since it concerns a promising new cytochemical principle for both light and electron microscopy. The basic idea is to alter established histochemical methods by utilizing compounds that contain groups which react selectively with OsO_4. This principle may be illustrated by describing the method the authors developed for the demonstration of macromolecules that contain 1:2-glycol groups. These are oxidized in the usual way by periodic acid. The aldehyde groups thus formed, are reacted upon with thiosemicarbazide (i.e.: the hydrazino group of thiosemicarbazide ($H_2NNHCSNH_2$) condenses with the aldehyde groups). Subsequently, use is made of the fact that the thiocarbamyl moiety of the aldehyde-thiosemicarbazone complex has a strong reducing power for OsO_4. After rinsing, to remove the free thiosemicarbazide, the sections are treated with OsO_4-vapour. This results in the deposition of "osmium-black" at those sites, where the thiocarbamyl is present. The authors reported that they have been successful in demonstrating glycogen in liver and leucocytes, mucopolysaccharides in Descemet's membrane of the cornea and mucus in the goblet-cells of the intestine. By using other osmiophilic substances like mercapto- and diazothioether, they could show the ultrastructural location of a number of enzymes (esterase, alkaline phosphatase, cytochrome oxidase) in a variety of tissues. The "osmium-black" (chemically not yet well defined) that is produced at the reaction-sites, is reported to have a fine amorphous character, thus allowing high resolution microscopy. The reader is referred to the original report for further technical details.

The usefulness of the lead staining for preferentially increasing the electron-scattering properties of glycogen has been claimed by a number of authors. We have used such methods to our entire satisfaction for the studies to be reported in this thesis. The lead-containing stain-solutions used are described in chapter 3. Nevertheless, the staining procedures for lead are suffering from the lack of clear-cut criteria for performing the procedure and, which is even more serious, from an almost complete lack of knowledge concerning the chemical mechanism - if any - of the glycogen-lead affinity. Without this knowledge, it remains a trial-and-error method, in which the results are easily influenced by a number of variables that are not recognized as being essential in the application of the procedure. Especially in the case of glycogen, however, this may easily lead to erroneous interpretations. Since glycogen has no affinity for osmium,

this cytoplasmic constituent will not be detectable (or detectable only through the presence of electron-transparent areas) in the case of an unsuccessful staining. Indeed, we have often observed that a number of cellular constituents, like mitochondrial membranes or RNA-particles, contrast very well when the sections are treated with, for example, the Millonig stain, whereas glycogen (known to be present from the PAS-positivity of adjacent control sections) did not show any contrast at all. The hasty conclusion might be drawn from such pictures, that glycogen is present in a non-particulate form. However, after replacement of the stock staining solutions by newly prepared ones, glycogen could be shown to be present in the particulate form, in all these instances.

We know of one report, in which it was suggested that glycogen lost the particulate form. Remmer and Merker (1963), while studying the increase in drug-metabolizing enzyme activity in its relation to an increased smooth ER-content, noticed that "die bisher als Glykogen gedeuteten, durch Bleihydroxyd-fixierung (!) erkenntlichen Partikeln verschwanden fast völlig, obwohl der Glykogengehalt der Leber und auch der Mikrosomenfraktion sich nicht verminderte". Since this statement suggests that there might exist a non-particulate form of glycogen, we repeated this experiment, but were not able to confirm the findings of these authors. Livers of rabbits, treated every second day with a dose of 50 mg phenobarbital per kg bodyweight (total amount administered 400 mg/kg), showed normal glycogen levels as well as normal lead-stainable glycogen particles in their cytoplasm.

Failure to stain glycogen adequately was in most instances due to an insufficient amount of lead in the staining solutions. When a stock solution is used, repeated filtration of the stain (as is recommended for the Karnovsky and Millonig stain) quickly detoriates the staining results. As will be shown in chapter 3, this is due to the fact that filter-paper (a polysaccharide as well!) absorbs large amounts of lead from the solutions. The same holds true of course for the staining of grids enclosed in filter-paper, which is recommended by Normann (1964) to prevent precipitates. The addition of saccharides to the staining solutions, as recommended in the prescriptions of Themann (1963) or Dorn (1965), does not seem advisable for the same reasons. Of primary importance is, of course, the fact that it is not yet known with certainty whether the lead associates with the polysaccharide-component of the protein-glycogen complexes or with the protein-part of it. Our attempts to clarify this question, as well as our studies concerning the chemical mechanism of the lead staining, are reported in chapter 3.

2-5. STRUCTURAL PRESERVATION OF GLYCOGEN IN DEPENDENCE ON THE HISTOLOGICAL PROCESSING OF THE TISSUE

It has already been mentioned above that the use of a particular embedding medium might influence the structural form in which the glycogen particles are encountered in the sections. Before it can be decided which form gives the most reliable representation of the form that occurs naturally, one must understand the causes through which these differences arise. A serious disadvantage of methacrylate is that, during polymerization of this acrylic plastic, tissue components may become seriously disrupted or distorted through a process called "polymerization-damage". It seems unlikely, however, that this phenomenon occurs located so accurately in the cell, that it can be held responsible for the "disrupted" appearance of the "membrane-remnants containing" glycogen-units after methacrylate embedding. On the contrary, the effects of polymerization damage are highly variable and unpredictable.

A second disadvantage of methacrylate is that it is known to be susceptible to sublimation during the electron-bombardment. Reimer (1959) could show that even under moderate conditions of illumination (10^{-2} A sec/cm^2) the methacrylate sections loose some 50-60% of their mass and this effect is already attained within a fraction of a second. Moreover, groups of molecules that are easily split off, are lost. The surface-tension phenomena during the sublimation can deform seriously the cellular ultrastructure; phospholipid membranes are usually damaged to such an extent that the unit membrane configuration can no longer be resolved. Such sublimation effects are far less serious when epoxy-resins, like Epon 812, are used. In general, one might therefore expect that the structural preservation of glycogen will be better when Epon embedding is employed. Nevertheless, these remarks cannot fully explain why one comes across such obvious differences. (See fig.2 and 3; as may also be seen from fig.8, which represents a methacrylate-embedded, permanganate stained section, a number of glycogen particles show the electron-transparent centre). To understand this appearance of the glycogen particles as vesicle-like structures, one must take into account that the glycogen particulates are strongly hydrated complexes, as was already shown by Lazarow (1942). According to him this peculiar hydration property, must even be taken as supporting the concept of storage of water with glycogen in the cell (for each gram of glycogen 2-3 g of water are stored, too). We observed that it was necessary to dry isolated particulate glycogen (cf. chapter 4) for 12 hours at 100°C under high vacuum, to obtain a constant weight of the sample.

If water is so firmly bound in these particles, it seems rather im-

probable that all the water is removed during the routine ethanol or aceton dehydration of the tissue. Indeed, if particulate glycogen is treated with absolute ethanol, some water remains, as may be judged from subsequent loss of weight in drying of the sample (after initial drying over P_2O_5 for 12 hrs) under the high temperature - high vacuum - conditions mentioned above. Since methacrylate is known not to mix with water, it is conceivable that the plexiglass cannot fully penetrate the particle and thus leaves the central part probably somewhat more accessible to the effects of the electron beam. On the other hand, water is readily miscible with Epon (Luft, 1961), which might explain the fact that similar phenomena are not found upon Epon-embedding.

The supposition that glycogen particles are not fully dehydrated and thus not completely impregnable with methacrylate, might also explain the observation of Cedergren and Holme (1959), who noticed the presence of a number of "holes" in the cytoplasm of Escherichia coli cells with a high glycogen content. Since the size of these holes compared well with the particle size of glycogen, isolated by alkaline hydrolysis, the view was expressed that the holes represented the sites of glycogen granules (dissolved out of the sections during their preparation). Comparable observations were made by Boothroyd (1963), studying Trichinella larvae. He could show that the "glycogen-vesicles" in sections, previously irradiated in the microscope, became holes in the sections after treatment with lead-acetate. This observation strongly supports the supposition that glygogen deposits are more sensible to electron beam effects than are other cellular constituents.

The experience gained from a number of studies on the repartition of glycogen in various organs and tissues made Revel (1964) state that adequate preservation of glycogen is to be expected only when glycogen is widely dispersed in the cell or is surrounded by "foreign" material (protein). According to him, the usefulness of Millonig's phosphate-buffered fixative must be explained by the fact that this fixative prevents extraction of the cytoplasmic matrix. One might argue equally well that the more crowded the glycogen is in the cell, the less effective the dehydration of the cell areas in question can be, whereas the effects of the phosphate addition are probably to be related to "specific ion effects" as described by Wood and Luft (1965). One might think, for example, of an interference with the maintenance of the electrostatic equilibrium in the glycogen particles, accomplished either by water dipoles or by ions (Lazarow, 1942). But further studies will be required before definite statements can be made.

2-6. THE FINE STRUCTURE OF GLYCOGEN

Although the number of studies on the fine structure of glycogen

that inspire confidence is still small, the evidence available points to the usefulness of accepting, for purposes of description, two basic levels of structural organization, each with a tissue-specific occurrence, (Drochmans, 1962; Bavia, 1963; Revel, 1964).

In the mammalian liver a complex, rosette-like form is encountered, for which Drochmans proposed the name of "alpha particle". These α-particles are described as closely packed aggregates of smaller sub-units, which represent the second basic type or "beta-particle". The beta-particles are 150-400 Å in diameter and more or less spherical. Glycogen in mammalian tissue other than liver is present in this beta-particle form. Some examples of this type are given in figures 11 up to and including 14. Fig.11 is a section of a polymorphonuclear neutrophil from human bone marrow. The glycogen particles in this cell-type show no tendency towards gathering in a particular cellular region. Another example of a beta-particle like glycogen deposition is given in fig.12. This micrograph presents parts of epithelial cells, taken from kidney-tumor tissue which is maintained by subcuteneous transplantation in Balb/C mice. Glycogen-containing cells are relatively scarce in this tumor. Here, too, the β-particles lie scattered throughout the cytoplasm. There is no wide difference in size from the ribosomes present in the cell at the bottom of the micrograph.

Fig.13 represents a section through a rat skeletal muscle. The glycogen particles lie predominantly in the inter-myofibrillar space, in the neighbourhood of the sarcosomes and the sarcoplasmic reticulum elements. A small number of glycogen particles is present between the filaments of the myofibrils, though without an apparent preference-location.

A tissue that is able to store glycogen in substantial amounts during development, is the vertebrate skin. Fig.14 represents a section of the stratified epithelium of fetal human epidermis (age: 20 weeks). Large amounts of simple glycogen particles are present in the cytoplasm, together with some swollen mitochondria. Note the regularity in outline of the glycogen particles, which do not show any sign of aggregation. The alpha particle level of organization is illustrated with a number of micrographs of insect tissue (fig.15 up to and including fig.18). Fig.15 is a section of a malpighian tubular epithelial cell, from the transitional zone between the distal and proximal halves of the tubule. The complex organization of the particles is apparent. Here, however, the particles show a distinct tendency to gather in circumscribed cell areas; they can be found by preference in the basal part of the cell. Even if the crowding in a given cell area is very high, the particles retain their individuality. In fig.15 the distance between the particles is sometimes no more than some tens of Angströms.

Whereas the malpighian epithelium is rather poor in chromidial or

basophilic substance, the columnar absorbing cells of the mid-intestine of Periplaneta are abundantly provided with ergastoplasm membranes, (see fig.16). This micrograph shows that the glycogen deposits in these cells take the same aspect of alpha particulate configuration. The uniformity in diameter of these particles (varying from about 100 to 130 mμ), is very striking.

At this rather low magnification, no facts are revealed that are not covered by the description of these alpha-particles as rosettes "that can be likened to a somewhat rounded bunch of grapes" (Revel, 1964). At higher magnification, (fig.18) however, it appears that there is more which can be defined in these structures, albeit that an unequivocal interpretation is hard to give. In fact, several particles clearly stand out as star-like structures, with rod-like elements radiating from the centre. These rods sometimes run parallel to each other; in other cases they give the suggestion of joining in the more central part of the glycogen deposit. The sub-units, situated more centrally, often appear as small circles or squares. This should perhaps be related to the angle under which the electron beam "sees" these sub-elements.

An interesting observation is revealed in fig.17. Between the cells of the tubular epithelium in the proximal half of the tubules, that have a normal "transparent" cytoplasm, cells may occur with a very electron-dense cytoplasm. This phenomenon is reminiscent of the occurence of "Schaltzellen", which also have a dense cytoplasm, in the collecting tubules of the vertebrate nephron. The alpha particles present in this dark cell are distinctly smaller than those of the normal transparent cells surrounding it. In this particular case a range in diameter from 55-78 mμ was found. Little can be said with certainty about this observation, but it is likely that a relation exists with the degree of hydration of these dark cells. To judge from their electron-density, the amount of osmium-reducing materials present per unit-volume of cytoplasm must be considerably higher than in the transparent cells. The dark cells are in all probability young cells, as can be deduced from the frequency with which centrioles are encountered in these cells. In fig.17, a centriole occupies a position in the top of the dark cell, encircled by the mitochondria.

It is of course also possible that for some unknown reason the enzymatic equipment of the dark cells is inadequate to allow these particles to grow beyond a certain limit. Whatever the reason may be, this observation endorses the view, expressed by Revel (1964), that the classification of glycogen into two main types is useful in describing the appearance of the glycogen deposits, but must not be interpreted too rigidly, since the two morphological types may merely be two extremes of a graded spectrum. We will return to the problem of glycogen fine structure in chapter 4.

2-7. DIFFERENTIAL STAINING OF RIBONUCLEOPROTEIN PARTICLES AND GLYCOGEN PARTICLES

As already indicated in the introduction, it is sometimes extremely difficult to differentiate between RNA-containing particles (ribosomes) and glycogen of the beta-particle type. When these ribosomes are joined to the assembling unit in protein synthesis (polyribosomes or polysomes), their aspect somewhat resembles the alpha-particle form of glycogen. We have wrestled with this problem especially when undertaking the investigation described in chapter 8. We have therefore tried to combine in one staining procedure the preferential affinity for uranyl-ions of structures containing nucleic acid with the growing knowledge of the mechanism of the glycogen-lead interaction, as it is described in the next chapter.

Nucleic acids in fixed tissues can, with relative specificity, be stained by indium (Watson and Aldridge, 1961, 1964), by bismuth (Albersheim and Killias, 1963) or by uranyl-salts (Huxley and Zubay, 1960, 1961; Zobel and Beer, 1961; Stoeckenius, 1961). We have used aqueous uranyl-acetate solutions, since the procedures for indium- or bismuthstaining are far more complicated. Evidence has been brought forward that the specificity of the uranyl-staining is much increased when organic fixatives are used (Watson and Aldridge, 1961); therefore these studies have been performed on glutaraldehyde-fixed tissue.

Since the exposure to the electron beam prevents the subsequent uptake of stain, it is impossible first to study or photograph the effect of one staining and subsequently apply the second stain. Our procedure has been to collect two successive sections separately on the two halves of one grid (or on grids with a central hole). Both sections are first stained with a half-saturated aqueous uranyl-acetate solution, pH 5. Subsequently, the sections are treated with a lead-acetate solution, half-saturated, at pH 7,3. After 20 to 30 min one of the sections is removed from the staining solution and rinsed, whereas the second section is treated for another 15 min with a lead-acetate solution at pH 10 to 11 or with a Reynolds solution. After rinsing, the two halves of the grid are placed in one specimen-holder and notwithstanding some trouble owing to grid-bars that overlie part of the sections the corresponding cell areas are photographed. The figures 19 and 20 represent two sets of successive sections, treated as described. The transparent regions in the one figure are shown to contain particulate glycogen in the adjacent section. The treatment of the sections after uranyl-acetate staining with the lead-acetate stain considerably increases the electron density of the ribosomes, but shows no contrast-enhancement of the glycogen. Most probably glycogen is not stained at this pH, since the solution does not contain negatively charged

lead ions. The positive metal ions present are probably attached to the uranyl-acetate, which is known to be absorbed by the nucleoprotein components as the singly charged $UO_2(Ac)^+$ (or as the $UO_2(Ac)_2$ species; Huxley and Zubay, 1961). If required sections can, of course, be processed by only one of the two procedures (see fig.21).

2-8. DISCUSSION

The studies described in this chapter show that the procedure of staining thin sections with lead can be used satisfactorily as a method for demonstrating glycogen at the subcellular level. Yet the method lacks specificity.

In every tissue we were able to study, we found the glycogen present in a particulate form, either as simple particles or as complex structures. Several technical points, however, may interfere with the results that can be obtained. The type of embedding-medium that is employed is of primary importance in the preservation of the ultrastructure of particulate glycogen. Difficulties encountered in differentiating glycogen particles from cellular elements containing nucleoprotein, could be overcome by making use of the preferential affinity of uranyl ions for elements containing nucleoproteins. The varying results that were encountered at the outset in applying the lead-staining method induced the study, to be reported in the following chapter, of the chemical mechanism of the glycogen-lead affinity.

CHAPTER 3

PRESERVATION OF GLYCOGEN IN ELECTRONMICROSCOPIC SPECIMEN AND MECHANISM OF STAINING WITH LEAD

3-1. INTRODUCTION

The reliability of any particular histochemical technique depends among other things on whether or not the substance under examination is preserved in situ without unplanned modification or loss during the histological processing of the tissue. Especially in this respect, the preservation of glycogen has long been and still is puzzling histochemists. There consists an extensive number of publications on this subject (see for example, Lillie, 1948), though still without a consensus of opinion. A variety of fixatives has been recommended as well as special precautions during section treatment, for example celloidin-covering of the slides.

Recently Kugler and Wilkinson (1964) have studied this problem through a quantitative approach. The mode of action of ten different tissue fixatives, recommended for correct glycogen fixation, was evaluated. The amount of glycogen present after fixation was estimated both quantitatively and histochemically and compared with the amount originally present in the fresh tissue. In none of these cases was the recovery better than 90%; in some fixatives it was even as low as 40%. It was moreover indicated that some solutions (e.g. absolute alcohol) modify the existing glycogen in such a way as to render it unsuitable for histochemical detection.

Since the electron microscopist has only a very few types of fixatives available that warrant adequate preservation of overall tissue ultrastructure, the question arises to what extent these fixatives serve the purpose of optimum glycogen fixation. The results of some preliminary studies will be reported in section 2 of this chapter.

A second point of importance in the precise application of a histochemical technique is the chemical mechanism that causes the substance to take up the dye and thus to contrast in the section. Leaving aside the rather academic point whether or not the staining of glycogen with lead in electron microscopy deserves to be denominated a

"histochemical" technique, the preferential affinity of glycogen for lead salts has been experienced by many (Revel, Napolitano and Fawcett, 1960; Millonig and Porter, 1960; Karnovsky, 1961; Daems and Persyn, 1962; Themann, 1963; Reynolds, 1963; Baker, 1963; Revel, 1964; Wood and Luft, 1965).

However, nothing definite is known about the chemical mechanism behind this "trial-and-error" experience and experimental approaches to this problem are lacking.

Since it is not even established in what ionic form lead is present in the various staining solutions at the pH most commonly employed, we made some preliminary studies, the results of which are given in section 3 of this chapter.

3-2. PRESERVATION OF GLYCOGEN BY ELECTRON MICROSCOPIC FIXATIVES

In order to make a quantitative study of the preservation of glycogen after fixation in the most commonly used fixation-fluids, livers of normally fed rats were removed, immediately cooled and cut free-hand in slices approximately 2-3 mm thick. Part of the slices was used for direct quantitative estimation of glycogen (for methods: see chapter 6); after wet weight estimation the remaining slices were fixed in either 2% OsO_4, 6,25% glutaraldehyde or 4% formalin.

Since glycogen is located predominantly in the perivascular regions of the liver lobes, the distribution of the slices was chosen in such a way as to account for this uneven distribution of glycogen in the liver.

After fixation (2 hrs) the slices were rinsed, dehydrated and subsequently digested in KOH 30%.

In a first series of determinations, the results of which are given under A in table 1, it proved very difficult to purify the glycogen from the osmium-fixed slices. After KOH-digestion the glycogen was collected by alcohol-precipitation. When aethanol is added, the reduced osmium, in the fixed tissue presumably present as (hydrated) osmium-dioxyde, is precipitated in colloidal form together with the glycogen (Bahr, 1954).

In an attempt to overcome this difficulty, the osmium-fixed slices were in a second series (B) of determinations first rinsed and then put in hydrogen peroxide (2%, 2 hrs).

Treatment of osmium-fixed tissue-sections with hydrogen peroxide or periodic acid is known to reduce the contrast, presumably because the reduced osmium is re-oxidized and can therefore be eliminated (Marinozzi and Gautier, 1961). However, even after this treatment the glycogen precipitate was not found to be completely free from osmium.

TABLE I

Preservation of glycogen in liver tissue
after fixation with osmiumtetroxide, glutaraldehyde or formalin.
For further explanation see text.

Ser.	Unfixed	Osmium-tetroxyde 2%	Glutar-aldehyde 6,25%	Formalin 4%
A	4,78 5,21	1,91	5,04	4,52
	4,49 5,23	2,69	4,66	4,36
	5,05 4,64	2,48	4,88	4,57
	4,85 5,52	3,32	4,77	4,43
	mean 4,97	2,60	4,84	4,47
B	5,45	4,30	5,50	5,28
	5,72	4,78	5,20	5,37
	5,56	5,10	5,45	5,03
	5,46	5,08	5,40	5,21
	mean 5,55	4,82	5,39	5,22

As follows from these results, the recovery of glycogen after fixation of the tissue in glutaraldehyde or formalin amounts to about 97% and 90 - 94% resp.; a recovery which is better than any of those arrived at with a series of 10 fixatives, recommended for optimum preservation of glycogen in light microscopy (Kugler and Wilkinson, 1964). The less satisfying results after osmium-fixation cannot be taken to indicate that osmium-treatment of the tissue causes a substantial loss of glycogen. The presence of even a small amount of osmium interferes with colour-development in the anthrone-reaction. As a matter of fact several investigators have praised the qualities of osmiumtetroxyde for glycogen-fixation (Biava, 1963, Padykula and Richardson, 1963), but their statements especially concern the fact that osmiumtetroxyde preserves glycogen more precisely on its intracellular sites than other modes of fixation.

3-3. THE CHEMICAL MECHANISM OF STAINING WITH LEAD

Watson (1958), who introduced several methods for staining thin sections with heavy metals, had already noticed that the alkalization of a saturated lead-acetate solution resulted in an increased contrast. He therefore propagated the use of his alkaline lead-hydroxide solution

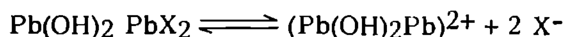
(pH 8,15) as the most generally useful of all methods described by him. Indeed it has become common practice to use lead-containing solutions and several authors could affirm that the intensity of staining increased when the pH of the solutions was increased (Dalton and Zeigel, 1960; Lever, 1960; Karnovsky, 1961; Millonig, 1961; Reynolds, 1963). Since, however, the "leadhydroxide", prepared according to the directions given by Watson (1958), is extremely unstable when exposed to air, several more or less complicated procedures or apparatuses have been developed for the exclusion of air or carbon dioxide during staining (Peachey, 1959; Parsons and Darden, 1960; Feldman, 1962; Normann, 1964; Dorn, 1965).

Complexing agents like tartrate (Millonig, 1961) or citrate (Reynolds, 1963) were used with more success to achieve a stabilization of lead salts.

It may be concluded from the above mentioned pH-dependency of the staining-effect, that the chemical mechanism of staining in all the various methods will be the same (Reynolds, 1963). Nothing, however, is known about this mechanism and, as has been pointed out already, there is even no agreement on which ionic form of lead is present at high pH-values.

According to Dalton and Zeigel (1960), treatment of sections with highly alkalized solutions results in a more complete ionization of the sulfhydryl-, phosphate- and carboxyl-groups present in the tissue components. This availability of more free ionic groups should result in an increased binding of Pb^{2+}-ions. Reynolds (1963) proposed a strongly alkaline lead-citrate solution (pH 12). According to this author "basic salts" of the general type $Pb(OH)_2PbX_2$ are formed upon addition of alkali to divalent lead salt in aqueous solution.

Such compounds are thought to ionize as:



$$Pb(OH)_2\,PbX_2 \rightleftharpoons (Pb(OH)_2Pb)^{2+} + 2\,X^-$$

The divalent cations thus formed contain two atoms of lead and so twice as much lead would be bound on the tissue binding sites (believed to be cysteine, orthophosphate and pyrophosphate groups and additional lead-sequestering sites formed during fixation) when compared with the binding of "monomeric" divalent Pb^{2+}-ions at pH 7.

When the pH of the lead-citrate solution is increased to pH 14 the intensity of staining decreases; according to Reynolds because in that case the cationic forms of lead present at pH 12 are converted into anionic hydroxy-plumbite ions.

Themann (1963) assumes that the Pb^{2+} - ions in staining are reduced to metallic lead. The influence of the pH on the staining-intensity was explained by a facilitated reduction at higher pH, since the redox-potential $Pb^{2+}/Pb^{\emptyset}$ decreases with increasing concentration of

OH-ions. An experimental point that seems to support this assumption is the fact that treatment of the stained sections with acetic acid does not result, with the exception of glycogen, in a decrease of the contrast. Whereas lead hydroxide and lead carbonate are soluble in acetic acid, metallic lead is not. The fact that the contrast of glycogen diminishes after acetic acid treatment brought Themann so suppose that glycogen binds the lead in a different way ("Innerkomplex Salz").

Karnovsky (1961), on the other hand, thinks it unlikely that after fixation positively charged groups are available for ionic binding.

According to him negatively charged plumbite ions, like $Pb(OH)_3^-$ or $Pb(OH)_4^{2-}$ are responsible for the much more rapid and intense staining at high pH. He suggests a hydrogen bonding of the plumbite ions to groups in the tissue. In support of this hypothesis is Karnovsky's observation that ureum treatment of the lead-stained section causes a decrease in staining intensity.

Daems and Persijn (1963) distinguished three types of lead-staining. The membranes in osmium-fixed tissue are believed to stain with lead due to the presence in these membranes of reduced osmium-tetroxyde. It is known through the work of Riemersma and Booy (1962) that OsO_4, upon reduction at the unsaturated bond of the fatty acid chains, is deposited as OsO_2 in the polar regions of the phosphatids.

Through the acidic properties of this OsO_2, the affinities of lead (as a positive ion) for membranes was understood. The staining of nucleoproteins was supposed to be due to the negative groups of the nucleoproteins. As to the staining of glycogen, Daems and Persijn assumed without definite reasoning that initially some lead is attached to the hydroxyl-groups by chelating. Thereupon additional lead should aggregate around this primarily attached lead, thus resulting in a coarse precipitate that, according to them, characterizes the staining of glycogen with lead.

In the experiments, to be described below, the following staining solutions, which are commonly employed in our laboratory, were used.

1. Karnovsky, B. Pb0 in sodium cacodylate, alkalized. Method of preparing:
   To 15 ml of 10% sodium cacodylate in distilled water Pb0 is added in excess; the mixture is gently boiled for 15' and subsequently filtered. Two ml of the filtrate are diluted with 8 ml of 10% sodium cacodylate. Then 1 N NaOH is added drop by drop (about 6 drops) while the mixture is thoroughly stirred. Ph 11,9.
2. Millonig's K-Na-tartrate stabilized lead hydroxide solution. Method of preparing:

A stock solution is made up, containing:

NaOH	20 gm
K-Na-tartrate	1,0 gm
H_2O to	50 ml

To prepare the stain, 1 ml of this stock solution is added to 5 ml of a 20% lead acetate Pb $(CH_3COO)_2 . 3H_2O$ solution, stirred, diluted 5 times with distilled water and filtered. Ph 12,1.

3. Reynold's lead-citrate solution.
Method of preparing:
1,33 gm lead nitrate ($Pb(NO_3)_2$), 1,76 gm sodium citrate ($Na_3C_6H_5O_7.2H_2O$) and 30 ml distilled water are vigorously shaken. After some time 8,0 ml 1 N NaOH is added and the suspension is diluted to 50 ml. After thorough mixing the lead citrate dissolves. Ph 12,5.
For comparison a
4. Watson's lead-acetate solution (Ph 5,9) was studied too. The following experiments, for the sake of brevity numbered 1 to 11, were carried out to gain some more insight in the mechanism of lead-staining of glycogen.

1. A first point that had to be settled concerns the question whether the affinity of particulate glycogen for lead must be attributed to the polysaccharide-component or possibly to the "surface-coat, containing protein" as suggested by Mercer (1963). This point was checked up in a simple manner by the following experiment. A number of easily sedimentable polysaccharides (starch), amylopectin (British Drug Houses), cellulose-powder (Schuchardt), inulin (Warner-Chilcott) and particulate glycogen (N.-content: 0,22%, P not detectable, no ashes: for method of isolation: chapter IV) were mixed in test-tubes with a 5 x diluted Millonig lead-solution. After half an hour the polysaccharides were centrifuged down and the stain solution decanted. The polysaccharides were then resuspended in 5 ml 0,01 N NaOH for washing. This was repeated three times; in every instance the second and third wash-medium - upon addition of $(NH_4)_2S$ proved to be free from lead. After the third washing, the polysaccharides were resuspended in 0,01 N NaOH. Upon addition of $(NH_4)_2S$, all polysaccharides reacted immediately by staining black, except for the cellulose-powder that showed a brown colour. From these findings it follows that it is in all probability the polysaccharide component in particulate glycogen which achieves the staining-effect. Moreover, it appears that one may study the polysaccharide-lead interaction phenomena by using, instead of particulate glycogen, less expensive polysaccharides.

2. In order to find out whether the polysaccharide-lead binding is mo-

mentary or time-consuming (as is suggested by the interpretation of glycogen-staining, given by Daems and Persijn, 1963), equal amounts (5 mg) of starch were suspended in a series of 5 test-tubes, each containing 15 ml of Millonig's or Reynolds' lead-solution. A similar series was made with cellulose. After thorough mixing, one sample was processed immediately, the remaining ones were stirred for 5, 10, 30 and 60 min. resp. After centrifuging down the polysaccharide, the amount of lead present in the supernatant fluid was determined quantitatively. The method used was a modification of the colorimetric one after Treadwell and Hall, Analytical Chemistry, vol.II, 1949. Exactly 5 ml of the supernatant fluid is shaken twice with 5 ml of a solution that contains per liter chloroform 100 mg diphenyldithiosemicarbazone (dithizone). The combined chloroform-phases are washed with a 0.01 N NaOH solution, until the washing fluid is colourless. The chloroform-phase is thereupon filtered and the filtrate read at 520 mμ. The method could be simplified in this way, since in our case Pb was the only heavy metal present and it was present, moreover, in a complex binding. Consequently the lead did not precipitate as leadhydroxyde. It was found that in all supernatant-fluids of the starch-series equal amounts of lead were present. This was also the case in the cellulose-series. Therefore the reaction between starch and lead (or cellulose and presumably also glycogen) must have been a momentary one; the amount bound by the polysaccharide does not increase with time. These findings make it unlikely that glycogen-staining proceeds by a sequence of events, as suggested by Daems and Persijn.

3. When a solution of soluble starch or commercial glycogen was mixed with Reynolds' staining solution, an insoluble polysaccharide-lead complex precipitated. This is of importance since it makes it improbable that (part of the) glycogen, upon treatment of thin sections with lead-solutions for staining, is dissolved out. Inconstant staining results are therefore in all probability due to considerable variation in the amount of lead present in the various staining solutions. The following experiments were undertaken to get an idea of this aspect.

4. Lead solutions were made according to the prescriptions of Reynolds, but instead of sodium-citrate we used NH_4OH, KCN, K-Na-tartrate and EDTA-Na. Normal Reynolds was used, too. After preparing these mixtures, they were centrifuged as long as was necessary to obtain a clear supernatant. The amount of precipitate was largest with NH_4OH, whereas KCN > K-Na-tartrate > Na-citrate = EDTA = 0.

EDTA always gave a clear solution, whereas with sodium-citrate a precipitate was sometimes formed initially that appeared to dis-

solve in short time. The amount of lead that stays in solution is therefore virtually zero when NH_4OH is used, and is in KCN < K-Na-tartrate < Na-citrate = EDTA. The amount of lead that stays in solution is therefore apparently dependent on the type of stabilizing agent that is employed.

5. The experiments to be described now, were carried out in order to study the electric charges of the lead ions, as they are present in the various staining solutions. Volumes of 3 ml alkaline lead solutions, prepared with resp. KCN, K-Na-tartrate, Na-citrate or EDTA were subjected to electrophoresis on agar-plates (7 V/cm). The agar-plates were made from 2% agar in Na-acetate-NaOH buffer, pH $\pm$ 12, or from 0,9% agar in veronal buffer, pH 8,5. After 30 min of electrophoresis the agar-plates were treated with $(NH_4)_2S$. The KCN- and K-Na-tartrate-lead solutions had moved towards the positive pole, leaving some lead at the starting-spot; the citrate- and EDTA-lead mixtures also moved towards the positive pole, but without leaving part of the lead behind.

Since during the course of the experiments these agar-plates could hardly be prevented from being exposed to air, which involved the risk of $PbCO_3$-formation, the experiments were carried out again, this time with the use of a cataphoresis apparatus after Baker (1958). This apparatus consists of a U-tube partly filled with a glycine-NaOH buffered aquous agar gel, pH 12,0. After the agar had set in the tube, care being taken it did not come unstuck from the wall, the staining solutions were poured into each limb to exactly the same height. Two small U-tubes, also filled with agar, were dipped upside down, with one limb into the staining solution (via a stopper to exclude the air) and with the other into two small electrode-beakers, each containing a 10% aqueous $CuSO_4$-solution and either the negative or the positive pole. A D.C.-power supply (from L.K.B., Stockholm) was used at 100V, 30mA. It was found that a Millonig stain, as well as a Reynolds and an "EDTA-stain" all showed a descent in the cathode-limb in response to the current, whereas no descent was found in the anode-limb. This behaviour of the lead shows that in any of these alkaline lead solutions, the lead ions are present negatively charged. A saturated lead-acetate solution, on the contrary, showed a movement towards the negative pole. These findings thus seem to support Karnovsky's supposition that negatively charged ions are much more effective in staining thin tissue sections.

However, another point that emerged from these experiments was that, after the current had been passing for $1\frac{1}{2}$ hours, Millonig's stain ($tartrate^{2-}$) had moved 12 mm, whereas Reynolds' solution ($citrate^{3-}$) had moved 21 mm and the EDTA-lead solution even 36 mm. When the solutions were diluted twice, virtually the same results were obtained.

These findings show - as was to be expected - that the cataphoretic behaviour of the lead in these solutions was independent of concentration, but depends on the type of stabilizing agent used, since the complex with the most polyvalent chelating agent penetrated deepest. The conclusion may be drawn that lead is present as a negative complexion: its concentration depending on the type of complexing agent present (see 4). Moreover, there seems to be no binding of the lead to the carboxylic groups - fully ionized at this pH - of the agar.

6. In order to get some insight into the firmness of the polysaccharide-lead binding, the following test-tube experiments were carried out. Starch and cellulose were treated with a Millonig staining solution; then washed three times with 0,01 N NaOH as described under 1. Subsequently the polysaccharides were treated with resp.:

1) 1% glucose in 0,01 N NaOH,
2) 5% glucose in 0,01 N NaOH,
3) 1% sucrose in 0,01 N NaOH,
4) 5% sucrose in 0,01 N NaOH,
5) 0,2 M acetic acid,
6) 5% Urea in 0,01 N NaOH,
7) 0,05% EDTA-Na in 0,01 N NaOH,
8) 2% K-Na-tartrate in 0,01 N NaOH,
9) 2% tri-Na-citrate in 0,01 N NaOH,
10) a saturated alkaline dithizone solution.

The polysaccharides were washed twice with these solutions, afterwards once with 0,01 N NaOH and resuspended in aqua dest. The washing media as well as the residue were checked on the presence of lead by means of adding $(NH_4)_2S$.
The findings are summarized below:

Sol.nr.	1	2	3	residue
1	-	-	-	+++
2	-	-	-	+++
3	-	-	-	+++
4	-	-	-	+++
5	+	$\pm$	$\mp$	$\mp$
6	-	-	-	+++
7	+	$\mp$	-	-
8	-	-	-	+++
9	-	-	-	+++
10	$\pm$	$\pm$	$\pm$	+

These findings show that the bond between Pb and the polysaccharides employed (starch, cellulose) can be broken by treatment with acid and EDTA, and to some extent by dithizone but not by glucose, sucrose, K-Na-tartrate, or citrate. In the case of EDTA it may be assumed that the complexing activity of the polysaccharide is superseded.
This will only partially be the case with dithizone. The results of the next experiment support this assumption.

7. Starch in aqua dest. was shaken about 20 min with alkaline lead solutions, prepared with resp. KCN, K-Na-tartrate, Na-citrate or EDTA (5 times diluted but otherwise prepared as mentioned under 4) as well as with an alkaline Pb-dithizone solution. After being centrifuged down, the polysaccharide was washed 4 times with 0,01 N NaOH. The second washwater was already negative, when tested for the presence of lead. Upon treatment of the starch with $(NH_4)_2S$, the polysaccharide appeared to have picked up a considerable amount of lead from the KCN, the K-Na-tartrate and the Na-citrate solutions, but far less from the Pb-dithizone solution and nothing at all from the EDTA-lead mixture.
From these findings it is evident that starch is capable of superseding the complexing agents CN^-, tartrate^{2-}, and citrate^{3-} (or else capable of binding these complexes as a whole), but not versenate^{4-}. The same apparently holds true for glycogen, since treatment of thin sections with any of these solutions resulted in an effective staining of glycogen, except for the EDTA-mixture. When pre-stained (Reynolds) sections were treated with 0,05% EDTA in aqua dest., the glycogen particles lost all contrast. The same effect was noticed when stained sections were processed with 2,5% TCA or 2,5% acetic acid. This effect of an increased H^+-ion concentration will be discussed further down.

8. Attempts to subject an amylum solubile-lead complex (Reynolds) to electrophoresis on agar-plates, were rather unsuccessful. Since the complex is almost insoluble, much diluted solutions had to be used. At the spot where the lead was present on the anodic side, no amylum solubile could be demonstrated (tested with I_2 in KI). Since I_2 in KI cannot stain at high pH, the agar plates were neutralized beforehand. This, however, might have caused solubilization of the complex.
The amylum had not moved electro-endosmotically towards the negative pole either; presumably since the complex cannot penetrate the agar.

9. Since the literature gave no indications that alcoholic OH-groups - even at high pH - can act as ionogenic complexing agents, the importance of the OH-groups of the polysaccharides in the lead-

binding reaction had to be studied. A first point concerns the question whether the alkaline lead solutions are capable of oxidizing the OH-groups, as does for example HIO_4 in the PAS-reaction. This point was studied as follows. Starch and cellulose were incubated for 3 days at room-temperature and in darkness, with an alcoholic HIO_4-sol. Other samples were incubated for 3 days with Millonig's or Reynolds' solutions. After incubation the polysaccharides were first washed 3 times with 0,05 NaOH; then 3 times with 0,2 M acetic acid and lastly 3 times with aqua dest. An idea of the degree of oxidation was got by adding Schiff's reagens. The results are summarized below:

polysaccharide	treatment	$(NH_4)_2S$ react.	Schiff
starch	HIO_4	not tested	+++
starch	alc.lead	-	-
cellulose	HIO_4	not tested	$+\pm$
cellulose	alc.lead	-	-

It follows that the alcaline lead-containing solutions have no oxidizing effect on the polysaccharides tested. When HIO_4 -oxidized starch (incubation-period: 7 days, washing as above) was treated with Millonig's or Reynolds' staining solution, considerably less lead was bound by the polysaccharide. This could be judged from the colour-development after reacting with $(NH_4)_2S$ (light-brown in the case of oxidized starch; black-brown with normal starch). However, HIO_4-oxidation of OsO_4-fixed liver-sections did not diminish the taking-up of lead by glycogen.

10. In view of the question, whether the polysaccharides are capable of superseding the complexing activity of tartrate or citrate during staining, or otherwise are capable of binding the lead-containing complexing agents as a whole, starch, that was previously stained with Reynolds' solution and washed with 0,01 N NaOH, was analysed for the presence of citrate with the Denigès-procedure (Hoppe-Seyler: Handb. Physiol. Path. Chem. Analyse, III, 551, 1955). No positive reactions could be obtained. Moreover, the respective lead-containing washing-media, obtained from starch-samples that were stained with Millonig's tartrate-lead- and Reynolds' citrate-lead solutions resp. (and thereupon washed with acetic acid in order to release the lead again from its binding to the polysaccharide), after alkalization to pH 12, showed identical cataphoretic behaviour of its lead-content and no dependency on the type of complexing agent that was originally present.

From this evidence, it may be concluded that the polysaccharides

are capable of taking over the lead from the tartrate- or citrate-lead complexes during staining.

11. Further study will be necessary to elucidate the point why a number of polysaccharides (glycogen, starch, amylopectin), are capable of taking over the lead from negatively charged complex ions, whereas others can perform this only to some extent (cellulose), and still others (agar, pectin, acid mucopolysaccharides) do not stain at all. The fact that agar and acid mucopolysaccharides do not stain after treatment with strongly alkalized lead solutions indicates that negatively charged groups are of minor importance.

3-4. DISCUSSION

From the evidence presented in section 3 of this chapter, the conclusion may be drawn that in all lead-containing solutions of high pH, used for staining thin sections, the lead is present as a negatively charged complex-ion. The electrophoretic behaviour of the lead in any of these complexes runs parallel to the valency of the complexing agents used. Polysaccharides, like starch, amylopectin, cellulose, inulin and glycogen are capable of taking over the lead from a number of these complexes, viz. from the cyanide, the tartrate- and citrate-complexes and to a certain extent also from a Pb-dithizone complex. The lead, present in an EDTA-lead complex, cannot be taken over by any of the polysaccharides mentioned. It has been reported (Saltman et al. 1962) that reducing sugars, like fructose, can form soluble stable complexes with a series of metal ions at alkaline pH. No evidence, however, has been presented to show that alcoholic OH-groups - even at high pH - possess ionogenic complexing activity. It seems probable, therefore, that the only type of bond governing the polysaccharide-lead interactions, must be the H-bond, which is presumably present between the electronegative oxygen of the OH-group and the negatively charged Pb-ion ($Pb(OH)_3^-$ or $Pb(OH)_4^{2-}$). Several arguments point in that direction, such as the decrease in Pb-binding capacity after oxidation of the OH-groups; the precipitation of amylum solubile or glycogen upon addition of a Pb-complex (presumably because the hydration-water of the OH-groups, necessary for solubility, disappears); perhaps also the decomposition of the polysaccharide-lead complex in the presence of H^+-ions, since this may cause the negative lead-ions to become positive.

Possibly also in support of this supposition is the observation that negatively charged lead moves out of a polysaccharide-Pb complex, when an electric field is applied (cf. 8).

The observation that neither glucose nor sucrose interferes (cf.6)

in the concentrations used, is presumably due to the fact that the concentrations are relatively low, when compared to the enormous concentration of OH-groups which the polysaccharides have in their volumes.

From the fact that neither a 5% urea solution in 0.01 N NaOH (nor the OH-ions themselves: 0.01 N NaOH!) are capable of decomposing the polysaccharide-lead complex, it may be concluded that the H-bonding to Pb, as operative in these "stained" polysaccharides, is rather strong.

To what extent steric aspects are of importance remains yet to be studied.

CHAPTER 4

CHARACTERIZATION OF ISOLATED PARTICULATE GLYCOGEN AND OF ITS DEGRADATION BY CHEMICAL PROCEDURES

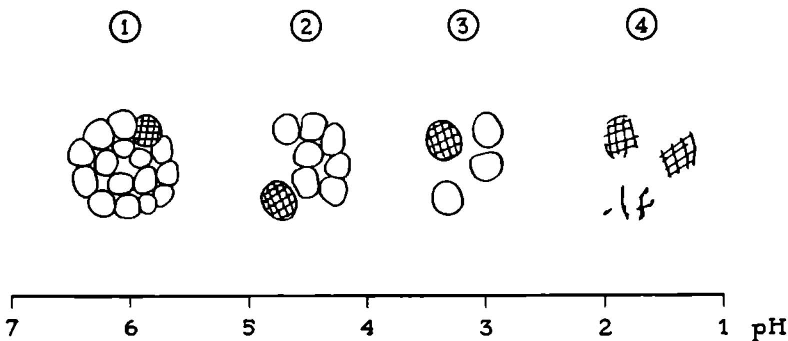
4-1. INTRODUCTION

The evidence presented by the electron microscopic examination of glycogen-containing tissues (cf. chapter 2), seems to support Lazarow's supposition (1942) that "particulate glycogen", as isolated by differential centrifugation, is a pre-existing cellular component and is not formed artificially during the extraction procedure. Indeed, in vivo glycogen might behave as a heavy cellular component that can be thrown down by ordinary centrifugal forces, as was shown by Claude (1948). After centrifugation of intact Amphiuma liver at 18.000 x g, he found that the glycogen, instead of the nucleus or the mitochondria, had accumulated at the centrifugal pole. The most valid way to answer the question whether the high molecular weight glycogen, as isolated by means of differential centrifugation, is nearly representative of native glycogen, is of course to establish that this material is identical in morphology (and staining characteristics) with the intracellular glycogen (Revel, 1964).

Such studies were made by Drochmans (1962). He isolated particulate glycogen from rat liver and studied its mode of structural organization by means of the negative staining technique. This method consists of staining not the particles themselves but their surroundings. Other samples of particulate glycogen were embedded in the usual way and studied by positive staining with permanganate.

Drochmans found the particulate material to consist of complex units, which showed a pattern of organization comparable with that of the structure encountered after staining in situ. Three structural levels were discernible, each with a particular size and morphology. The largest units - designated alpha particles - measured 60-200 mμ and were lobed in outline. They were composed of smaller units, the β-particles, which measured 20 to 30 mμ in diameter. This second stage of organization was described as resulting from the

"regular disposition in a frame" of still smaller rod- or filament-like structures, measuring 3 x 20 mμ and denominated: γ-particles. Upon treatment of this particulate glycogen with acids (acetic acid or phosphotungstic acid) a stepwise degradation of the α-particles occurred, releasing at definite pH-values the two types of constitutive units. Whereas between pH 7,0 and 5,0 no changes in the structures were observable, at pH 4,5 - 3,0 the α-particles completely dissociated and released in a pure form the second organizational stage: the β-units. At still lower pH-values (2,5-1,7) these β-particles released the asymmetric γ-particles. This last element was taken to constitute the glycogen molecule, obtained by chemical extraction. A schematic depiction of Drochmans observations is given in textfig.3.



Textfig.3. Effect of decreasing pH-values on the structure of rat liver particulate glycogen. After Drochmans, 1963.

However, since Drochmans' statements concerning the three level organization of the complex glycogen particles are based mainly on morphological observations, it seems necessary to use still other experimental approaches, e.g. sedimentation analyses, before the description given can be considered valid. Part of Drochmans' observations are made on material that was embedded in methacrylate and stained with $KMnO_4$. It was shown above (chapter 2) that this processing may evoke results that cannot be taken to be fully reliable. Especially with regard to the supposed existence of a γ-level of organization, Drochmans' study is not convincing. The author does not present a picture of the dispersed asymmetric γ-elements in negative stained preparations. Moreover, before the conclusion can be made that these γ-elements are the "glycogen-molecules, obtained by chemical extraction", it is at least necessary to study the effects of KOH or TCA, in stead of acetic acid or phosphotungstic acid, on the α-particles, since it was not proved by Drochmans that these extrac-

tion-solutions, which are normally used, have comparable effects on the particulate glycogen. The studies to be reported in this chapter were undertaken in order to shed some more light on this problem by extending Drochmans' morphological observations with analytical ultracentrifuge studies.

4-2. EXPERIMENTAL PROCEDURE

4-2-1. Animals

Particulate glycogen was isolated from the livers of young adult, female Wistar rats. They were given a standard diet (Hopefarms) and water *ad libitum*.

4-2-2. Apparatus

Preparative ultracentrifugation was carried out with a Servall RC-2 refrigerated centrifuge; the analytical runs were made with a Spinco E ultracentrifuge, equipped with Philpott-Svensson optics and a temperature-control system.*) Turbidity measurements were made with a Vitatron colorimeter, using a 520 mμ filter.

4-2-3. Isolation of particulate glycogen

The method used for the isolation of particulate glycogen was the "precipitation-centrifugation" method, developed by Drochmans (1963). Some slight modifications were introduced in an attempt to increase the recovery somewhat. Actually, the procedure gave recoveries that were no better than 10-15%. This lack of quantitative recovery, due to differential sedimentation losses, excludes of course the possibility of studying the whole spectrum of molecular weights (Orell et al. 1964). However, as this aspect was of less importance in view of the purpose of this study (which was to give an analysis of the pattern of organization of the complex alpha-particles and to study the effects of classical extraction-media on these structures), we did not make extensive attempts to achieve quantitative recovery.

Isolation procedure: After killing the animals by stunning, the livers were quickly excised and cooled to 0-4 C°. All further operations were carried out at this low temperature. After being weighed, the livers were pressed through a sieve with 1 mm borings in order to eliminate connective tissue. The pulp was

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*) The analyses were performed by Mr.Combé, Laboratory for Organic Chemistry, Agricultural College, Wageningen.

then minced in a mortar for about 10 min. and subsequently brought to pH 5,2 by suspending it in a 0,1 M K-phthalate solution, adjusted to pH 4,8 with 0,1 M NaOH. By adding about 1 gm of homogenized tissue per 2,8 ml of buffer, the desired pH was obtained. After standing for 5 min, the agglutination had proceeded to such an extent that a five minutes centrifugation at 1100 x g was sufficient to eliminate all cell-debris. The supernatant fraction was subsequently centrifuged for 30 min at 35.000 x g in the Servall centrifuge in order to obtain at the centrifugal pole a transparent firm pellet of particulate glycogen, covered with a loose layer of microsomal material. The glycogen-pellet was washed twice with 0,001 N NaOH and afterwards 2 to 3 times with aqua dest. After lyophilization, the particulate glycogen was stored above P_2O_5 in an desiccator.

4-2-4. Negative staining technique

Small drops of the glycogen samples were placed on carbon-reinforced formvar-coated grids. After two to three minutes excess fluid was removed with filter paper and a drop of 1% phosphotungstic acid (PTA) (pH 7,4, adjusted with 0,01 N NaOH) added. Excess staining solution was removed again with filter paper. Special care was taken to ensure optimum wettability of the supporting film.

4-3. ELEMENTARY CHEMICAL ANALYSIS OF PARTICULATE GLYC OGEN

Samples of particulate glycogen, obtained by means of the precipitation-centrifugation (prec. centrif.) method, were analysed for their carbon-, hydrogen- and oxygen-content, as well as for total nitrogen, phosphate and sulfur*). As it is known that glycogen particulates are highly hydrated complexes, the samples were thoroughly dried before analysis (12 hours at 100 C° under high vacuum).

This method of drying to constant weight caused a 7,1-7,3 per cent decrease in weight of the samples that had previously been stored at room temperature over P_2O_5.

The results are given in table 2; for comparison the theoretical values for a polysaccharide of the glycogen-type $(C_6H_{10}O_5)_n$ are also given.

It can be seen from this table that the C and O values are somewhat lower than the theoretical values, whereas the H-value is somewhat higher. Comparable findings were obtained by Lazarow (1942) and Drochmans (1963). The total N content in this particular sample was rather high; other preparations showed values less than 0,1%, depending on the number of washings that were performed.

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*) I would like to thank Drs.J.Walters for performing the sedimentation runs and Dr.A.Lansink for much helpful discussion.

TABLE 2

Chemical composition of particulate glycogen, isolated by the precipitation-centrifugation procedure. Results expressed as mg per 100 mg particulate glycogen.

	C	H	O	Ash	Total N	Total P	Total S
Particulate glycogen	43.63	6.39	47.89	none	0.22	0.01	0.1
Calculated for $(C_6H_{10}O_5)_n$	44.44	6.22	49.34	-	-	-	-

In view of the known regular association between glycogen and proteins and in view of the possible function of the glycogen-protein interaction for the maintenance of the complex structural organization of the alpha particles, no further attempts were made to purify the samples.

4-4. STRUCTURAL ORGANIZATION OF PARTICULATE GLYCOGEN AS OBSERVED IN NEGATIVE STAINING

Our investigations into the fine structural organization of undegraded particulate glycogen, as studied on negatively stained preparations, gave results that are to a great extent comparable with the findings obtained by Drochmans (1962). The large structures measure 60 to 200 mμ (fig. 1 and 22) and represent the structural level of the first order. The mamillated outline of these α-particles strongly suggests that they are constituted from smaller sub-units: the β-particles. The dimensions of these sub-units seem to be rather uniform: from 25 to 30 mμ. Isolated β-particles are encountered only occasionally. This must, in all probability, be related to the fact that only a small portion of the total amount of glycogen present in the living tissue, is recovered when use is made of Drochmans' isolation-method. According to Orrell et al. (1964) all glycogen particles with sedimentation coefficients of less than 500 S are lost.

If the particles are studied at high magnification (fig.23, 240.000 x) a still finer structural pattern becomes discernible.

According to Drochmans, this fine structure of the third level results from the regular disposition in two perpendicular planes of small, rod-like filaments (3 x 20 mμ) within the beta-particles. These rod-like elements were called γ-particles. Sometimes one can indeed discern small filament-like structures (fig.23, arrows) but their re-

gular occurence is not apparent. In our preparations at such high magnifications, the alpha-particles as a whole display a fine stippled aspect provided the structure has flattened sufficiently. Upon close examination one discerns large numbers of small 25 Å particles. In our opinion, however, it seems inappropriate to give a definite interpretation of this observation. Although the whole structure has flattened out considerably in this particular case, the thickness that is passed by the electron beam must be considerable, when compared with the dimensions of the "25 Å particles". The fact that structural elements of these dimensions, if present at all, will overlap considerably, makes it impossible to give an interpretation of the spatial arrangement that is based on these observations.

Another point that has attracted our attention is the fact that the smaller the alpha-particles are, the more easily they are penetrated by the electron beam. Moreover, the peripheral parts of an alpha-particle are, in the main, more transmissive than the central parts, whereas in these central areas the outlining of beta particle sub-units is only incidentally detectable. Moreover, in a number of these flattened particles a central "hole" is visible: see fig.22, arrows.

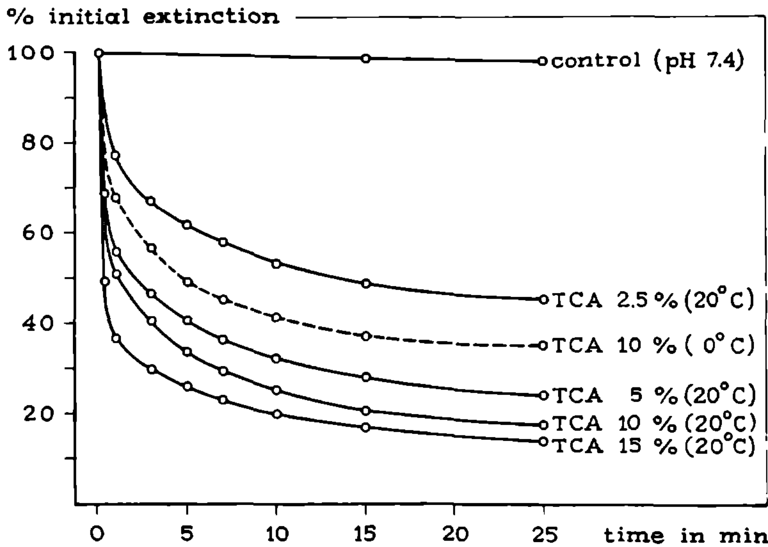
These facts are difficult to combine with the view of simple association of beta particles to a morula-like "bunch of grapes" (Revel, 1964) because, if one adheres to this view, one might expect the central parts to be thicker after drying on the supporting film. It is useful to point out, in this connection, that many particles in thin-sectionmicrographs also show a prominent crowding at the periphery (see fig.18). We shall discuss this point in section 4-6 below.

4-5. EFFECTS OF TCA AND KOH-TREATMENTS ON THE TURBIDITY OF SUSPENSIONS OF PARTICULATE GLYCOGEN

Suspensions of particulate glycogen exhibit a high turbidity, even at very low concentrations. Commercial glycogen solutions, on the other hand, show only a faint opalescence when the concentration rises above 5%. This high turbidity is due to the presence of very large particles in the suspensions. This makes it possible to study the effect of various treatments on the approximate size of the particles by simple transmission measurements. The transmission of incident light through a solution that contains large particles, will be lowered by scattering-, reflection- and absorption-phenomena. In the case of turbid colourless solutions, the lowering in the intensity of the incident beam after passing through the solution can be taken to be in a qualitative sense a function of particle size. It follows that simple transmission- or extinction-measurements (as an alternative for light-scattering measurements) yield a sufficiently reliable qualitative information about

changes in the size of particles, when aliquots of particulate glycogen are subjected to a variety of treatments.

Textfig.4 presents the "turbidity" (i.e. extinction)- measurements, plotted as per cent of initial extinction, when particulate glycogen suspensions (final concentration: 1 mg/ml) are treated with TCA of various concentrations at room temperature 20°C. It can be seen from this figure that the turbidity declines sharply when TCA is added; so sharply, indeed, that proper zero time readings could not be obtained within the time needed for mixing the glycogen solution with the acid. The zero times given were derived from readings that were gained by measuring the extinction of samples of particulate glycogen, which were introduced directly into phosphotungstic acid solutions (PTA, 2,5%, pH 7,4, adjusted with 0,01 N NaOH).



Textfig.4. Effects of TCA-solutions of various concentrations on the turbidity of particulate glycogen suspensions (1 mg per ml).

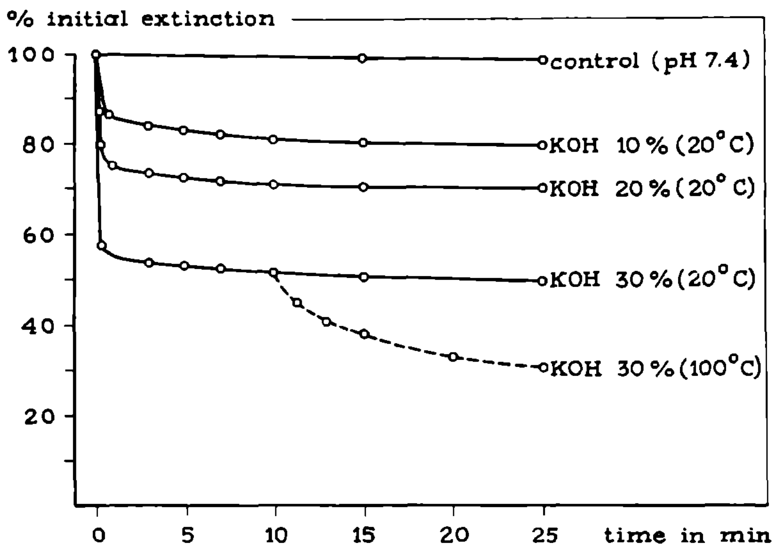
Here, too, we used PTA, this being the contrasting material chosen in the negative staining experiments, and which, moreover, was very useful as a heavy "reference-particle" in the sedimentation analyses.

It may also be seen from textfig.4 that the more the concentration of TCA is increased, the more prominent are the effects registered. In all cases a rapid initial decrease in turbidity is followed by a gradual continuous decline. However, the effect of increasing the concentration from 2,5% to 5% is more effective than the increase in concentration from 5% to 15%; a 15% TCA-solution at room-temperature

practically abolishes the turbidity in less than an hour. The influence of temperature was studied in that the readings of the 10% TCA-treatment (the most commonly employed concentration for the extraction of glycogen from tissues) were also made at 0°C.

Although apparently this lower temperature somewhat decreases the rate of attack on the particles, there is an effective decrease in turbidity as well, indicating a drastic decrease in particle size.

When these findings are compared with the effects of KOH-treatment (see textfig.5), it appears that some differences exist. With KOH, too, there is a pronounced initial effect. This effect is already attained within the first minute after mixing the glycogen solution with the alkali, but afterwards the degrading influences work at a far lower rate. The effect of boiling the particulate glycogen-KOH 30% mixture is also indicated in textfigure 5. It can be seen that this rise in temperature accelerates the degradative influences of KOH.



Textfig.5. Effects of KOH-solutions of various concentrations on the turbidity of particulate glycogen suspensions (1 mg/ml).

From these findings it may be concluded that both TCA and KOH cause a rapid decrease in turbidity of particulate glycogen solutions.

If estimated by proper light-scattering measurements, the changes in turbidity of high molecular weight glycogen can be taken to be roughly proportional to the changes in the average molecular weight of the preparations. L a s k o v and M a r g o l i a s h (1963) found that boiling of a phenol-extracted rat liver glycogen with a weight-average molecular weight of 132 x 10^6 caused a diminution in turbidity of 42%

and a decrease in molecular weight of 35% (to 86 x 10^6).

Our measurements cannot be considered informative in this respect. In interpreting our results, one should take into account the fact that light-scattering (and this also applies to extinction) depends among other things on the refractive index of both the particles and the medium, and is related to the quotient: $n_{particle}/n_{medium}$.

The n_{medium} is different from one reading to another since the refractive index of a solution of TCA or KOH increases with concentration. To get an idea of this: the n_{water} = 1,333; the n for a 10%, 20% and 30% KOH-solution resp. 1,353, 1,372 and 1,390. We have no exact numbers available for TCA, but the values will be about the same as those for acetic acid or perchloric acid: 1,337; 1,340 and 1,344 resp. for a 5%, a 10% and a 15% solution of these acids. From these data it follows that there will be an abrupt and distinct decrease when the particulate glycogen samples are mixed with the KOH, whereas this will be only of minor importance in the case of TCA-treatment.

Moreover, one must bear in mind that $n_{particle}$ will also change when the glycogen particles are brought into contact with concentrated alkali, because concentrated alkali will increase the degree of hydratation of the complex structures. In this respect, too, the effects of TCA will be of minor importance.

Bearing these facts in mind, the TCA- and KOH-curves need a correction. The path of the TCA-curves will change little; their initial steep slope will be somewhat less pronounced, but they retain their curve-like character; i.e. they point to a change in particle-size right from the start of the contact between glycogen and acid.

The KOH-curves, however, will show a distinctly different path, since the initial shift, as indicated in textfig.5, is really artefactual (if taken as being an indication for changes in particle-size). The KOH-curves will show approximately a straight line just below the control curve. Only the shift induced by temperature (100°C) will retain its original slope. Therefore the complex alpha particles do not show a distinct decrease in particle size when brought into contact with concentrated alkali; the temperature-dependency should be understood from the more general lability, especially in the presence of oxygen, which these structures demonstrate with regard to temperature.

The TCA-effect on particle size must certainly be related to the acid-lability of the glucosidic linkages. A rupture of only a few of such bonds in a molecule may lead to a drastic decrease in particle size and a corresponding drop in turbidity. Given the fact that one starts with very big particles and assuming that this acid hydrolysis of the glucosidic bonds occurs in a random fashion, then the (corrected!) slope of the TCA-curves becomes understandable. Initially, when there are large particles, one random hydrolytic cleavage will have a radical effect on the average particle size (see page 84), but since

the particles become progressively smaller and more numerous, the same number of cleavages per unit of time will have a progressively smaller effect on the particle size (Stetten and Stetten, 1957). Of course, one might think of the initial steep slope of the TCA curves as representing the destruction of "structural proteins" in the particles, but this remark has a speculative character, since little is known with certainty about the role proteins play in the maintenance of the particulate organization ("surface-coats") of this high molecular weight glycogen. Moreover, since TCA causes protein-precipitation rather than a hydrolysis at low temperatures, a TCA-effect on proteins will rather cause an increased solidity of such presumed structural-protein bonds.

4-6. CHANGES IN SEDIMENTATION COEFFICIENT DISTRIBUTION UPON TREATMENT OF PARTICULATE GLYCOGEN WITH ACIDS

Drochmans' statements about the three-level organization of rat liver particulate glycogen seem to obtain firm support from his observations that treatment of the complex alpha structures with acids caused a stepwise dissociation into the constitutive units. The compound alpha particles, according to him are not affected by a decrease of the pH from 7,0 to 5,0. Beyond this pH the alpha particles begin to dissociate and at pH 3 all the alpha particles have completely released their constitutive beta particles. At pH-values below 2,0 these beta-particles were found to release in their turn the rod-like gamma elements.

Drochmans amplified these statements with a study of the decrease in opalescence of the glycogen suspensions at the various pH-values in the acidic range. The "relative opalescence" *) -pH curve showed four different slopes, each corresponding to a definite stage in the dissociation of the complex alpha particles: no changes in turbidity from pH 7,0 to 5,0; a slow decrease from pH 5,0 to pH 4,0; a steep drop between pH 4,0 and pH 2,5 and again a slower decrease below pH 2,5. The stepwise dissociation could be induced both by acetic acid and phosphotungstic acid and was therefore thought to depend solely on the pH of the medium and not on the type of acid used. One might take such a stepwise dissociation to indicate that the dissociation of the alpha particles into the beta units is caused by the

*) The "relative opalescence" data were obtained by comparing the turbidity of the samples at the various pH values with the turbidity of a dilution scale; i.e. a series of glycogen suspensions at decreasing concentrations from 1.-0.02%. The author does not state how the turbidity-measurements proper were made.

cleavage of another type of bond than is the case with the dissociation of the beta particles into the gamma elements. A stepwise dissociation, with the formation of discrete sub-units, can be detected only if the method chosen gives information about the particle size distribution. Turbidity measurements cannot be considered valid for this purpose. A study of the sedimentation behaviour, however, gives information about the particle size distribution and, in the case of the release of discrete sub-units with a substantial difference in molecular weight, a sedimentation run will give direct evidence about the number as well as about the relative amounts of the component parts. The studies to be reported in this section, were undertaken with the intention of tracing the effects of treatment with acids on the particle size of particulate glycogen by means of sedimentation analysis.

All sedimentation measurements were carried out with 2,5% PTA (adjusted to pH 7,4 with 0,01 N NaOH) as the solvent. The sedimentation constants are given as $S_{app.} = S_{(20,\ PTA)}$ at c = 1 g/100 ml. Since we were mainly interested in the number of components present, no corrections were made for concentration (except for one particular sample) or type of solvent used. After the treatment with acid solutions of PTA, the degradative influence was stopped by simply adjusting the pH to 7,4. After the TCA-treatment, the glycogen was precipitated with aethanol and resuspended in PTA.

The high turbidity of the suspensions of particulate glycogen hinders the photographing of the refractive index gradient curves and excludes the possibility of exact measurement of the relative amount of each component by measuring the area below the curves. Consequently only approximative information could be obtained in this respect. For the same reason some difficulty was encountered in determining the exact maximum of the peak of the heavy component, since this peak is half buried in the dark part of the diagram.

Each time a particulate glycogen isolation was performed, a run was made of the material obtained. The UC-diagrams of these 'untreated' samples showed in all cases one broad peak, indicating an extremely wide molecular weight distribution. The sedimentation coefficients varied from 800 to 1000 S; one particular run is presented in UC-diagram 470 with $S_{app} = 957$. All the index gradient curves, representing this high molecular weight component, showed the presence of spikes, which indicate the presence of very large aggregates of varying size. As was already noted above, these untreated samples contained mainly the complex alpha particle type of glycogen, pictured in the figs.1, 22 and 23.

The sedimentation results, obtained on particulate glycogen samples that were treated for varying periods with PTA or TCA at different pH-values, are brought together in table 3. The PTA-solutions were

TABLE 3

Sedimentation results of particulate glycogen samples, suspended in 2.5% PTA, pH 7.4, after treatment for varying periods with PTA at different pH-values or with TCA.
The S-values are given as $S_{app} = S_{(20\ PTA)}$ at c = 1 g/100 ml.

Run nr.	Treatment	Sedimentation coefficients	
		S_1	S_2
373	-	950	-
379	1 hr 2.5% PTA, pH 7.0	930	-
380	1 hr 2.5% PTA, pH 5.0	927	-
381	1 hr 2.5% PTA, pH 3.0	840	163
478*	5 hrs 2.5% PTA, pH 1.7	492	102
382	24 hrs 2.5% PTA, pH 1.7	+	72
470	-	957	
472*	1 min 2.5% TCA, pH < 1.0	874	156
473*	5 min 2.5% TCA, pH < 1.0	885	139
474*	15 min 2.5% TCA, pH < 1.0	770	138
477	15 min 10.0% TCA, pH < 1.0	487	92
513*	24 hrs 2.5% TCA, pH < 1.0	+	82
507*	72 hrs 2.5% TCA, pH < 1.0	-	40
375*	Commercial glycogen (BDH)	-	110
511*	7 days 8 M urea	923	-
531	1½ hrs boiling with KOH 30%	+	97

* These runs were continued until the PTA-peak occurred.

adjusted to the desired pH with 0,01 N NaOH.

It can be seen from the results, obtained with PTA at pH 7,0 and 5,0 that the alpha particles are relatively stable in this pH-range, since no appreciable changes in molecular weight occurred. This fact was already made use of by Drochmans (1962), since in his

precipitation-centrifugation method for the isolation of particulate glycogen, a decrease in pH below pH 5,0 is avoided.

PTA-treatment of the alpha particles for one hour at pH 3,0, however, caused the apparence of a second peak in the UC-diagram (cf. UC-diagram 381).

From negatively stained EM-preparations of this sample (see fig. 24) it followed that this second lower molecular weight component is formed by the beta particles, which have now been released from the alpha structures.

If the PTA-treatment is conducted at a still lower pH-value (1,7) and for as long a time (5 hrs), as is claimed by Drochmans to be appropriate for the complete dissociation of the beta particles into their constitutive gamma elements, it appears that both the heavy and the lower molecular weight components have undergone a distinct decrease in S-value, but also in this case a third component did not come through in the UC-diagram (cf. run nr. 478).

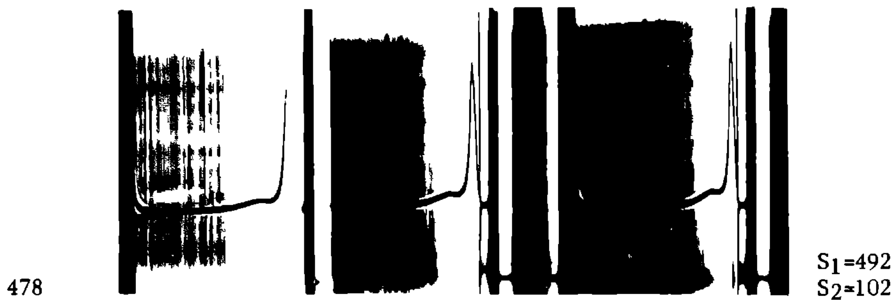
Negatively stained preparations from this last sample (fig.24) revealed the presence of numerous beta particles. Moreover, one might take the appearance of the alpha particles present on this micrograph to be reminiscent of the disintegration of these structures. When the sedimentation coefficient of the second component of this latter sample (S = 102) is compared with that of a commercial glycogen sample (run nr.375, rabbit liver glycogen BDH, KOH-extracted.), it appears that our data contradict the statement that the 'asymmetric gamma elements' constitute the glycogen molecules, obtained by chemical extraction (Drochmans, 1963).

Even after a 24 hours' prolongation of the PTA-pH 1,7 impact, which causes an almost complete disappearance of the alpha particles, no indications were found for the existence of a third component with an appreciably different molecular weight. Whereas the S-value of the beta particle component, after this component had been acted upon for 4 hrs by PTA, decreased from 163 to 102, a treatment lasting 19 hrs longer only causes a decrease from 102 to 72 S. In interpreting this observation one should take into account the fact that there is a distinct increase in concentration of the second component during the process of degradation of the alpha particles. Sedimentation constants are dependent, among other things, on concentration; according to Larner et al. (1958) the S_{20}-values for glycogen are a function of c^2. They proposed the relation $S_{(20)c} = S_{(20)0}(1 - kc^2)$, where k has the value 0,152. In order to get some idea of this, a concentration-dependency determination was performed, use being made of a particulate glycogen sample that had been treated for 3 days with 2,5% TCA, pH 0,95.

We have chosen this material, because in these preparations the

UC-diagram of 10 mg particulate glycogen/ml 2,5% PTA, pH 7,4 untreated. (1-10,589-2/2)*

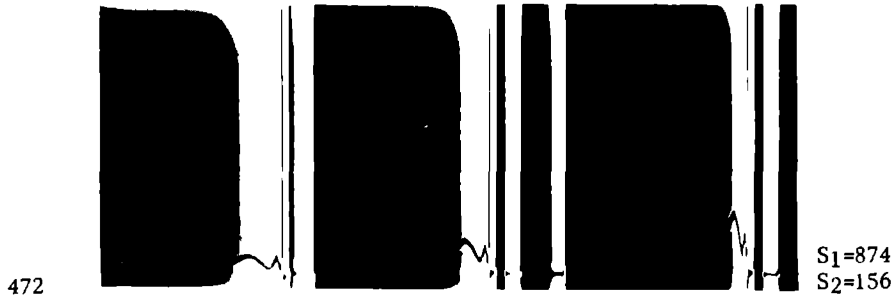
UC-diagram of 10 mg particulate glycogen/ml 2,5% PTA, pH 7,4 after treatment for 1 hr with 2,5% PTA, pH 3,0. (4-6,995-8/8)*



UC-diagram of 10 mg particulate glycogen/ml 2,5% PTA, pH 7,4 after treatment for 5 hrs with 2,5% PTA, pH 1,7. (2-10,589-2/2)*

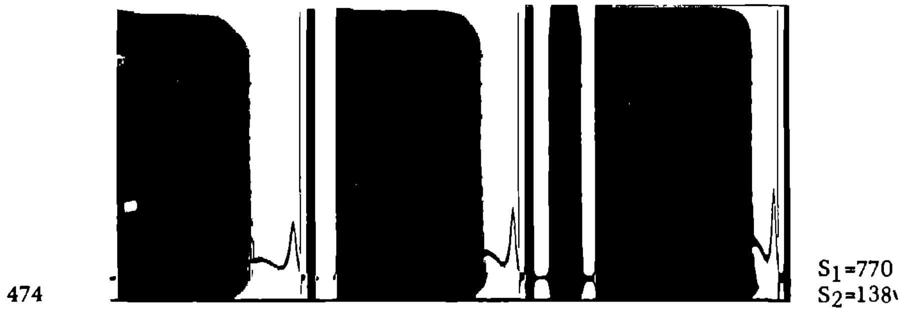
* (x-y-z) = photos taken x min after reaching a speed of y RPM at time intervals of z min.

472



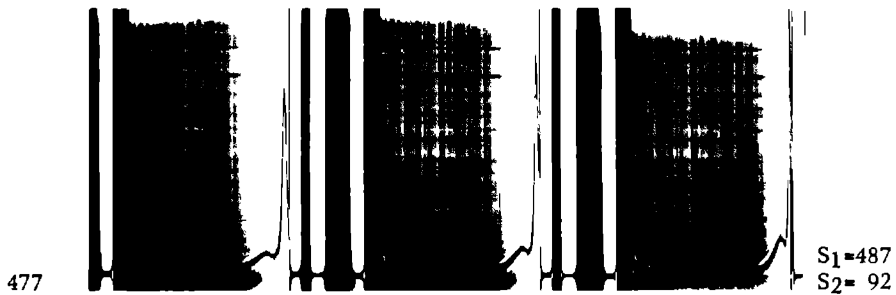
UC-diagram of 10 mg particulate glycogen/ml 2,5% PTA, pH 7,4 after treatment for 60 sec with 2,5% TCA, pH 0,95. (2-10,589-2/2)*

474



UC-diagram of 10 mg particulate glycogen/ml 2,5% PTA, pH 7,4 after treatment for 15 min with 2,5% TCA, pH 0,95. (2-10,589-2/2)*

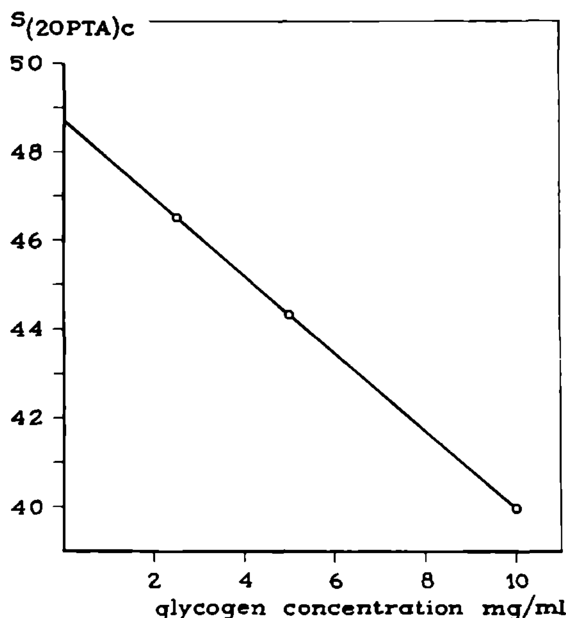
477



UC-diagram of 10 mg particulate glycogen/ml 2,5% PTA, pH 7,4 after treatment for 15 min with 10% TCA, pH 0,45. (4-10,589-2/2)*

* (x-y-z) = photos taken x min after reaching a speed of y RPM at time intervals of z min.

alpha particles are no longer present. The extrapolation plots of the $S_{(20\ PTA)c}$-values versus the glycogen concentration are given in textfig.6.



Textfig.6. Concentration-dependency of glycogen.

It can be seen from these data that the sedimentation constant varies about 2% for a 10% change in concentration. Textfig.6 supports the statement by B r y c e et al. (1958) that the dependency of the sedimentation constant on concentration is represented by a linear function. According to them the relation can be expressed by $S_{20} = (S_{20})_0(1-kc)$; the average value of k being: 0,10 $\pm$ 0,02 and c expressed in g/100 ml. However, if the sedimentation constants of the second components in the runs UC 381 (163S), UC 478 (102S) and UC 382 (72S), which are examined only at 'total' glycogen concentration c = 1 g / 100 ml, are re-calculated for values $S_{(20\ PTA)0}$ on the basis of the latter formula, there remain distinct differences in the sedimentation values of the second component particles in these runs. Of course, these corrections are only approximative, since the relative amount of the second component particles has changed from one run to the other. Nevertheless, we are inclined to think that these observations can be understood better by assuming that the beta particles are subject to a slow random degradation, without the release of a distinct third component.

Since our interest was likewise directed towards the effect of classical glycogen extraction media on the particle structure of particulate glycogen, a comparable series of studies was made of the effect of TCA. It follows from table 3, that a period as short as one minute of contact with a 2,5% TCA-solution is sufficient to bring about a second peak in the UC-diagram. (cf. UC-diagram 472). If this period of contact with the acid is extended to 15 min the number of beta particles released is increased considerably. This follows from a comparison of the peak-heights in question. (cf. UC-diagram 474). A treatment of 15 min with TCA 10% is sufficient to cause a sharp fall in turbidity; see the UC-diagram of run nr.477. As with all other diagrams the second component is always present in the completely "transparent" part of the diagram, from which it follows that these beta particles contribute little or nothing at all to the turbidity of the particulate glycogen solutions, which is therefore mainly due to the presence of the alpha particles. This raises some doubt as to the reliability of the "relative opalescence" data (see page 74), using a dilutionscale of particulate glycogen, in which the alpha particles are present throughout.

After boiling a particulate glycogen sample for 1,5 hrs with KOH 30%, the heavy component peak had almost completely disappeared; the beta particle population present after this treatment showed an S-value of 97.

A treatment of the samples with a 8 M urea solution for one week did not cause changes that were detectable by sedimentation analysis or by electron microscopic examination; cf. run nr.511. Similar findings were made by Orell and Bueding (1958).

4-7. DISCUSSION

Several data are now available in literature to support the opinion that the type of method used for the extraction of glycogen from tissues, is of primary importance with respect to the particle size that is finally obtainable. If glycogen is extracted by alkaline digestion of the tissue, no marked differences in the physical properties of glycogens from various sources are detectable.

To illustrate this point, the work of Bueding et al. (1964) may be mentioned. They used a cold-water method to isolate liver glycogen from patients with various types of glycogen storage diseases. Any one of the samples showed characteristic sedimentation diagrams, so that it was possible to recognize the particular type of storage disease from these diagrams. However, when any of these glycogen samples were exposed to the degradative influences of hot alkali, the characteristic differences disappeared, while a marked reduction in molecular weight was observed.

After alkaline digestion, the glycogen samples mostly show high polydispersity with molecular weights between 1 and 10×10^6 (Greenwood, 1956; Manners, 1957; Bryce et al., 1958).

Although it is a generally accepted view that glycogen is relatively stable in hot concentrated alkali it was shown, in a recent study by Stetten and Katzen (1961), that the treatment of TCA-extracted glycogen with hot concentrated alkali caused a reduction in molecular weight, even under anaerobic conditions. The glycogen was converted into a polydisperse system of relatively stable polysaccharinic acids, whereas a small amount of isosaccharinic acid was set free.

When cold TCA is used as the extraction medium, the particle sizes one finds are usually higher, having molecular weight values up to 100×10^6 (Stetten et al. 1956, 1958; Bryce et al., 1958; Remarque, 1958).

Lazarow's method of isolating the glycogen by centrifuging a liver homogenate in cold 0,85% NaCl-solution, as well as Drochmans' "precipitation centrifugation" method might be supposed to be even less drastic methods, since they yield particles with molecular weight values of about 10^9.

Precise knowledge of the influence which the classic extraction-media exert on glycogen molecular size, is of importance in considering the relation between the structural image of glycogen as encountered in electron microscopic specimens and the structural image that is arrived at on the basis of chemical or physical studies.

If for the moment we may restrict the discussion to the compound α-particle type of glycogen, it appears that the electron microscopic in situ aspects of these structures - both with regard to dimensions and apparent fine structure - correspond closely to the appearance in negatively stained preparations of cold water extracted, chemically well defined, high molecular weight glycogen. This fact strongly supports the supposition that this high molecular weight material approaches the state of glycogen in the living cell more closely than does the glycogeh that has been in contact with TCA or boiling KOH; these classical extraction media were found to cause an immediate degradation of these structures, especially the first.

From these findings another conclusion may be drawn, namely that the tissue processing, as applied in the electron microscopic histology, permits one to arrive at a reliable representation of the in vivo form of glycogen. Consequently, the differences between the complex form of glycogen (as encountered, for example, in liver cells and the epithelial cells of insect malpighian tubules or intestine, see chapter 2) and the simple particles (as present, for example, in muscle) can be taken to represent real in vivo differences.

The question remains which are the real causes behind these different appearances of *in situ* glycogen in various tissues. Lazarow (1942) thought of the large molecular size glycogen as being the result of a protein-bonded aggregation of smaller sub-units. The studies, reported in this chapter, have indeed shown that these big structures, when brought into contact with acids at sufficiently low pH-values, (or after being treated with boiling conc. alkali) immediately begin to release a component with a distinctly different and much smaller average molecular weight. One is inclined to consider this lower molecular weight component to represent the "sub-unit" particles (β-particles), which are held together by proteinaceous aggregation. Especially the negatively stained images of the complex particles, due to their mamillated outline, are very suggestive of an association of smaller sub-units. However, the studies by Orrell et al. (1958, 1964) make it unlikely that proteins can be held responsible for the aggregation. The complex, cold water extracted glycogen particles of *Ascaris* did not show a change in molecular weight average or distribution after a thorough purification of the glycogen sample resulting in a protein-content of less than 0,03 per cent. They found moreover, that incubation with a number of agents that are known to rupture protein- or hydrogen-bonds (like urea 8 M, guanidine 8 M, thiocyanate 2 M, lithium bromide 8 M, and non-ionic, anionic or cationic detergents like Tween 1%, sodium dodecyl sulphate 1% or cetyltrimethyl ammonium bromide 1%) did not cause changes in the molecular weight distribution. Repeated precipitation with aethanol and drying, as well as repeated freezing and thawing did not affect the molecular size and distribution either.

Irreversible degradation could only be obtained after treatment of the samples at low pH values or by heating them above 60-70°C. The authors concluded from these results that proteins cannot be considered responsible for the aggregation of the sub-units, which must be held together by some form of a still unknown chemical bond. This bond must already exist in the living cell, which follows from the fact that the molecular weight distribution curve only changed under the impact of factors other than those operative during the extraction procedure. In their method, use is made of a mixture of chloroform and octyl alcohol, which causes a precipitation of proteins (in the interphase), whereas polysaccharides remain in the aqueous phase.

One should bear in mind that the concepts "aggregates" and "sub-units" are as yet poorly defined in this context. Indeed, the electron microscope pictures are very suggestive of the presence in the large "aggregates" of small "sub-units". Nevertheless, the use of these loaded words is not justified as long as it has not been defined more

precisely (1) in what respect these sub-units are "units" (molecules); (2) which type of bond holds these sub-units together in the "aggregates". If, for example, the associating bonds are ordinary glucosidic linkages, then no real objections can be made to consider the whole particle as one molecule.

The electron microscopic information concerning the real presence of "sub-units" must therefore be considered critically. One should be aware of the fact that the EM-pictures of the complex structures, studied either in thin sections or in negatively stained preparations, result from the perpendicular "projection" of the whole structure. We should like to attribute special significance to the observation, made both on thin sections and on negatively stained preparations, that the central areas often appear less densely packed than the peripheral parts. This indicates that the amount of polysaccharide material in the peripheral parts must be higher than in the central areas.

If one think of a "construction" of the complex alpha particles, that is, on its own level, in accordance with the depiction, given in textfig.2; i.e. if one considers the alpha particle as a giant molecule (when this depiction is raised to a 3-dimensional level; at the same time abandoning the principle of strictly regular branching), then such a concept can explain most of the phenomena observed:

1. the central part of the molecule will be less "dense" than the peripheral parts.
2. the ramifications on one stem can give the idea, after the particles have dried out, of being semi-circular sub-units. This may certainly happen when these ramifications stick out of the structure in an irregular way.
3. Upon treatment with acids, the centre will be most liable to cleavage of the glucosidic bonds.
   It is also at the centre that the effects on particle size will be greatest and give rise to the release of the presumed "subunits".
4. There is no third-level unit, as may be evident from our researches.
5. There is no need to contribute a structural function to the adhering (enzyme)-proteins.

It remains for further study to answer the question why one encounters this alpha particle only in the liver of mammalians and in a number of tissues of lower animals, whereas the other mammalian tissues contain the smaller beta particles.

PART II

CHAPTER 5

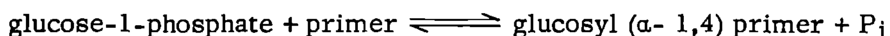
SURVEY OF LITERATURE ON CELLULAR METABOLISM OF GLYCOGEN

It is becoming more and more apparent that in the study of metabolic processes, knowledge both of the biochemical factors and of the cellular structural relationships involved is indispensable.

Accordingly this chapter, conceived as an introduction to the second part of this thesis, will be divided into two sections. The first section deals with the biochemical reactions involved in glycogen synthesis and breakdown, and the second with the cytological aspects of glycogen metabolism as observed in electron microscopic studies.

5-1. BIOCHEMICAL ASPECTS OF SYNTHESIS AND DEGRADATION OF GLYCOGEN *

Until some years ago the phosphorylase-reaction:



$$\text{glucose-1-phosphate} + \text{primer} \rightleftharpoons \text{glucosyl } (\alpha\text{- } 1,4) \text{ primer} + P_i$$

was generally held to account for both glycogen synthesis and degradation in vivo. This view dates back to the discovery, by Cori and Cori, of the enzyme phosphorylase in animal tissues and the discovery by Cori, Schmidt and Cori in 1939 that the reaction is readily reversible in vitro. Since these pioneering studies, several reports have been published, the results of which made it difficult to accept the view that glycogen synthesis is catalyzed by phosphorylase merely as a reversal of glycogen degradation.

These objections, as listed by Stetten and Stetten (1960), concern:

1. the equilibrium of the reaction

The direction which the reaction takes is dependent on the relative concentrations of inorganic phosphate and glucose-1-phosphate. Excess inorganic phosphate acts in the direction of glycogen breakdown, excess glucose-1-phosphate

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* The systematic names of the enzymes mentioned in this chapter are placed together in the legend of textfig.7. Those not given in this figure, are indicated in the text.

in the reverse direction. At pH 7 equilibrium occurs when the ratio P_i : G-1-P equals 3,5 : 1. It was found, however, that in tissue the ratio is always higher than 3. For instance Larner et al. (1959) reported rapid glycogen synthesis in normal and insulin-treated rat diaphragm-muscle with ratio's P_i : G-1-P in the order of 250-305:1. In order to overcome this difficulty, it has been postulated that the ratio on the site of glycogen-synthesis in the cell might be quite different from the "overall ratio" which is found after biochemical handling i.e. tissue-extraction. This, however, does not seem valid because phosphorylase is known to occur "in soluble form" in the cytoplasm; i.e. the enzyme is present in the same cytoplasmic space as is glycogen.

2. hormonal influences on glycogen-metabolism

It has been found that administration of epinephrine to muscle and epinephrine or glucagon to liver invariably causes glycogen degradation. These hormones are known to act by increasing the phosphorylase-activity. It is reasonable to expect that the activation of an enzyme which catalyzes a reversible reaction, under the appropriate conditions would demonstrate this reversibility. The unidirectional effect of phosphorylase-activity enhancement remained unexplained.

3. effect of alteration of intracellular ion-concentrations

Rat liver slices incubated in the presence of high concentrations of Na^+ showed a more rapid rate of glycogenolysis than did similar slices incubated in high potassium medium (Cahill et al. 1957). Net glycogen-synthesis was only observed when phosphorylase-activity was low. In the high sodium medium the increased rate of glycogenolysis was due to elevated levels of phosphorylase activity.

4. synthesis of saccharides by transglucosylation reactions involving nucleotides

In the last 15 years sugar nucleotides (sugar or sugarderivatives, esterified through the glucosidic hydroxyl to the β-phosphate of a nucleoside 5'-pyrophosphate) have been shown, - largely through the work of Leloir and his collaborators - , to occupy a central position in carbohydrate metabolism. Their role in a number of sugar interconversions has been clearly established, as well as their functioning as glucosyl donors in many transglucosylation reactions. Thus Leloir's studies (1951) on galactose-metabolism, i.e. the transformation of α-D-glucose-1-phosphate to α-D-galactose-1-phosphate, led to the isolation of uridine diphosphate D-glucose (UDPG) as a co-factor.

The other important function of sugar nucleotides, namely their role as glucosyl donors in the formation of complex saccharides through transglucosylation reactions, was indicated in the studies of Dutton and Storey (1953), who found that UDP-glucuronic acid (UDPGA) was the glucuronic acid donor in the synthesis of a glycoside, β-O-aminophenol glucuronide. The synthesis of the disaccharide trehalose-phosphate from UDPG and glucose-6-phosphate with a yeast enzyme was achieved shortly afterwards by Leloir and Cabib (1953).

For additional information on the role of sugar nucleotides in carbohydrate metabolism the reader is referred to a recent review of this rapidly expanding field of research: Ginsburg, 1964. The most important aspect of their biological function is presumably the division of carbohydrate metabolism into separate anabolic and catabolic pathways, thus creating the possibility of independent control mechanisms.

In 1957 Leloir and Cardini reported the presence, in liver

homogenates, of an enzyme which catalyzed the transfer of glucose from UDPG to glycogen primer. In this report an absolute requirement for primer was stated, as indicated by the fact that no UDP was formed when no polysaccharide was present.

In a subsequent paper (Leloir et al. 1959) it was shown that an analogous enzyme in rat muscle preparations similarly could synthesize glycogen by a transglucosylation reaction, in which UDPG acts as the D-glucosyl donor

$$\text{UDPG} + \text{primer} \longrightarrow \text{glucosyl } (\alpha - 1,4) \text{ primer} + \text{UDP}.$$

Purification procedures, though rather unsuccessful, tended to separate the enzyme activity from phosphorylase-activity. The stoichiometry of the reaction followed from the fact that for each mole UDPG that was utilized in the test system, about 1 mole UDP was formed and 1 mole glucose was added to glycogen.

That an α - 1,4 type bond was indeed formed in this reaction could be proved by using UDPG- ^{14}C to yield radioactive glycogen. This glycogen, upon β-amylolysis, lost all the radioactivity, while maltose-^{14}C was set free.

Phosphorylase treatment of the labelled glycogen yielded glucose-^{14}C-1-P.

The primer requirements of the enzyme were about equal to those of muscle phosphorylase: the best primer being glycogen. Primer activities of glycogen showed a steady decrease after treatment of the glycogen sample with phosphorylase, β-amylase and α-amylase respectively.

An interesting point found was that the partially purified enzyme preparations could be markedly activated by adding glucose-6-phosphate and, to a lesser extent, by fructose-6-phosphate.

Experiments with glucose-^{14}C-6-P, however, showed that this glucose was not incorporated into glycogen. The possibility was considered that G-6-P might serve as the primary acceptor of the glucose residue, but no indication in favour of the intermediate formation of a disaccharide phosphate (to serve as a donor to glycogen) could be found.

These findings from muscle preparations were confirmed by the studies of Villar- Palasi and Larner, 1958; Robbins, Traut and Lipmann, 1959; and Hauk and Brown, 1959.

Villar-Palasi and Larner (1958) showed that UDPG in muscle tissue was formed from UTP and G-1-P according to:

$$\text{UTP} + \text{G-1-P} \rightleftharpoons \text{UDPG} + \text{pyrophosphate (PP)},$$

the reaction being catalyzed by the enzyme uridine diphosphate glucose pyrophosphorylase.

This evidently makes the uridine-linked glycogen-synthetic route

independent of inorganic phosphate and thus the unfavourable equilibrium for synthesis through the phosphorylase reaction is circumvented.

Robbins et al. (1959) found the UDPG-glycogen synthetic activity in pigeon breast-muscle and rat-skeletal muscle to be greatest in a particle fraction with sedimentation characteristics of microsomes. Because this fraction contained high concentrations of glycogen and almost half of the phosphorylase-activity, the possibility was considered that the enzyme was strongly adsorbed on glycogen. The fraction had to be supplemented for optimum activity by a nondialyzable supernatant factor, Mg^{++} and phosphate.

These authors discuss the thermodynamic advantages of glycogen synthesis by way of UDPG and found this route to be very favourable: the conversion to glycogen being greater than 99 per cent.

Association of the enzyme with particles was found for the rat liver preparations as well (Leloir and Goldemberg, 1960). By separation from the particulate glycogen, a 20- to 30-fold increase in specific activity was obtained. The enzyme appeared to be bound to the particulate glycogen as an enzyme-substrate complex, since on treatment of the (high molecular) particulate glycogen with solutions containing (low molecular) KOH-treated glycogen, followed by centrifugation, there remained considerable activity in the supernatant. The same pH optimum (8,4) and about the same Km value ($4{,}8 \times 10^{-4}$M) as for the muscle enzyme were found for the liver enzyme.

The concentration of glucose-6-P, necessary for half the maximum activation was found to be 0,6 mM for rat muscle (Leloir et al. 1959) and liver (Leloir and Goldemberg, 1960), which shows that in vivo G-6-P concentrations (conc. in liver and muscle range from 0,1 to 0,5 mM; Steiner and Williams, 1959) might be important in regulating the glycogen synthesis. We will discuss this point further down.

All this evidence clearly indicates that the uridine-linked pathway rather than phosphorylase might serve the synthetic route to glycogen. However, to find an unequivocal method to assign the intracellular events to one enzyme or the other presents considerable difficulties. Fortunately, nature itself offers unexpected collaboration. Strong support for the operation of the UDPG-mechanism in vivo was gained by the discovery of a human McArdle type myopathy (Cori type V), characterized by rapid fatigue, which could be alleviated by the administration of glucose or lactic acid. In this glycogen storage disease (Cori type V) only the muscles are involved (Mommaerts, Illingworth, Pearson, Guillory and Seraydarian, 1959; Larner and Villar-Palasi, 1959; Schmid, Robbins and Traut, 1959). In the structurally non-aberrant muscles, glycogen levels were normal or slightly raised, whereas the glycogen deposited

had normal structural characteristics. While phosphorylase activity was virtually absent, phosphorylase-kinase and phosphorylase-phosphatase (P.R.-enzyme) activities were normal, thus illustrating the point that the absence of phosphorylase was specific (Mommaerts et al. 1959). As there were, moreover, no differences from normal in the activities of amylo-1,6-glucosidase, phosphoglucomutase and UDPG-pyrophosphorylase (Larner and Villar-Palasi, 1959) and of UDPG-glycogen synthetase (Schmid et al. 1959), the most obvious explanation is that glycogen in this myopathy is synthetised via the UDPG-route.

In support of the assumption of a cyclic mechanism of glycogen synthesis and breakdown (see textfig.7) are also the findings on the type VI glycogenosis or Hers-disease (a still relatively ill-defined condition with a suggested defect in phosphorylase-activity, Hers, 1959, 1961; Schmid, 1964) and those on the recently described "glycogen storage deficiency disease" (Lewis, Stewart and Spencer-Peet, 1962; Spencer-Peet, et al. 1964). The dominant findings in the latter, apparently inherited, condition are the deficiency in liver glycogen synthetase, combined with very low levels of liver glycogen and an inability to maintain a normal blood glucose level during fasting. In one of the more thoroughly studied patients the phosphorylase and UDPG-pyrophosphorylase activities were normal while glucose-6-phosphatase activity was only slightly increased.

A study of the quantitative distribution of the enzymes concerned in correlation with the glycogen content of various rat tissues (muscle, liver, heart, kidney, spleen, lung, brain, testis, intestinal mucosa, fat), revealed no data that were incompatible with the assumption of a cyclic UDPG-linked mechanism for synthesis and a phosphorolytic mechanism for degradation of glycogen (Villar-Palasi and Larner, 1960), which is now a generally accepted theory (Touster, 1962).

Here too, however, the tendency to overemphasize the uniformity of metabolic pathways might to some degree divert our attention from biological variability. By using Takeuchi's histochemical methods to demonstrate phosphorylase-, branching enzyme- and UDPG-glycogen synthetase activity, Hess and Pearse (1961) could show that in rat skeletal muscle the phosphorylase and branching enzyme are most active in the large white fibers, whereas UDPG-glycogen synthetase with a very great activity, is concentrated in the small red type fibers. Under normal conditions the white fibers contain more glycogen than do the red ones, but upon adrenalectomy the red fibers retain their levels, whereas the white fibers become depleted (De

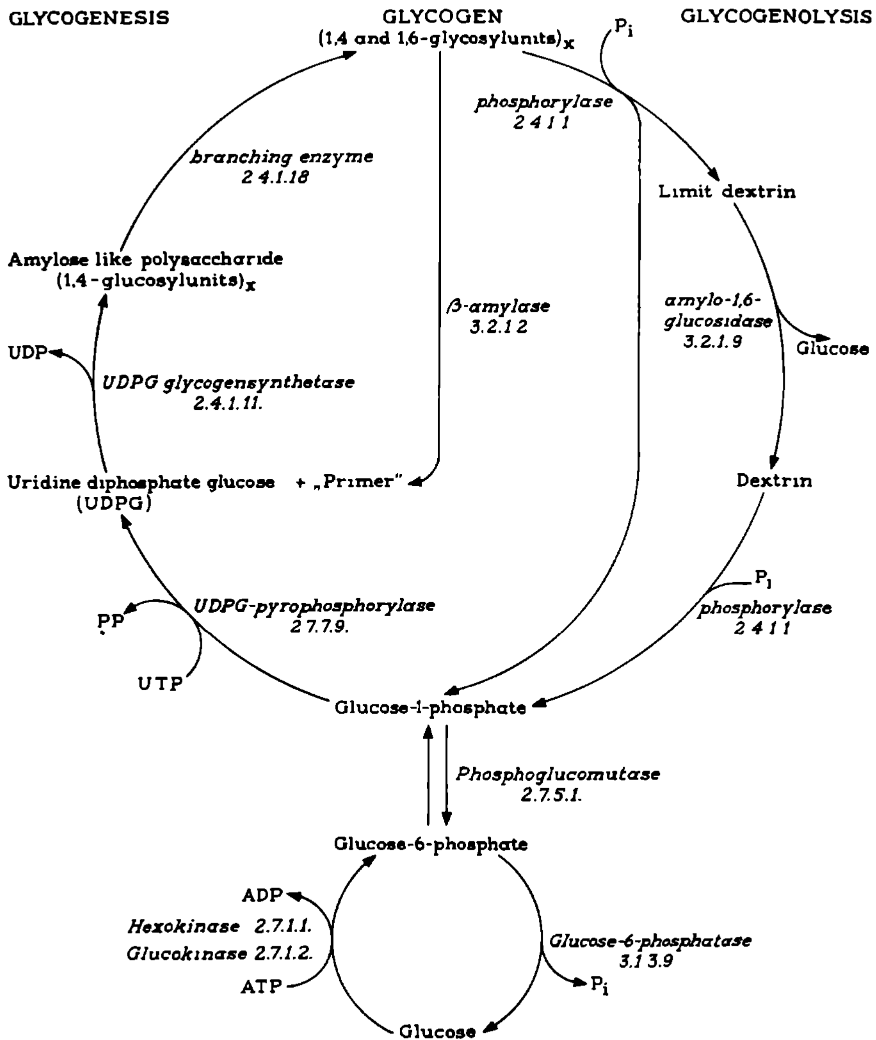
Textfig.7. PATHWAY OF GLYCOGENESIS AND GLYCOGENOLYSIS ►
In this scheme only the trivial names are given;
the systematic names are listed below.

E.C.number	Trivial name	Systematic name
2.7.1.2.	glucokinase	ATP: glucose 6-phosphotransferase
2.7.1.1.	hexokinase	ATP: D-hexose 6-phosphotransferase
2.7.5.1.	phosphoglucomutase	α-D-glucose-1,6-diphosphate: α-D-glucose-1-phosphate phosphotransferase
2.7.7.9.	UDPG-pyrophosphorylase	UTP: α-D-glucose-1-phosphate uridyltransferase
2.4.1.11.	UDPG-glycogen synthetase	UDPglucose: α-1,4-glucan α-4-glucosyltransferase
2.4.1.18.	branching enzyme	α-1,4-glucan: α-1,4-glucan 6-glycosyltransferase
2.4.1.1.	α-glucan-phosphorylase	α-1,4-glucan: orthophosphate glucosyltransferase
3.2.1.9.	amylo-1,6-glucosidase	amylopectin 6-glucanohydrolase
3.2.1.2.	β-amylase	α-1,4-glucan maltohydrolase
3.1.3.9.	glucose-6-phosphatase	D-glucose-6-phosphate phosphohydrolase

Man, personal communication). Bo (1962) easily obtained glycogen synthesis in vitro from G-1-P in rabbit uterus, but this polysaccharide could not be identified histochemically after incubation with UDPG. Biochemical analysis showed no UDPG-glycogen synthetase activity in the uterus of ovariectomized or ovariectomized-hormone (estrogen and progesterone)-treated rabbits, whereas the enzyme could readily be demonstrated in the skeletal muscle of the tongue (Bo and Smith, 1964). Bo concluded that in the uterus glycogen synthesis does not follow the UDPG-pathway.

It is beyond the scope of this survey to describe all properties of the enzymes, which take part in this glycogen cycle. For information on this point the reader is referred to the reviews by Stetten and Stetten (1960) and Manners (1962).

Because it has been suggested (Leloir and Goldemberg, 1960) that the separation of the synthetic and degradative pathways provides a scheme for delicate hormonal and metabolic regulation, some recent studies concerning this aspect will be reviewed briefly further down.



Very little is known about the nucleotide specificity of the (nucleotide-sugar-glycogen) glucosyl-transferase reaction. By histochemical methods Cohen and Wolfe (1963) succeeded in demonstrating glycogen synthesis in cryostat sections from skeletal muscle, taken from a variety of species, by incubating these sections with thymidine diphosphate glucose (TDPG). In comparison with UDPG as the substrate the quantities of glycogen that were deposited, were much smaller, suggesting that the rate of this TDPG-glycogen transglucosylase-mediated reaction was considerably lower. TDPG can act as a glucose-donor to glycogen under in vitro test-conditions as well,

as was shown by Kornfeld and Brown (1962) using rabbit skeletal muscle preparations and TDPG-^{14}C. Here too, however, TDPG reacted slowly at about 1/20 the rate of UDPG. In view of these experiments it seems improbable that TDPG should act as the natural substrate in glycogen synthesis. On the other hand, Recondo and Leloir (1961) found that the analogous enzyme in starch synthesis, originally studied with UDPG as a glucose-donor, reacted about ten times as fast with adenosine diphosphate glucose (ADPG), whereas no reaction followed with inosine diphosphate glucose, cytidine diphosphate glucose or guanosine diphosphate glucose. As there is known to exist an ADPG-pyrophosphorylase in plant tissue, which seems to be different from UDPG-pyrophosphorylase, ADPG and UDPG may be regarded to react in plants with the same enzyme, thus suggesting that both sugar nucleotides might play a role in starch synthesis in vivo.

In these experiments no starch synthesis was obtained with ADP-maltose, ADP-galactose and UDP-galactose. Tests with muscle and liver enzyme preparations showed ADPG to be about only half as active as the uridine analogue.

From the evidence given it may be concluded, as was done by Kornfeld and Brown (1962), that the requirements of the enzyme with respect to the nucleotide portion of the substrate are less specific than its requirements with respect to the sugar portion, but further studies to elucidate this point will be necessary.

The effect of glucose-6-P on the activity of UDPG-glycogen synthetase has been the subject of several studies (Villar-Palasi and Larner, 1960, 1961; Steiner et al., 1961; Rosell-Perez et al., 1962; Friedman and Larner, 1962, 1963; Kornfeld and Brown, 1962; Traut and Lipmann, 1963; Hizukuri and Larner, 1964).

Villar-Palasi and Larner (1961) suggested that the synthesis of glycogen in isolated rat-diaphragm muscle was mediated by two forms (with different activities) of the synthetase enzyme. Extracts of diaphragms, previously incubated with insulin and glucose, showed greater enzyme activities than extracts prepared from control diaphragms and tested without the addition of glucose-6-P. However, when glucose-6-P was added in excess, the enzyme activities in both extracts were increased and found to be equal. The increased enzyme activity after insulin pretreatment appeared to be independent of the presence of glucose in the incubation medium, which is of importance because the administration of glucose is known to increase the glucose-6-P content of skeletal muscle. To explain the activation by insulin, it was postulated that insulin might be active in regulating the interconversion of the two forms.

Steiner et al. (1961) could show that the synthetase activity in extracts from livers of alloxan-diabetic rats was significantly higher

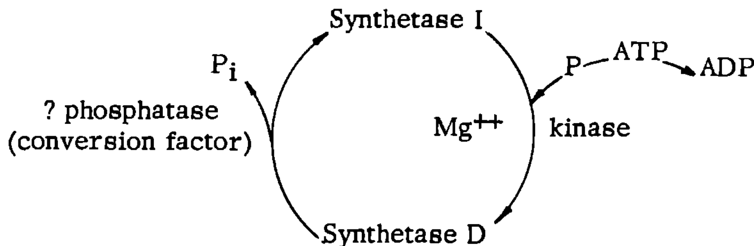
than in those from normal livers, provided that glucose-6-P was used in the assay mixture. On the other hand, when glucose-6-P was omitted, the enzymatic activity was significantly lower in the diabetic group. Injection of insulin into diabetic rats 2 to 4 hours before killing them caused a marked increase in maximum activity (that is: when tested with excess glucose-6-P) and an even more marked increase in the glucose-6-P independent activity. Whereas prednisolone increased the glycogen- and glucose-6-P content of the liver, it did not affect synthetase-activity. These findings were taken to indicate that the UDPG-pathway to glycogen-synthesis could be regulated by hormonal influences, which either affect the amount of active enzyme or the presence of important activating substances. The two forms, whose existence was suggested by Larner and co-workers, were afterwards prepared in a crude form from rat skeletal muscle (Rosell-Perez et al. 1962).

In this report kinetic studies were presented that clearly differentiated these two forms with regard to activation by glucose-6-P and Mg^{++}.

One form (I from Independent form) did not require glucose-6-P, but was strongly stimulated by Mg^{++}. The other form (D-form) was dependent on the presence of glucose-6-P; stimulation by Mg^{++} only occurred in the presence of this sugar phosphate.

Subsequent reports from the same laboratory (Friedman and Larner, 1962, 1963) showed that both forms of the muscle synthetase enzyme were interconvertible. The conversion of the I-form to the D-form required ATP and Mg^{++}. The reaction involved incorporation of the terminal phosphate from ATP into the enzyme, as could be shown by using ^{32}P-labelled ATP. This phosphorylation was enzymic in nature and was presumably effectuated by a proteinphosphokinase. As shown by the loss of ^{32}P as P_i during the D to I conversion, this reaction was a dephosphorylation, but the mechanism was not further clarified.

The interpretations given can be depicted as follows:

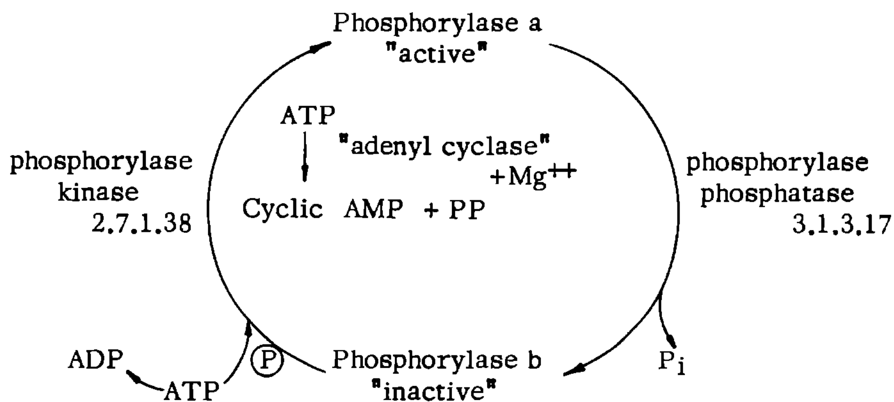


Because many phosphorylases have also been found to exist in two forms with different activity (an almost inactive b-form) dependent

for activity on the presence of adenosine monophosphate and an active form, phosphorylase a, which is highly active in the absence of A.M.P.) an interesting analogy between the two enzymes, which regulate the tissue glycogen content, becomes apparent. Considerable attention has been given to these phosphorylase systems, especially with regard to their activations and inactivations.

The reader is referred to the reviews by Stetten and Stetten 1960); Brown and Cori (1961); Krebs and Fisher (1962) and Manners (1962) for detailed information. Only a brief summary is given below.

Whereas the active liver phosphorylase is merely the phosphorylated inactive enzyme (Wosilait and Sutherland, 1956), the active muscle phosphorylase is a tetramer, which is hydrolytically converted to the dimeric b-form. With this cleavage of the molecule through a specific phosphorylase phosphatase (phosphorylase phosphohydrolase E.C.3.1.3.17) 4 molecules of inorganic phosphate are released per molecule of phosphorylase a. The conversion of phosphorylase b to a is effectuated by a phosphorylase kinase (ATP: phosphorylase phosphotransferase E.C.2.7.1.38) through the incorporation of 4 moles of phosphate per 2 moles of phosphorylase b. This phosphate becomes attached, through an ester linkage, to the serine residues of the enzyme protein (Fisher et al. 1959). The b to a conversion has an absolute metal-(Mn^{++} or Mg^{++}) and nucleotide requirement, ATP being the best phosphate-donor. It was found that the kinase requires a cyclic nucleotide (adenosine-3',5'-cyclic monophosphate=3',5'-cyclic adenylic acid) for stimulation. This cyclic nucleotide is generated from ATP in the presence of Mg^{++} and an enzyme system "adenyl cyclase" (Sutherland et al. 1962). Leaving apart some apparently fundamental differences between liver and muscle phosphorylases, (e.g. the muscle enzyme is immunologically distinct from that of liver), the reactions involved in activation and inactivation of the liver enzyme may, according to Harper (1963), be summarized as follows:



The evidence given above indicates that the interconversions of both the synthetase-system and the phosphorylase-system proceed via phosphorylation- and dephosphorylation reactions, albeit that the interconversion reactions for activation lie in opposite directions. As to the possibility of a common control mechanism for both systems, Friedman and Larner (1963) assume two possibilities:

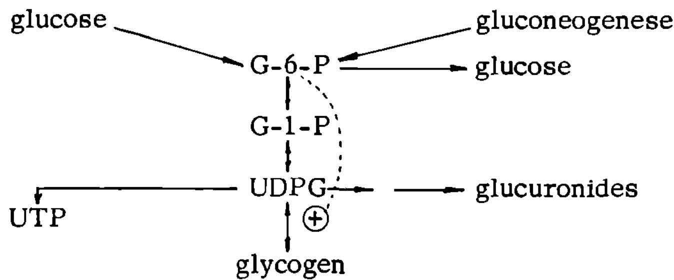
1) simultaneous regulation of both systems through the control of a common kinase (or phosphatase).
2) control through a mechanism which would act in an identical manner on two separate but similar kinases (or phosphatases).

> ......"either mechanism of control would then lead to an acti-
> "vation of one of the glycogen-metabolizing enzymes and a co-
> "incident inhibition of the other,......
> "this would mean that the synthesis of glycogen would be in-
> "activated when its breakdown was activated, and vice versa.
> "Belocopitow (1961) already has presented evidence in
> "preliminary form in general agreement with these specula-
> "tions. His results indicated that 3', 5'-adenylic acid inhibited
> "synthetase activity but activated phosphorylase."
> (experiments with rat muscle diafragm)

Though indeed such a concept is in itself very attractive, there is still a complete lack of experimental evidence in favour of it. It is still not known whether or not the liver synthetase is also subject to such phosphorylation-dephosphorylation reactions. Belocopitow's findings regarding the inhibitory effect of ATP and 3'.5'-AMP could only be partially substantiated by the studies of Traut and Lipmann (1963). These authors, among others, studied the effect of ATP pre-incubation on the "intrinsic" (independent) and "maximum" (with standard saturating concentration of G-6-P) activity of highly purified synthetase from lamb skeletal muscle. In these studies synthetase and phosphorylase were to a great extent separated in the final test-preparations. Such precautions were not taken by Belocopitow. This, however, is of special importance if one measures synthetase activity through the rate of incorporation of ^{14}C-glucose into glycogen, the effect of which can be counteracted by increased phosphorylase activity when crude preparations are used. Whereas Traut and Lipmann did find a significant ATP-effect in less purified enzyme fractions - as did Belocopitow -, the maximum activity in the highly purified preparations remained unchanged and here only a decrease in intrinsic activity occurred. Moreover, when the purified preparations were used, cyclic 3'.5'-AMP had no effect either on the intrinsic or on the maximum synthetase activity. This might be interpreted, however, to indicate that a conversion enzyme dependent on 3'.5'-AMP was eliminated during the purification-procedure.

Another interesting point follows from the studies by Traut and Lipmann (1963). The authors gave evidence that the G-6-P dependent form has a special binding site for this activator, which means that G-6-P can alter the conformation of the enzyme in a freely reversible way. This conclusion was based on the fact that G-6-P caused a change in the "pH-profile", counteracted the inhibitory effect of p-chloromercuribenzoate and increased the affinity of the enzyme for the substrate UDPG. These findings point to the possibility of a "feedback control of the synthesizing enzyme by the level of a precursor" (Northcote, 1964).

Sols et al. (1964) have outlined this initial-substrate activation of glycogen synthetase (textfig.8). Such a control system is regarded to be a significant counterpart to the principle of end-product inhibition.



Textfig.8. Initial substrate activation of glycogen synthetase.
After Sols et al. (1964).

As Traut and Lipmann found that prolonged incubation with G-6-P and a sulfhydryl compound increased the intrinsic activity to a stable level, yet another mechanism may play a role in controlling the enzyme-activity.

The rat liver enzyme has recently been studied by Hizukuri and Larner (1963, 1964). Conversion of the original G-6-P dependent form to the I-form was obtained, but no findings were presented that would characterize this conversion as a dephosphorylation-reaction. Indications were found of two different ways of conversion from the D to the I form, one including an inactive intermediate.

Both enzyme-forms were characterized with regard to dependency on pH, UDPG-concentration and nucleotide-sensitivity. Whereas the D-form was inhibited for about 50% by 6.7 x 10^{-3}M ATP, ADP and AMP, the I-form was virtually insensitive to these nucleotides.

In partially purified preparations the D to I conversion followed on incubation with Mg^{++} and SO_3^{--}, but highly purified enzyme required the addition of a heat-labile subcellular fraction, containing

the "conversion factor". This factor was separated from the glycogen-associated enzyme by ultrasonic desintegration and centrifugation of the particulate glycogen. The fraction containing the conversion factor had very little synthetase activity. Electron microscopic examination showed this fraction to be rich in smooth-surfaced vesicles, most probably derived from the vesicles of the endoplasmic reticulum that are known to occur under certain conditions in glycogen-rich cell areas (see section 2 of this chapter).

These last findings indicate the importance of being acquainted with the cellular topographical aspects of glycogen metabolism. The relevant literature will briefly be reviewed in section 2.

5-2. ULTRASTRUCTURAL IMPLICATIONS OF GLYCOGEN METABOLISM

Recent electron microscopic research has lead to a divergency of opinion about the cellular fine structure associated with glycogen synthesis and breakdown. This is due in part to the lack of clear-cut criteria for electron microscopic identification of glycogen. A second cause is, no doubt, the fact that one is forced to draw conclusions from a series of naturally static E.M.-pictures about a cellular process that essentially must be a dynamic one.

Fawcett (1955) was the first to observe that compact masses of "small vesicular and tubular" elements appeared in liver cells of rats that recovered after a period of fasting. It was assumed that the development of this agranular reticulum represented a stage in the regeneration of the granular reticulum, which had been depleted by fasting.

Porter and Bruni (1959) were about the first to surmise that this smooth-surfaced endoplasmic reticulum (SER) was involved in glycogenesis and glycogenolysis. After administration of 3'-Me-DAB (3'-methyl-4-dimethylaminoazobenzene) to rats, the liver cells were depleted of glycogen within 4 to 7 days, whereas in former glycogen-areas a hypertrophic SER was present. As it is apparent that this carcinogen gradually destroys the capacity for cellular glycogen storage, meanwhile causing a strong hypertrophy of agranular reticulum, the authors supposed this SER-system to be involved in glycogen-metabolism. In order to explain the hypertrophy, it was thought that the drug was metabolized within the SER-elements. Some metabolite, thus formed, might become attached to the protein-part of the membrane, which would result in a displacement of certain enzymes or enzyme-systems involved in glycogen-metabolism. The authors presumed that the cell could counteract this enzyme "intoxication"

through some feed-back mechanism, by forming more membranes of the agranular type.

Lafontaine and Allard (1964), in a comparable study, also interpreted the simultaneous disappearance of glycogen with the hypertrophy of the agranular reticulum after the ingestion of 2'-Me-DAB as a functional relationship between the SER and glycogen.

A study of the behaviour of this SER-system during glycogenesis and glycogenolysis as induced by starving and refeeding-experiments was undertaken by Millonig and Porter (1960). In the liver of control animals, fed ad libitum, there is no distinct topographical relationship between glycogen and endoplasmic reticulum, though some smooth tubules or vesicles may lie intermingled with the glycogen. Rough-surfaced ER seldom lies between the glycogen deposits. Upon fasting the liver cells decrease in size, but there is no clear increase in amount or volume of the SER. Upon refeeding, however, this situation changes immediately. Parallel with an increase in glycogen content, there is a distinct rise in the total number of smooth-surfaced ER vesicles in the glycogen areas. Under continuous feeding the amount of SER-vesicles decreases again, whereas the glycogen-fields become more extensive; the resulting situation therefore equals the control-picture. The authors also deduced from this morphological evidence that the SER is somehow associated with glycogen metabolism, but whether or not this proliferation of smooth ER profiles during the storage phase reflects some role of this system in the process of storaging deserves further investigation.

In a later comment on this study, Porter (1961) listed a number of findings in support of the view that the SER is not an obligatory associate of glycogen synthesis. To illustrate this point: large stores of glycogen may be encountered within the nuclei of livercells, for example in diabetes mellitus (l.c.) and in glycogen storage diseases (Sheldon et al. 1962). Moreover, a number of tissues other than liver (for example, brown adipose tissue of the mouse and chick glycogen body), contain very high concentrations of glycogen with virtually no cytoplasmic membranes (Revel et al. 1960).

Porter therefore believed that the SER is functional on the degradative pathway of glycogen metabolism. Themann (1963), in a comprehensive monography on the electron microscopy of glycogen in cell-metabolism, made a comparable study on mouse liver. He points out that in control livercells there is an inverse relationship between glycogen-content and ribosome-content (see also: Itikawa, 1962), whereas glycogen is never found in the ergastoplasmic areas of the hepatocytes. After 14 hours of fasting, glycogen has disappeared for the greater part. This depletion parallels a change in the ultrastructural appearance of the endoplasmic reticulum. The lamellar arrangement of the E.R. is transformed into a vesicular one. After

24 hours of fasting these phenomena are still more pronounced and more wide-spread. Moreover, these vesicular elements of the reticulum strongly dilate, and give the cytoplasm of the hepatocyte a highly vacuolized appearance. Meanwhile the ribosomes on the membranes disappear, whereas no changes are observed in the ultra-structure of the nuclei or the mitochondria. In those cellular areas where under normal conditions glycogen is present, a close-meshed network of tubules now appears, which is considered to belong to the SER, though clear-cut smooth contours are absent. Continued fasting induced pathological changes and necrosis ("aggregation" of cytoplasm, fatty degeneration etc.). It has been shown by others (Price et al. 1952; Porter and Bruni, 1959) that these extensive masses of tubules of the agranular reticulum correspond to the hyaline inclusions seen under the light microscope and are already well-known in a number of pathological conditions (Lafontaine and Allard, 1964; Steiner et al. 1964).

After refeeding, re-synthesis of glycogen particles can be observed in the immediate neighbourhood of the vacuolized SER-vesicles. Under the feeding-regimen the cells become filled with glycogen before the vacuolized SER regresses "in order to make way for the lamellar RNA-particle-studded ergastoplasm". Themann compared these findings with the ultrastructural modifications of heart muscle cells under hypoxaemic or anoxaemic conditions, which are known to induce glycogenolysis. Here, too, an "activation" of the sarcoplasmic reticulum occurred with multiplication into vacuoles. Moreover, the mitochondria and the myofibrillar arrangements became disorganized.

Return to normal conditions restores within a short time the normal ultra-structural aspects of all organelles involved. During this restoration-phase no clear-cut interrelationships between sarcoplasmic reticulum and glycogenesis were traced. Comparable results were obtained in the guinea pig retina. Here glycogen-storage and depletion can be induced by appropriately changing the time of exposure to light. Contrary to the conclusions of Porter and his co-workers, Themann deduced from his experiments that the SER is not directly involved either in glycogen-synthesis or in glycogen-breakdown. The "ER-activation" observed is interpreted as the expression of a specific physiological function of this system in the carbohydrate-metabolism of the liver. It is thought that the SER might serve to control cellular exchange of glucose, i.e. uptake from, and/or secretion into the extracellular space.

It is useful to point out that Themann's final conclusions were to a great extent guided by the results of a study by Luck (1961). The latter elaborated the sedimentability of the synthetase enzyme with the microsomal fraction, as was indicated in the studies by Robbins et al. (1959); a discovery which gained special importance in view of the

regular association between glycogen deposits and smooth structural elements in the intact liver cell. A study of the distribution of the enzyme in liver cell fractions showed that this distribution could be correlated with the glycogen content of each fraction. A purified glycogen fraction still contained, on examination in the E.M., the vesicular glycogen-associated structures. Upon sub-fractionation, Luck succeeded in separating the synthetase-activity and the glycogen from the DPNH-cytochrome c reductase and the membrane components. The author therefore concluded - as did Leloir and Goldemberg (1960) without morphological control - that the liver enzyme was bound to glycogen and not to the structural elements which are present in the glycogen fractions.

However, Anderson, Cedergren and Muscatello (1963) arrived at an opposite statement regarding the muscle enzyme. By actual isolation of the smooth membrane system of the frog skeletal muscle it was shown that the synthetase was localized in this sarcotubular system.

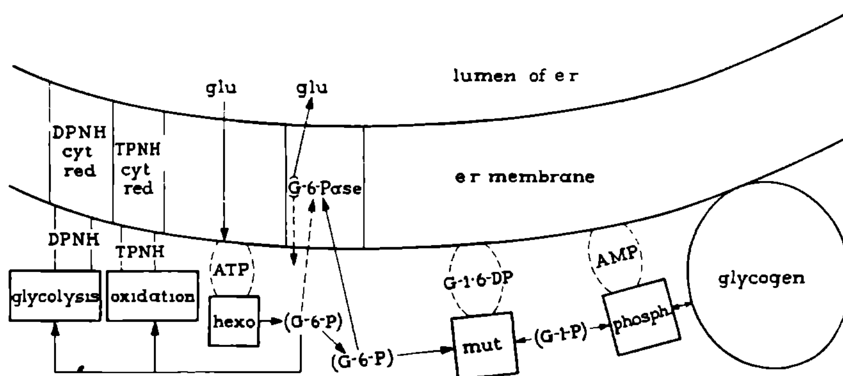
It should be noticed that this sarcotubular system is regarded by most authors to be analogous to the SER of other cell types, although several tissue-specific properties (e.g. stimulus-propagation) of these membranes have been found. A point that deserves further consideration, is that Anderson-Cedergren and Muscatello claimed that the fraction containing the sarcotubular vesicles was rich in desmoglycogen. Electronmicroscopic study of these membranes, however, revealed no structures that could be taken to represent this desmoglycogen. After treatment of the sample with EDTA small aggregates appeared of about 50 Å, which were taken to be glycogen. But this statement, based on purely morphological observation, needs more convincing evidence.

Siekevitz (1959) drew up the following schematic presentation of the possible role of the smooth ER in some aspects of carbohydrate-metabolism (see textfig.9). The author himself denominated this scheme as an entirely theoretical one;

> "purposefully exaggerating the morphological component of "cellular metabolism in the hope that, while I will undoubted-"ly overshoot my mark, this will not happen in every case."

The depiction is based on the following:

1. glucose-6-phosphatase is essentially a microsomal enzyme (De Duve and Berthet, 1954).
2. part of the DPNH- and TPNH- cytochrome c reductase also is localized on the microsomal membranes.



Textfig.9. Schematic representation of the relationship of the smooth endoplasmic reticulum to carbohydrate metabolism. From Siekewitz, 1959, 'Regulation of cell metabolism'; Ciba Foundation Symposion, Churchill, London.

3. the assumption that (a) either there is an, at least temporary, continuity between the ER-cisternae and the exterior of the cell with a resulting equilibrium of glucose concentration between the ER-lumen and the extra-cellular space; or (b) that glucose enters the cell by pinocytotic activity and subsequently is channeled into the ER-lumina. In both cases the intra-cisternal lumina can be regarded to be "extra-cellular".

4. the assumption that the ER-membranes throw up a "barrier" for free exchange of glucose between this ER-lumen and the intra-cytoplasmic space.

The ER-barrier would achieve that glucose cannot get into or out of the cytoplasmic matrix without contacting a transforming-enzyme. Immediately upon inside-passage, glucose is thought to be phosphorylated at the cytoplasmic side of the ER-membrane by ATP and hexokinase (glucokinase!).

The glucose-secretion into the lumen of the ER is believed to be under the control of the membrane-associated glucose-6-phosphatase and this situation would serve a dual purpose.

On the one hand, this is thought of to regulate the cellular output of glucose. Reduction of glucose concentration in the blood would cause a decrease in the amount of glucose within the ER-lumina. This would led to increased G-6-Pase activity which would cause an increase in glycogen breakdown. The "ER-activation" during glycogen-depletion, taken by Themann (1963) to indicate a specific physiological function of the ER, could therefore merely be the expression of increased glucose-sequestration in the ER-lumina on transport for

export. Another functional meaning of the membrane-association of G-6-Pase might be the removal of phosphorylated glucose from contact with glycolytic enzymes, when necessary in the regulation of intra-cellular glucose metabolism.

Other enzymes like hexokinase, phosphoglucomutase and phosphorylase, known to be "soluble", were thought of to become "activated" at the ER-membrane surface. The co-factors of these enzymes would act by binding the enzymes onto the membrane-surface.

To complete this concept, Siekevitz suggested that the hormones, which are known to be involved in the control of carbohydrate metabolism, do not act on the enzymes *per se*, but control their intracellular movements in bringing enzyme, substrate and cofactors together at a suitable surface.

CHAPTER 6

CHANGES IN GLYCOGEN RESERVES OF THE FETAL AND NEONATAL RAT LIVER

6-1. INTRODUCTION

The accumulation of large amounts of glycogen in embryonic tissues is well known since Claude Bernard (1859) made the first studies of this subject. Less well-known is the fact that the repartition of glycogen in the various embryonic tissues changes during fetal life. Whereas in almost all species studied, the glycogen-levels in lung and heart are highest when about half-way through gestation and decrease towards the end of fetal life, there is a strong increase of glycogen in skeletal muscle and liver tissue towards the end of pregnancy (Shelley, 1960). The factors responsible for these changes in glycogen-repartition are unknown. It has been suggested that the appearance of glycogen in a given fetal tissue coincides with the development in that tissue of the enzymes necessary for glycogen synthesis and that later changes in glycogen concentration may be governed by changes in enzyme activity (Nemeth, et al. 1954; Shelley, 1960). The experiments to be described in this chapter were carried out in order to obtain quantitative data on the glycogen content of developing fetal rat liver and on the changes in this glycogen content at birth. Several reports exist on this subject (Jacquot, 1955; Stafford and Weatherall, 1960; Ballard and Oliver, 1963; Dawkins, 1963), but part of them give data about total tissue carbohydrate. Therefore the actual data on the beginning of glycogen-accumulation differ from one author to the other. Because, however, this point is of special importance in view of the correlation to be made with changes in the patterns of glycogen metabolizing enzymes (chapter 7), the estimations have been made anew. Another important point is that most of the physiological or biochemical studies of liver development simply neglect the fact that the fetal liver is a mixed cell population, in which the ratio of hematopoietic to hepatic tissue changes throughout fetal development.

Therefore, some additional aspects of rat liver growth have been studied, especially with regard to the involution of hematopoietic tissue during the last third of gestation; the results will be presented in this chapter.

Data were collected from both the normal and the homozygous Gunn-strain of Wistar rats. The latter seemed of special interest in view of the very low levels of glycogen that have been reported to occur in the livers of adult homozygous Gunn-rats (Halac and Stuart, 1960).

6-2. EXPERIMENTAL PROCEDURE

6-2-1. Animals

Young adult Wistar albino rats and Gunn-strain rats (local stock) were used. They were fed on a standard diet (Hopefarms) and water *ad libitum*. The data on oestrus and conception were determined by vaginal smears. The gestation period was found to be 22 $\pm \frac{1}{4}$ days.

6-2-2. Glycogen determination

Glycogen was determined according to the prescription of Good, Kramer and Somogyi (1933), using the anthrone-reagent (Seifter et al. 1950). Remarque's (1958) prescriptions were carefully followed. According to the latter the fixed "desmo"-fraction of liver glycogen is practically constant, regardless of the total amount of glycogen present. The livers of a series of 10 Wistar rats were analyzed for lyo-glycogen and desmo-glycogen fractions. The animals were killed by stunning, they were then decapitated and bled for about 10 sec. The livers were quickly excised, blotted and immediately squeezed between blocs of dry-ice. Powdering of the livers was performed with a Waring-blendor. The TCA-extractable glycogen was collected by suspending and subsequently homogenizing about 300-500 mg of liver-powder in a pre-cooled Potter-Elvehjem tube, containing 10 ml TCA 10%. After 1 hr the suspension, kept at $0^{o}C$ throughout, was centrifuged 5' at 3000 g. This extraction was repeated twice. For the liberation of the desmofraction, the sediment thus obtained was boiled $\frac{1}{2}$-1 hr with 30% KOH. "Total KOH-glycogen" was determined by immediate immersion of about 500 mg of liver powder into KOH 30%. Glycogen was collected from the solutions by ethanol-precipitation. Two glucose standards, as well as two blanks, were included in each set of readings.

The results are given in table 4.

From these data one may conclude that the procedures used gave results that well agreed with those reached by Remarque. He found the desmo-fraction to be 0,46 $\pm$ 0,03 g per 100 g wet tissue for animals under non-standardized conditions of housing (temperature, number of animals per cage) and feeding, and 0,36 $\pm$ 0,02 g per 100 g wet tissue for animals under standardized circumstances of life.

TABLE 4

Lyo- and desmo-glycogen fractions
in livers of adult female Wistar rats.
All animals were kept under controlled conditions.
Each number is the average of two determinations.
Results are expressed as g of glycogen/100 g wet tissue.

Rat no.	Desmo-fraction	Lyo-fraction	Sum of fractions	Total KOH	Lyo-fraction / sum of fract. x 100%
1028	0.37	4.79	5.16	4.93	92.9
1029	0.40	3.14	3.53	3.88	88.9
1030	0.46	4.00	4.46	4.39	89.7
1031	0.36	5.50	5.87	5.74	93.7
1032	0.35	4.58	4.93	4.52	92.9
1034	0.45	4.91	5.36	5.25	91.6
1035	0.39	3.22	3.62	3.23	88.9
1036	0.45	3.46	3.91	3.69	88.5
1037	0.44	2.93	3.37	3.50	86.9
1038	0.48	3.43	3.90	3.92	87.9
Mean	0.42	4.00	4.41	4.31	90.2
Stand. dev.	$\pm$ 0.046	$\pm$ 0.887	$\pm$ 0.873	$\pm$ 0.812	$\pm$ 2.38

6-3. CHANGES IN LIVER GLYCOGEN LEVELS OF FETAL AND NEWBORN WISTAR RATS

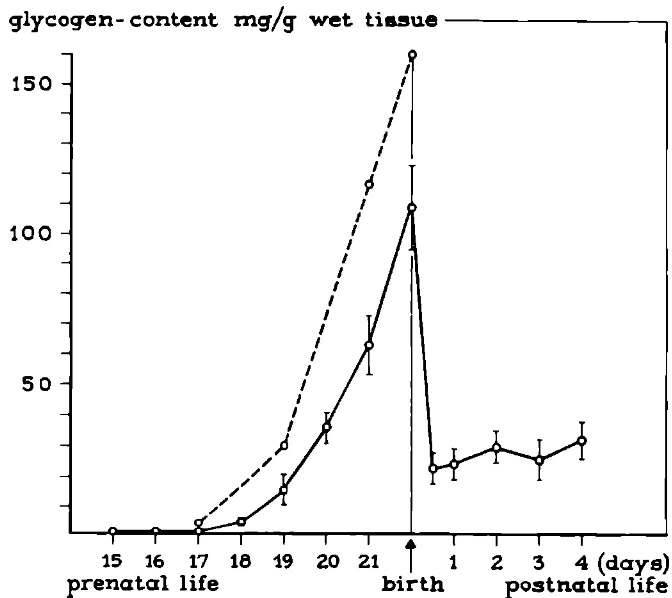
In table 5 the changes in liver glycogen levels of fetal and newborn animals are given. In the earlier stages (15-18 days) the livers of litter-mates were pooled.

From these data it appears that there is already a small amount of glycogen present in the fetal rat liver as early as the 15th day of pregnancy. This level remains virtually constant until the 17th day of intra-uterine life. Accumulation begins rather suddenly between the 17th and 18th day. Storage then goes on with the phenomenally high

TABLE 5

Changes in total liver glycogen levels of fetal and newborn Wistar rats. Results given as mg glycogen/g wet tissue (mean and standard deviation).

Intra-uterine age (days)	Number of animals	Glycogen content	Postnatal age (hours)	Number of animals	Glycogen content
15	9	0,25 ± 0,087	1	13	89,9 ± 5,27
16	17	0,29 ± 0,094	2	12	80,5 ± 13,12
17	13	0,43 ± 0,393	6	5	31,5 ± 7,71
18	8	3,6 ± 1,08	12	6	22,9 ± 6,05
19	7	15,2 ± 4,81	24	12	21,9 ± 6,08
20	5	34,3 ± 5,60	48	3	28,9 ± 4,28
21	12	78,2 ± 10,63	72	9	25,2 ± 6,64
22 = 0 hours	10	107,9 ± 13,82	96	11	31,8 ± 6,92



rate of about 20-40 mg/g wet tissue/day and reaches a maximum of about 100 mg/g wet tissue at term. There is some variation in the values of the glycogen content found at the various ages. For the fetal stages this must certainly be related to imprecise knowledge of conceptual age. Moreover, it is possible that this variation is correlated with the number of litter-mates at a birth, though no strict correlation could be established in this respect.

Immediately after birth the liver glycogen content falls very rapidly, declining to about 30% of its initial value within 6 hours. The lowest levels were found at 12 hrs postnatal age. At that time the new-born animals had for the greater part begun sucking and were brought together in the nest, this being of importance in maintaining the body temperature.

6-4. RELATIVE LIVER WEIGHT DURING EMBRYONIC AND EARLY POSTNATAL GROWTH

In order to understand more fully this period of liver development some additional information on changes of weight in liver growth seems useful. According to Doljanski (1960) the study of the relative weight (organ weight per body weight) of an organ might give an indication of possible changes in the organ versus body relationships during growth.

We therefore determined the increase in liver weight (fig.11a) and body weight during the fetal and early postnatal growth period. From these data the indices of relative liver weight were calculated (fig.11b).

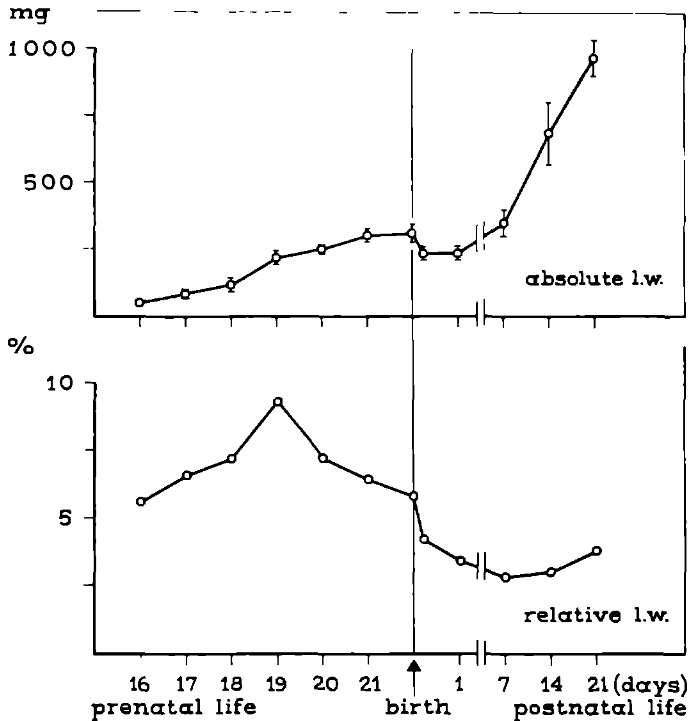
As can be seen from these figures there is a five- to six-fold increase in the absolute liver weight during the last six days of pregnancy, albeit that the curve levels near term.

The small, yet significant drop in weight of the liver immediately after birth is to be related mainly to the loss of glycogen. Liver weight in the weaning period then steadily increases to about 1.000 mg at the postnatal age of 3 weeks. Fig.11b shows that, despite the enormous accumulation of glycogen, the relative liver weight decreases from the 19th day of intra-uterine life onwards. This phenomenon must, no doubt, be related to the involution of hematopoietic tissue in the liver during this period. Indeed, as we shall see further down, there is a certain period in fetal liver development during which the hematopoietic elements greatly outnumber the hepatocytes.

◄ Textfig.10. Changes in liver glycogen content in the Wistar rat before and after birth. The full line represents the results, when expressed as mg/g wet tissue (mean ± standard deviation); the dashed line gives the "corrected" values, taking into account the shift in the ratio hepatocytes: erythropoietic cells.

Another reason for this decline in relative liver weight might be a decrease of the mitotic activity of the hepatocytes during the last days of pregnancy, possibly connected with the great glycogen metabolizing activity of these cells during this period. As a matter of fact it has been established that a high level of cellular metabolism delays the beginning of mitosis and therefore interferes with mitotic activity (Peter, 1947). Givol (1957; cited after Doljanski, 1960), found that the mitotic index (number of mitosis per 1000 cells) was as low as 1.3 on the second postnatal day, whereas at the age of 3 weeks the index was 4.5. It should be kept in mind, however, that a determination of the mitotic index, without further knowledge of the replication time and pattern, has only a limited significance in establishing the proliferative activity of cells during organ growth (Post, Huang, Hoffman, 1963; Wegener, Hollweg and Maurer, 1964).

Whether or not the presence of a large amount of paraplasmatic cell-constituents (in this case glycogen) might hinder the proliferative activity is still a controversial point (Jerusalem, personal communication).



Textfig.11. Liver weight and relative liver weight (liver weight to body weight ratio) of embryonic and newborn Wistar rats.

6-5. RATIO HEPATOCYTES: HEMATOPOIETIC CELLS IN THE DEVELOPING LIVER

During fetal life the liver is a very active site of hematopoiesis, mainly of erythropoiesis. Since this process is extra-vascular in nature (Sorenson, 1963; Dvorak, 1964), numerous foci of differentiating erythropoietic elements are diffusely arranged in the hepatic parenchyma. Accordingly, the "fetal liver" is not a homogeneous mass, but constitutes a mixed population of liver cells, cholangiole and bile duct cells, lining cells of the sinusoïds, including Küpffer cells and hematocytopoietic elements.

This intimate mixture of several different cell types is liable to a shift in the relative number of the component elements. Any analysis of a developmental process in one of the component parts, when studying or measuring the system as a whole (as biochemical handling does), has to take into account changes in the relative number of the component parts. Whereas the embryonic hemopoiesis in the liver has been the subject of an impressive series of studies and while the histological features have been explored extensively, the process has received very little attention from a quantitative point of view.

We have therefore gathered some quantitative data on the volume-ratio hepatocytes: haemocytopoiectic elements in the late embryonic and early postnatal liver.

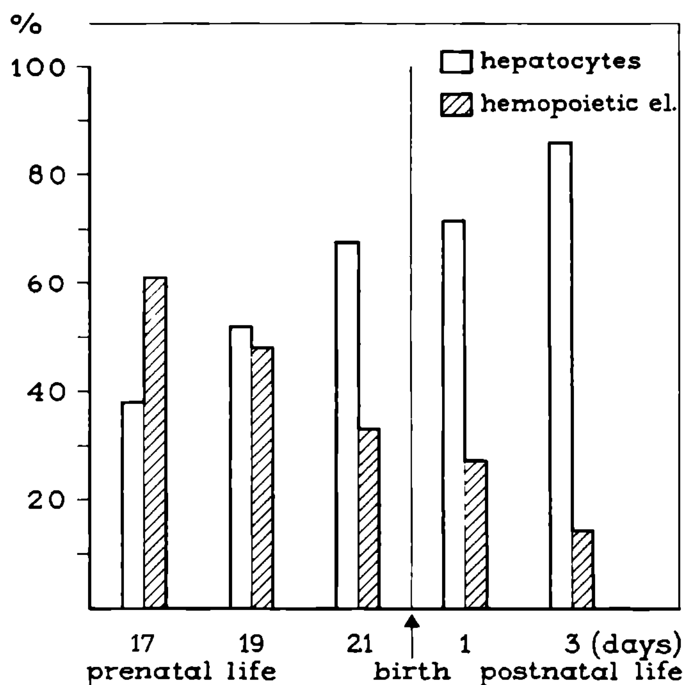
These data have been collected from relatively thin (3 μ) light-microscope sections, which have been analyzed by the "Punktzählverfahren", using a Zeiss Integrationsokular I (for details of the methods: see Hennig, 1957). The part of the "hits" on one particular component is directly proportional to the volume-portion of that component. These studies have been made from the 17th day of pregnancy on with two-day-intervals. The results are indicated in fig.12.

The hemopoietic elements amount to about 60% of the fetal liver volume (volume part of sinusoïds not included) on the 17th day of pregnancy. This percentage declines to about 30% at birth and to about 15% on the third postnatal day.

When one assumes that the hepatic cells and the blood-forming cells with an equal specific weight contribute to the organ weight, then a correction of the "glycogen-storage-curve" is possible in that the levels of glycogen found at the various stages can be calculated per g wet liver parenchyma tissue. When this is done it is found that from the 19th day of pregnancy on, there is a virtually linear increase in glycogen content to about 160 mg of glycogen per g wet liver tissue. That is about 3 to 4 times as much as is normally found in the adult rat liver (see fig.10, dashed line).

It is taken for granted in this line of thought that there is no glycogen present in the blood-forming cells of the fetal liver. This of course

might be incorrect since glycogen is known to occur in leucocytes, especially the neutrophilic granulocytes and, to a lesser extent, in the eosinophils (Gibb and Stowell, 1949; Biava, 1963). However, the fetal liver contains mainly erythroid cells and only very few granulocytes and megakaryocytes. Since, moreover, the amount of glycogen present in mature leucocytes is not more than about 1% (Remarque, 1958), an eventual error, especially in the latter gestational stages, will be very small indeed. The possible importance of this blood-cell glycogen in the earlier periods of liver carbohydrate-metabolism will be discussed later.



Textfig.12. Changes in the ratio hepatocytes: hemopoietic elements in the rat liver during the perinatal period.

6-6. LIVER GLYCOGEN LEVELS OF FETAL AND ADULT HOMOZYGOUS GUNN-STRAIN RATS

Since evidence had been brought forward that fetal tissue glycogen is affected by material nutritional conditions (Stuart and Higgins 1935; Shelley, 1961), it was of interest to study liver glycogen in Gunn-embryos.

TABLE 6

Liver glycogen content of homozygous Gunn-rat embryos

Intra-uterine age (days)	Number of animals	Glycogen content mg/g wet tissue
18	10	1.15 ± 0.41
19	10	10.62 ± 2.92
20	5	41.9 ± 9.99
21	9	75.8 ± 9.57

Homozygous animals of this mutant strain of Wistar rats, which are genetically deficient in glucuronyl transferase activity, are reported to have very low levels of liver glycogen (Halac and Stuart, 1960), whereas heterozygous Gunn-rats had normal levels.

The results of our estimations on the offspring of homozygous x homozygous matings are given in table 6.

From these findings it follows that homozygous Gunn-embryos in the last fifth of pregnancy accumulate an equally large amount of liver glycogen as do their normal congeners. Indeed, the neonatal homozygous animals do not develop the faintly yellow jaundice-colour, typical of their icteric condition, until they are one day old.

Much to our surprise, an analysis of the livers of adult homozygous rats did not show abnormally low glycogen levels: see table 7. The results of Halac and Stuart are given for comparison. All animals were killed on the same hour and in the same month in order to exclude possible diversions related to diurnal or seasonal variations (Von Mayersbach and Leske, 1963).

Halac and Stuart (1960) used the Wagtendonk-method (Wagtendonk, 1946) for the estimation of glycogen. This technique is a direct colorimetric one using the colour produced when glycogen is treated with iodine. The iodine-binding power as well as the absorption maximum of the iodine-glycogen complex is known to be related to the average chain length. The iodine-binding power of glycogen with CL 12 - 13 is only one quarter of that of 18-unit glycogen and about one tenth of that of amylopectin CL 20 - 23, (Manners, 1957). Therefore, the possibility was considered that in homozygous Gunn-rats an aberrant liver-glycogen might be present, possibly with a very low iodine-binding power.

We have therefore analyzed a series of 5 homozygous animals by both the Wagtendonk and the anthrone-procedure. The results are given in table 8 (expressed as g of glycogen per 100 g wet tissue);

TABLE 7

Lyo- and desmo-glycogen fractions
in livers of adult female heterozygous and homozygous Gunn-rats.
Each number is the mean of two determinations.
Results are expressed as g/100 g wet tissue.

	Rat nr.	Desmo-fraction	Lyo-fraction	Sum of fractions	Halac and Frank (1960) mg/100 mg	
Heterozygous	1045	0,42	5,54	5,96	nr. 1	6,516
	1046	0,36	3,76	4,11	2	4,903
	1047	0,47	4,41	4,88		
Homozygous	1040	0,52	3,58	4,09	1	0,280
	1042	0,54	3,93	4,47	2	0,076
	1048	0,46	3,08	3,55	3	0,340
	1049	0,52	4,53	5,06	4	1,074

TABLE 8

Comparison of liver glycogen content
of adult female homozygous Gunn-rats
as estimated by Wagtendonk's iodine procedure
and the anthrone-procedure

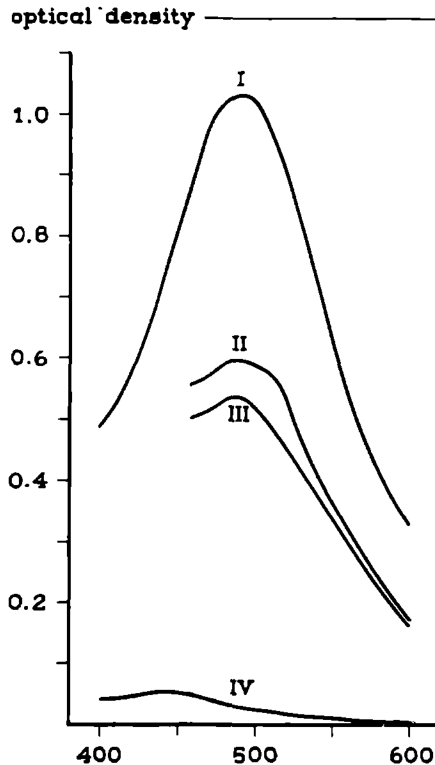
Rat nr.	Wagtendonk procedure	Anthrone procedure
1125	5.38	5.18
1128	4.73	4.09
1133	3.28	3.65
1135	4.02	4.69
1192	2.18	4.61

each number is the mean of at least two estimations.

With the exception of rat nr.1192, there was a fair agreement between the results obtained by the two methods. Moreover, the particulate glycogen, isolated by the method of Drochmans (cf. chapter 4), showed an iodine absorption-spectrum almost identical with that of

normal Wistar particulate glycogen: textfig.13.

These findings make it rather unlikely that a highly abnormal glycogen is present in the livers of our strain of homozygous Gunn-rats.



Textfig.13. Absorption-spectrum of iodine-glycogen-complexes in half-saturated $(NH_4)_2SO_4$ solution (procedure of Schlamowitz, 1951). I = Rabbit liver glycogen BDH. Lotnr.214620. II = Wistar rat, particulate glycogen. III = Homoz. Gunn-rat, particulate glycogen. IV = Iodine in half-saturated $(NH_4)_2SO_4$.

6-7. DISCUSSION

Three distinct phases characterize the differentiation of the fetal rat liver with regard to the functional state of its glycogen content. In the first phase of development, before the 17th day of pregnancy, only a very small amount is present in the liver. The second phase, from this intrauterine age to term, is characterized by a rapid increase in glycogen content, reaching a level which is 3 to 4 times as high as that found in the adult liver. The third period starts with a precipitous fall on the first day of neonatal life. Similar phenomena

occur in a variety of other species: monkey, sheep, dog, rabbit, guinea-pig (Shelley, 1961). It may therefore be concluded that the liver glycogen-stores, built up during the last part of fetal life, represent an important reserve for use immediately after birth. The most plausible hypothesis is to assume that the rapid mobilization of glycogen immediately after birth must be related to the necessity of maintaining the blood sugar level, now that the placental circulation has ceased. Indeed, Dawkins (1963) showed that this mobilization could be prevented by an exogenous source of glucose.

Dawkins studied the intrahepatic levels of glucose and glucose-6-phosphate during the early postnatal period and found a substantial loss of both substances. The blood glucose level, on the other hand, after an initial decrease from 40 to 20 mg/100 ml, had risen again at 6 hrs post partum.

The process of birth is accompanied by a profound change in the environment of the newborn animal. After birth the liver has to take over the homoeiostatic functions previously controlled by the placenta and one of these is the regulation of blood-sugar levels. Since the energy requirements of the newborn must be considerably higher than those of the fetus (maintenance of body temperature, breathing, etc.) the initial decrease in blood glucose level is easily understood. According to Dawkins (1963) the sequence of events after delivery might then be as follows: When the contacts with the maternal circulation are broken, the blood glucose level falls very rapidly on account of peripheral utilization. Since the liver cell is thought to be freely permeable to glucose (Sols, 1964) this fall in blood glucose concentration results in a decreased intrahepatic glucose concentration.

This in turn induces an immediate increase in glucose-6-phosphatase activity "by release of inhibition and synthesis of new enzyme protein". These last points will be discussed in more detail in chapter 7.

Another point, mentioned here only since it illustrates the importance of the fetal carbohydrate reserves for the newborn animal, is the established correlation between cardiac glycogen concentration and the ability to survive anoxia (Dawes et al., 1959; Stafford and Weatherall, 1960; Mott, 1961).

Much less is known about the importance for the fetus of its glycogen reserves. Already Bernard (1859) made the observation that the beginning of glycogen storage in the fetal liver correlates as to time with the decrease in the placental glycogen. This led him to suggest that the placenta serves the hepatic functions before the liver has acquired the capacity to fulfil the characteristic carbohydrate metabolic functions of the normal liver, i.e. to store glycogen and supply glucose for the extrahepatic cells of the organism. It is, however, still questionable whether the fetal liver glycogen behaves in the same

way as does the adult liver glycogen. That the fetal liver plays a definite part in glucose secretion has not been proved, though the experiments of Dawes et al., (1960) point that way.

In keeping with such a hypothesis is the statement of Goldwater and Stetten (1947) that the fetal glycogen is highly labile. Enrichment of the body fluids of pregnant rats with D_2O resulted in the rapid incorporation of deuterium into fetal glycogen. It was calculated from the change in the D-concentration that the 19 days'old fetus synthesizes per day a quantity of glycogen approximately equal to that which it contains at this stage of development; i.e. some 500 mg per 100 g of fetal weight per day. Since, however, a difference exists with the rate at which it was found to accrue, it is also apparent that glycogen is being destroyed in the fetus. Studies of the isotope incorporation rate are incommoded by an assumption that is difficult to prove, namely that the metabolite under consideration is metabolically homogeneous. Moreover, it should be kept in mind that the findings of Goldwater and Stetten concern the total fetal glycogen content. As a matter of fact Dawkins (1960) arrives at an opposite point of view with regard to the fetal liver glycogen. According to him it is characterized by a remarkable stability. Whereas in autolysing adult liver tissue (initial concentration 31,8 mg per g liver) glycogen has disappeared after 2 hrs, fetal liver glycogen declines much more slowly during autolysis. Oxidative phosphorylation, too, survived better in homogenates from autolysing fetal liver than in comparable preparations from autolysing adult liver.

Dawkins attributes this relative stability of the fetal liver glycogen to the low activity of glucose-6-phosphatase, one of the enzymes concerned in glycogen breakdown. He therefore thinks the situation in the fetal liver to be reminiscent of the conditions in glycogen storage disease (Cori type I, Von Gierke's disease) having a glucose-6-phosphatase enzyme defect. The progressively accumulated glycogen in the livers (and kidneys) of these patients is also abnormally stable after death.

We are inclined to accept from the evidence given above that the perinatal period offers a real opportunity to study hepatic glycogenesis and glycogenolysis separately, albeit that definite proof is hard to be given.

Another point that needs some discussion, is the fact that a small amount of glycogen is already present in the fetal liver before the accumulation-phase begins. In accordance with our results, Kornfeld and Brown (1963) found a small amount of glycogen (0,3 to 0,7 mg/g wet tissue) in the fetal guinea pig as early as the 46th day, whereas the accumulation begins suddenly on the 59th day of fetal life (term: 70 days). Upon isolation this glycogen proved to be a gly-

cogen of normal structure. This finding was thought to be of special interest since it had been suggested by Nemeth (1954) that a deficiency of branching enzyme (α-1,4 glucan: α-1,4-glucan 6-glucosyl-transferase, E.C.2.4.1.18) might be responsible for the low levels of glycogen before the 59th day. Kornfeld and Brown state that, if this were so, the early glycogen would have an abnormally long average chain length, which is not the case.

We would like to point out that this finding does not necessarily contradict Nemeth's supposition, since it has not been shown that the glycogen isolated really was present in the fetal liver cells. It might well be that this small amount of glycogen was derived from the blood cells present, which are either generated in the fetal liver or which invaded it.

CHAPTER 7

DIFFERENTIATION PATTERN OF SOME ENZYMES CONCERNED IN GLYCOGENESIS AND GLYCOGENOLYSIS DURING THE PERINATAL PERIOD

7-1. INTRODUCTION

It was shown in the preceding chapter that with respect to glycogen metabolism two periods of rat liver development appear to be critical. The first period begins on the 17th - 18th gestational day, when the factors involved in the initiation of glycogen deposition must have acquired appropriate maturation to be able to bring about the sudden and rapid accumulation of glycogen. The second period occurs at the time of birth and is associated with a precipitous loss of glycogen. Several arguments have been advanced in literature that led Nemeth and co-workers (1954) to assume that the onset of liver glycogenesis is due to some changes in the hepatic cell rather than to extrinsic factors such as circulating hormones. One argument put forward is that glycogen appears in other fetal tissue before liver. Furthermore, when chick embryo hepatic cells are brought into tissue culture before the glycogen storage starts and before the pancreatic islets have differentiated, the cultured cells acquire with time the ability to form and store glycogen (Doljanski, 1930). Moreover, when chick liver cells from the pre-glycogen-stage are transplanted onto the chorio-allantois of an embryo already arrived at the storage-phase, the transplanted cells fail to synthesize glycogen earlier than the control cells that have been left *in situ* (Dalton, 1937). It was shown by Nemeth et al. (1954) that slices from fetal guinea pig liver, when incubated *in vitro* with glucose or fructose as substrate, did not synthesize glycogen unless the livers had the "glycogenic" age.

In this chapter the problem will be studied whether the two periods of rat liver development that are critical with respect to glycogen turn-over, can be associated with the pattern of development of a number of enzymes which are known to be involved in glycogen metabolism.

7-2. EXPERIMENTAL PROCEDURE

7-2-1. Animals : see 6-2-1.

7-2-2. Reagent chemicals and enzymes

Glucose-6-P dehydrogenase (sp. act. 150 units/mg), phosphoglucomutase (6.5 units/mg), pyruvate kinase (125 units/mg) were obtained from Boehringer, as were phosphoenolpyruvate (tricyclohexylamine-salt), AMP, UDP, UDPG, G-1-P, G-6-P and NADP (all of them sodium salts). Amylopectin (starch) and glycogen (rabbit liver) were from British Drug Houses Ltd.

7-2-3. Enzyme estimations*

The livers were homogenized in 3 volumes of 0,25 M sucrose-0,001 M EDTA, unless stated otherwise.

The 1000 x g supernatant, or crude extract was analysed for the activities of:

1. PHOSPHOGLUCOMUTASE (α-D-glucose-1,6-diphosphate: α-D-glucose-1-phosphate phosphotransferase, E.C. 2.7.5.1.) according to a method, worked out at the laboratory for Chemical Cytology, Nijmegen University. Phosphoglucomutase catalyses the conversion of G-1-P into G-6-P. The latter is oxidized by NADP with glucose-6-phosphate dehydrogenase into 6-phosphogluconate and $NADPH_2$. The rate of NADP reduction as a measure for G-6-P production is estimated through the change in absorbancy at 340 mμ over 5 min. The assay-mixture contains: 165 μmoles Tris-buffer pH 7.7; 12.5 μmoles $MgCl_2$; 1.35 μmoles NADP; 2 μmoles EDTA; excess glucose-6-phosphate dehydrogenase and limiting amounts of the extract to be tested. The reaction was started with the addition of glucose-1-phosphate (final conc. 5 μmoles). Total volume 2.1 ml. The absorbancy increment was converted to enzyme activity in mμmoles G-6-P produced/mg protein/min.

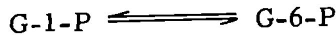
2. UDPG-PYROPHOSPHORYLASE (UTP: α-D-glucose-1-phosphate uridylyltransferase, E.C. 2.7.7.9.)

In the presence of excess pyrophosphate UDPG is quantitatively converted into UTP and G-1-P:

$$UDPG + PP \rightleftharpoons UTP + G\text{-}1\text{-}P;$$

* The author is very much obliged to Miss H.M.J.Jansen and Drs.H.W.B.Jansen for performing these enzyme estimations.

the reaction sequences further being



$$\text{G-1-P} \rightleftharpoons \text{G-6-P}$$

$$\text{G-6-P} + \text{NADP} \rightleftharpoons \text{6-PG} + \text{NADPH}_2 \text{ (as under 1)}$$

The increase in extinction was again measured at 340 mμ. The reaction medium contained: 150μmoles Tris-buffer pH 7.7; 12.5 μmoles $MgCl_2$; 1.3μmoles NADP; 2μmoles EDTA; excess glucose-6-phosphate dehydrogenase and phosphoglucomutase, 1 μmole UDPG and limiting amounts of enzyme. The reaction was started with the addition of excess pyrophosphate. Total volume 2 ml. Enzyme activity is expressed as m μmoles UDPG converted/mg protein/min.

3. UDPG-GLYCOGEN SYNTHETASE (UDP glucose: α-1,4-glucan-α-4-glucosyltransferase E.C.2.4.1.11), estimated by the rate of UDP-production from UDPG as described by Leloir and Goldemberg (1960). The reaction mixture contained: 0.25 μmole UDPG; 0.5 μmole G-6-P; 7.5 μmoles glycine-buffer pH 8.5; 0.25 μmole EDTA; 0.4 mg glycogen and limiting amounts of enzyme.
The total volume was 1.2 ml. Incubations were carried out for 10 min. at 37°C. The reactions were stopped by heating for 1 min in boiling water.
The UDP formed was measured by its phosphorylation by phosphoenolpyruvate (PEP) in the presence of pyruvate kinase. The pyruvate formed was assayed by the dinitrophenyl-hydrazine reaction. Activities are expressed as μmoles UDP produced/mg protein/min.

4. α-GLUCAN-PHOSPHORYLASE (α-1,4-glucan: orthophosphate glucosyltransferase, E.C.2.4.1.1.), measured according to the method, described by Sutherland (1955), by the rate of orthophosphate-production from G-1-P during the synthesis of glycogen. This is an estimation of total enzyme activity, because the phosphorylase-phosphatase is inhibited by NaF and the inactive form is activated by adding 5-AMP. A 2.5% homogenate was used. The assay-medium contained: 10μmoles G-1-P; 1% glycogen; 0.3 μmole AMP; 20μmoles NaF and enzyme. Total volume was 0.2 ml; incubation lasted for 20 min at 37°C. The reaction was stopped by adding 0.4 ml TCA. Inorganic phosphate was estimated before and after incubation, according to the method of Fiske-Subba Row. Activities are calculated as mμmoles P_i released/mg protein/min.

5. GLUCOSE-6-PHOSPHATASE (D-glucose-6-phosphate phosphohydrolase E.C. 3.1.3.9), estimated essentially as described by Hers (1959), by the rate of inorganic phosphate production upon hydrolysis of G-6-P. A 1% homogenate in 0.25 M sucrose was used. In order to gain information about the activity of unspecific (acid) phosphatases, homogenate (0.2 ml) and acetate buffer 0.1 M, pH 5.0 (0.02 ml) are in-

cubated for 5 min. at 37°C. This causes a complete and irreversible inactivation of the glucose-6-phosphatase. Then 0.2 ml 0.05 M G-6-P, pH 6.6 is added and incubation is continued for 1 hr at 37°C. The reaction is stopped by adding TCA (0.4 ml, 1 M). Simultaneously a test is performed without the pre-incubation at pH 5.0. The inorganic-phosphate is estimated as in the case of α-glucan phosphorylase. The activities are expressed as μmoles P_i released/mg N/hr.

6. BRANCHING ENZYME (α-1,4-glucan: α-1,4-glucan 6-glycosyltransferase, E.C.2.4.1.18), estimated according to Larner (1955), by the decrease in optical density of an iodine-amylopectin complex after branching enzyme activity. The homogenate was filtered through cotton to remove fat and floating particles. Enzyme 0.1 ml and 0.2 ml amylopectin 1% are incubated for 10 min at 37°C. The reaction is stopped by adding 0.3 ml 3% perchloric acid. After mixing the samples and letting them stand for five minutes, the samples are centrifuged. To a 0.2 ml supernatant aliquot are added 15 mg solid $NaHCO_3$, 1.0 ml Tris-buffer pH 7.9 and 1.8 ml water. Subsequently 0.15 ml I_2 in KI (0.2% in 0.4%) are added and extinctions are read at 570 mμ. Initial values are estimated by omitting the incubation.

7-3. LEVELS OF ACTIVITY OF SOME GLYCOGEN-CYCLE ENZYMES IN THE LIVERS OF ADULT WISTAR AND GUNN-RATS

In table 9 the activities of four glycogen-cycle enzymes in the livers of adult Wistar and homozygous Gunn-rats are compared. As it was shown by Burch et al. (1963) that between the adult male and female rat-livers there exist a number of modest but statisti-

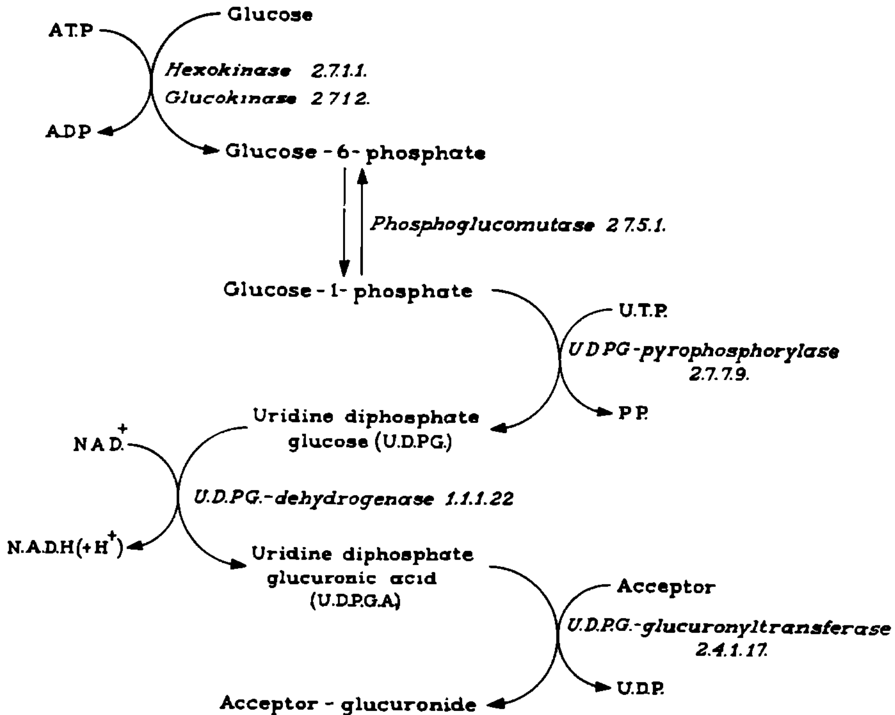
TABLE 9

Activities of glycogen-cycle enzymes
in the livers of adult female Wistar and homozygous Gunn-rats.
Activities given as mμmoles/mg protein/min.

	Phospho-glucomutase	UDPG-pyro-phospho-rylase	UDPG-glycogen synthetase	α-glucan phospho-rylase
Wistar	152 ± 14	165 ± 14.9	28.8 ± 3.5	78 ± 15.4
Gunn, homozygous	149 ± 14	163 ± 11.5	19.6 ± 2.3	78 ± 8.6

cally significant differences in the activities of a series of carbohydrate metabolizing enzymes, only female animals were used for these comparative studies.

It follows from these results that the activities of phosphoglucomutase, UDPG-pyrophosphorylase and α-glucan phosphorylase are essentially equal in the two strains of rats. This confirms the observations of Halac and Stuart (1960), who also found the phosphoglucomutase activities of homozygous Gunn-rats (which suffer from a recessively hereditary form of icterus) to be within normal limits. They observed only moderately reduced UDPG-pyrophosphorylase activity. It is known from the studies of Arias (1959) that the homozygous Gunn rats have also normal glucose-6-phosphatase and UDPG-dehydrogenase activity. The latter enzyme mediates the oxidation of UDPG to UDP-glucuronic acid (UDPGA): see textfig.14.



Textfig.14. Synthesis of glucuronides.

The only enzyme that is probably completely absent in these icteric rats is glucuronyl-transferase (UDPG-glucuronyltransferase, E.C. 2.4.1.17), which transfers the glucuronic acid moiety from UDPGA to bilirubin (Latha and Walker, 1957; Carbone and Grodsky, 1957; Schmid et al. 1958; Arias, 1959, van Leusden, 1963).

The distinct difference in the activity of UDPG-glycogen synthetase (table 9) between the two strains of rats is a rather surprising result in view of the fact that no differences in total liver-glycogen could be established between the Wistar and the homozygous Gunn rats (see chapter 6). We shall see further down (textfig.17) that the developmental increase in the activity of UDPG-glycogen synthetase in the two strains of rats runs more or less parallel during the last part of pregnancy. However, after birth the curves begin to deviate. The neonatal Gunn-rats have reached their low synthetase-levels already at the second post-natal day, whereas the normal Wistar animals maintain a rather high level during the first 3 to 4 days after birth.

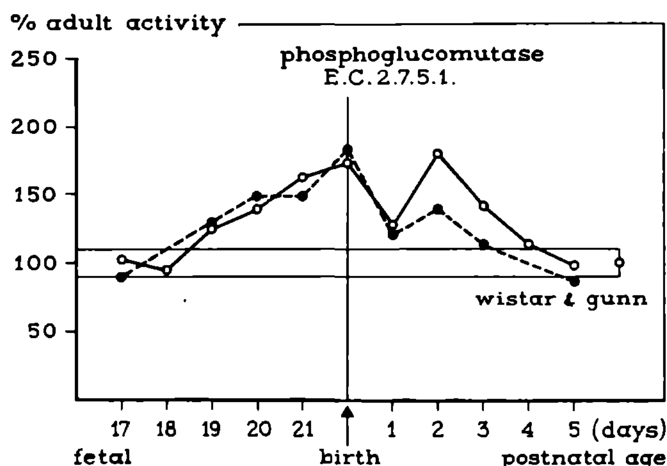
The UDPG-pyrophosphorylase activity in the two rat strains also shows a somewhat different behaviour during the early postnatal period. The time at which these differences become apparent therefore coincides with the development in these neonatal homozygous animals of their icteric condition; that is: with the increasing bilirubin-concentration in their serum and tissues. One might therefore think of an enzyme inhibition as explaining the low levels of UDPG-glycogen synthetase activity in the Gunn-rats but further studies will be necessary to elucidate this point.

7-4. CHANGES IN ACTIVITY DURING THE PERINATAL PERIOD OF A NUMBER OF ENZYMES, CONCERNED IN GLYCOGEN-METABOLISM

Textfig.15 up to and including 19 show the changes in activity of phosphoglucomutase, UDPG-pyrophosphorylase, UDPG-glycogen synthetase, α-glucan phosphorylase and glucose-6-phosphatase as a function of age from the 17th day of intra-uterine life until the 5th neonatal day.

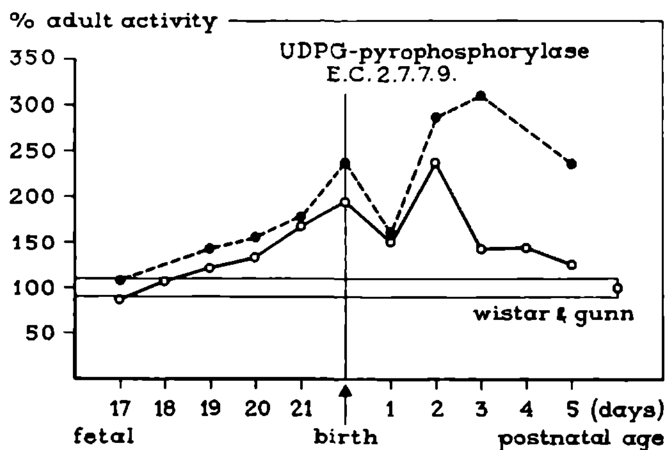
At the earliest stage we have measured, phosphoglucomutase already had adult levels of activity. There is an increase to about twice the adult level at birth.

This indicates a high capacity for glucose-assimilation of the fetal liver. We have registered a pronounced fall in activity in the 24 hrs neonatal animals, but there is no doubt that these values are artificially low. This should be related to the abrupt and strong increase in glucose-6-phosphatase activity during the first postnatal day. The



Textfig.15. Changes with age in phosphoglucomutase-activity of fetal and neonatal Wistar (o——o) and homozygous Gunn-rat (•——•) liver. The activities are plotted as per cent of adult activity.

phosphoglucomutase activity was measured by the rate of oxidation of glucose-6-phosphate to 6-phosphogluconate. Consequently, an increase in glucose-6-phosphatase activity interferes in the test-system owing to removal (hydrolysis) of part of the substrate. Similar considerations apply to the decrease in the activity of UDPG-pyrophosphorylase that can be observed on the first day postpartum. This enzyme, too, shows a high activity throughout the period studied, and in-

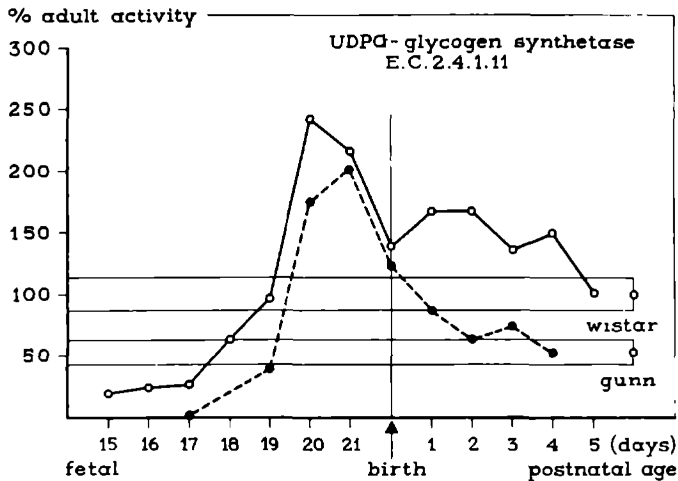


Textfig.16. Changes with age in UDPG-pyrophosphorylase activity of fetal and neonatal Wistar (o——o) and homozygous Gunn-rats (•----•) liver. The activities are plotted as per cent of adult activity.

creases from about adult levels on the seventeenth gestational day to 2 - 3 times these values at birth.

Whereas the curves, related to the two strains of rats, take about the same path in the prenatal period, a deviation occurs during the early postnatal period, when the enzyme from Gunn rats attains a distinctly higher level of activity than does the enzyme from Wistar rats.

Although the UDPG-glycogen synthetase activity is readily demonstrable in the Wistar fetal liver as early as the fifteenth day of gestation (7 m μmoles/mg protein/min), the activity remains at a nearly constant level until the seventeenth day. From that point on there is a very large augmentation to 69 mμmoles/mg protein/min. by the twentieth day of fetal life. This amounts to a tenfold increase in activity of the synthetase-enzyme during a period which coincides with the start and the subsequent high rate of glycogen accumulation in the fetal liver. A comparable developmental increase in the activity of this enzyme is found in the fetal Gunn-rat liver.

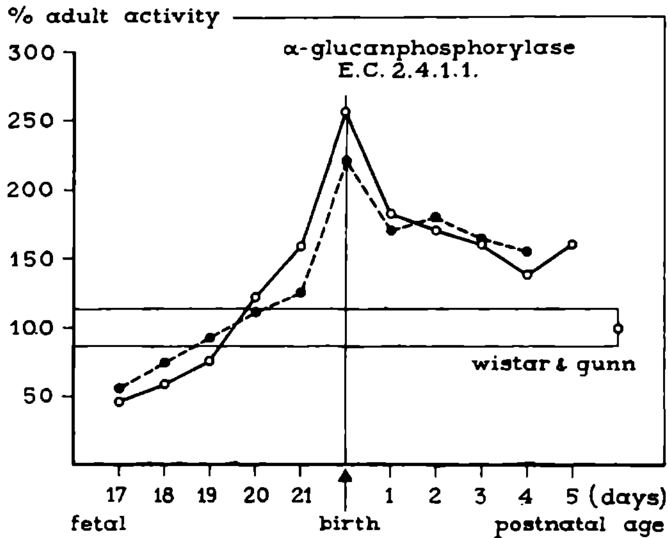


Textfig.17. Changes with age in UDPG-glycogen synthetase activity of fetal and neonatal Wistar (o——o) and homozygous Gunn-rat (•- - - -•) liver. The activities are plotted as per cent of adult activity.

After birth the activity of the synthetase-enzyme reaches the adult levels within about five days; the difference in the paths of the curves for Wistar and Gunn-rats has already been commented upon in the preceding section.

The α-glucan phosphorylase activity shows an identical pattern of development in the two strains of rats (textfig.18).

There is about a five-fold increase from the seventeenth gestational day to the time of birth. However, the steepest rise occurs during the last two antenatal days; that is: at a time when the glycogen synthetase has passed its peak values. This fact can be taken to prove that glycogen synthesis in the fetal liver occurs via the UDPG-pathway.



Textfig.18. Changes with age in α-glucanphosphorylase activity of fetal and neonatal Wistar (o——o) and homozygous Gunn-rat (•——•) liver. The activities are plotted as per cent of adult activity.

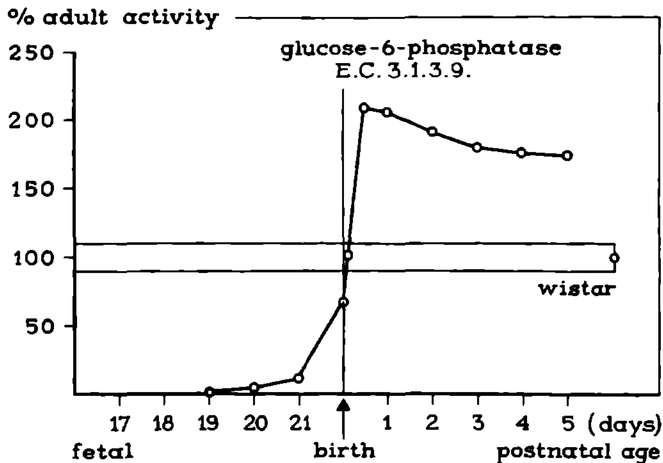
Although phosphorylase maintains a rather high level of activity during the early postnatal period, our measurements pointed to a decrease in activity immediately after birth. This result differs from those obtained by Kornfeld and Brown (1963) and Ballard and Oliver (1964). In the guinea pig and rat resp., they found a substantial rise in phosphorylase activity on the first postnatal day. The enzyme activity was measured in all these cases by the rate of orthophosphate production from glucose-1-phosphate during glycogen synthesis. One should be aware of possible unspecificity of the phosphorylase assay, due to orthophosphate production from other sources; for example from hydrolysis of glucose-6-phosphate by glucose-6-phosphatase, which shows a sharp postnatal rise.

The difference between our results and those of the authors mentioned, is probably to be related to the fact that we have used rather high concentrations of fluoride ions in the assay mixture, which are known to inhibit phosphatases (like phosphorylase phosphohydrolase) as well as phosphoglucomutase.

We have re-studied the glucose-6-phosphatase pattern of development since it was claimed recently (Burch et al. 1963) that this enzyme has peak values at four days after birth rather than at birth as was reported by others (Nemeth, 1954; Weber and Cantaro, 1957; Dawkins, 1961, 1963).

This point gains special importance in view of the supposition, made by Dawkins (1963, see chapter 6, paragraph 5), that low intra-hepatic glucose-concentrations as occurring immediately after birth, mediate in inducing the postnatal rise in glucose-6-phosphatase.

No enzyme-activity could be measured before the nineteenth day of intra-uterine life. At birth, the activity was 13.5 $\pm$ 2.1 μmoles/mg N/hr, which is 67% of the adult activity (20.2 $\pm$ 2.4 μmoles/mg N/hr). The activity increased to 17.7 $\pm$ 3.2 (89%) at 2 hrs after birth and to 41.5 $\pm$ 3.6 (207%) μmoles/mg N/hr at 12 hours post partem. Subsequently the activity decreases slowly; at the end of the period that was studied the values are still considerably higher than the adult levels: see textfig.19.



Textfig.19. Changes with age in glucose-6-phosphatase activity of fetal and neonatal Wistar rat liver. The activities are plotted as per cent of the adult activity.

Our results therefore confirm what Dawkins (1963) found, namely that the glucose-6-phosphatase activity increases within the first twelve hours after birth to values about twice the adult level.

Dawkins had already shown that the stimulus for the increase in the activity of glucose-6-phosphatase is not related to maturity at birth, but should be related to the event of delivery itself, since the effects of premature or postmature delivery - with regard to

this increase in the activity of glucose-6-phosphatase - were identical with those of a delivery at term (Dawkins, 1961). The increase in the activity of this enzyme immediately after birth coincides with the postnatal fall in liver glycogen content. The most plausible inference is of course to link these two phenomena together.

Coquoin - Carnot and Roux (1960), however, have pointed out that the interpretation cannot be so simple as would seem at first sight, because the glycogen content increases again on the 2nd-4th day after birth, whereas the glucose-6-phosphatase activity remains high during the early postnatal period. We will return to this matter in the following paragraph.

Our estimations of the branching enzyme activity yielded rather inconsistent results, the outcomes of duplicate estimations often varying greatly. In so far as they permit of a conclusion, however, one might infer from them that already at the earliest stage of liver-development that was measured (17th day of intra-uterine growth) a considerable activity can be registered. On these ground we do not think it plausible that a relative deficiency of the branching enzyme (and, consequently, an inadequate number of priming end-groups) should be hold responsible for the low levels of glycogen before the 18th gestational day.

7-5. DISCUSSION

In interpreting the results of enzyme estimations, it should be borne in mind that absolute enzyme activities, as measured under optimum in vitro conditions, do not necessarily give a true indication of the relative activities of the enzymes in the living tissue. A point particularly to be considered for a right interpretation of the data presented in this chapter, is that a comparison of enzymatic activities at different embryonal or neonatal ages is made difficult by the changes which occur in the ratio of hematopoietic to hepatic cells. Although we have gathered some quantitative data concerning the shift in this ratio (cf. chapter 6), these data cannot very well be used here, because little is known about the changes in the activity of glycogen metabolizing enzymes during the process of maturation of primitive blood cells.

Glycogen appears to be virtually absent from normal mature red blood cells. On the other hand, white blood cells are comparatively rich in this polysaccharide. The pathways, necessary for the formation of glycogen in erythrocytes, must, however, still be present as it has been claimed that glycogen can be demonstrated to accumulate in the red cells of patients with glycogen storage diseases (Sidbury et al, 1961; Manners, 1964).

This affects the exactitude of the statements that can be made. It is well known for example that the fetal liver shows a very high rate of glycolysis, and it has been claimed that this high glycolytic metabolism should be attributed to the erythropoietic function of this organ during fetal life. It is not possible, however, to settle this point unless direct measurements are made on the immature blood cells after separation from liver cells (Burch et al , 1963).

On the other hand, interpretation is less complicated in as far as qualitative differences are encountered in the enzymic equipment of the fetal and newborn or adult liver. Among the enzymes that we have studied, only the glucose-6-phosphatase is absent in the fetal liver and does not appear until shortly before birth. Because of its basic function in free glucose formation, glucose-6-phosphatase might be considered "to exert a considerable pull on glycogen catabolism in the liver" (Kornfeld and Brown, 1963). Indeed, all endogenous blood glucose originates via glucose-6-phosphatase, with the exception of the small amounts formed by the hydrolysis through the debranching-enzyme amylo-1,6-glucosidase or through the liver-amylase oligo-glucosidase pathway (Rutter et al. 1961). The accumulation of glycogen in the fetal liver might therefore be understood from the absence of glucose-6-phosphatase and the progressive decrease in the rate of glucose utilization in this organ via pathways other than glycogen formation. This supposition, originally formulated by Nemeth (1954), has led one to assume that the Cori-type I glycogen storage disease (or Von Gierke's disease), with a well-known deficiency in glucose-6-phosphatase, should be considered as the persistence during adult life of the fetal condition, which prevails during the last part of gestation.

Nemeth's supposition, quoted above, does not seem appropriate to explain why glycogen accumulation starts so suddenly between the seventeenth and the eighteenth gestational day. Our measurements have shown that there is about a tenfold increase in the activity of UDPG-glycogen synthetase between the seventeenth and twentieth day of fetal life; i.e. during a period which coincides with the start and subsequent high rate of glycogen accumulation. This makes it highly probable that the UDPG synthetase enzyme is the rate-limiting factor in prenatal glycogen storage, although - as Ballard and Oliver (1963) have pointed out - "a knowledge of enzyme-substrate affinities, enzyme turn-over numbers and the steady-state concentration of substrates in vivo would be required to define rigorously the rate-limiting step".

The activities of the enzymes phosphoglucomutase and UDPG-pyrophosphorylase reveal that the fetal liver is enzymatically well equipped for glycogen synthesis. It cannot be gathered from our measurements whether the enzyme α-glucan phosphorylase is active in vivo during

the prenatal period, since our assays concern estimations of total enzyme activity in which the inactive forms were activated. Nevertheless, we found that the steepest rise in α-glucan phosphorylase occurs at a time when the UDPG-glycogen synthetase has passed its peak values. This was interpreted as proving that glycogen-synthesis in the fetal liver follows the UDPG-pathway.

It was shown recently by Dawkins (1963) that the relationship suggested between the fall in liver glycogen after birth and the postnatal increase in glucose-6-phosphatase activity cannot be a direct one. This followed from the fact that administration of ethionine immediately after birth largely prevented the increase in glucose-6-phosphatase, but had no influence on the liver glycogen mobilization or on the postnatal decrease in intra-hepatic glucose-6-phosphate concentration. Injection of insulin also prevented the increase in glucose-6-phosphatase activity, but did not increase the rate of mobilization of glycogen. On the other hand, administration of glucose prevented liver glycogen from decreasing and glucose-6-phosphatase from increasing.

These findings justify the conclusion that the normal rise in activity of glucose-6-phosphatase is not essential for the mobilization of liver glycogen. Dawkins (1963) supposes that the rise in the activity of this enzyme is initiated by the same stimulus which is responsible for glycogen mobilization. (See Chapter 6.)

It is known from the studies of Langdon and Weakley (1955) that the level of assayable activity of glucose-6-phosphatase is decidedly influenced by circumstances which are attended by shifts in the direction of liver glucose metabolism. It is unknown whether these "adaptive" changes are due to an increase in enzyme synthesis or should be attributed to alterations in the ratio of active and inactive forms of a relatively fixed number of enzyme molecules, as seems to be the case with liver phosphorylase (Field, 1960). The above mentioned findings of Dawkins (1963) about the suppression effects of ethionine seem to suggest that the increase in glucose-6-phosphatase after birth must largely be due to synthesis of new enzyme protein.

Supporting evidence has been presented by Dallner (1964). Administration of actinomycin D to the newborn prevented the postnatal elevation of the glucose-6-phosphatase activity, thus suggesting that this process is dependent on the synthesis of messenger RNA.

On the other hand, it is known that surface-active agents, like Triton X 100 or deoxycholate not only render soluble much of the microsome-bounded glucose-6-phosphatase activity, but also cause a marked increase in the *in vitro* activity (Segal and Washko, 1959; Gosh, Kar and Chatterjee, 1963). Since the solubilized

glucose-6-phosphatase exhibits a seemingly greater affinity for its substrate than the membrane-bound form, this finding indicates that the affinity of the enzyme for its substrate varies according to whether the enzyme is bound or released. According to Dallner (1963) it is likely that the membrane structure constitutes a barrier for the access of the enzyme to the substrate, which can be removed by deoxycholate treatment. The question arises whether the abrupt postnatal increase of glucose-6-phosphatse involves striking changes in the morphology and disposition of the endoplasmic reticulum membranes. This, among other things, will be studied in the next chapter.

CHAPTER 8

CYTOLOGICAL DIFFERENTIATION OF THE RAT LIVER CELLS WITH SPECIAL REGARD TO THE GLYCOGEN-ENDOPLASMIC RETICULUM INTERRELATIONSHIPS

8-1. INTRODUCTION

A firm knowledge of the morphological and biochemical interrelationships within and between the various cellular elements is a prerequisite for arriving at an understanding of biological processes at the cellular level. However, special difficulties are encountered in raising the findings of the penetrating, yet rather rude biochemical studies to cellular reintegration.

Electron microscopy has revealed that all nucleated animal cells contain in their cytoplasm a system of lamellar and vesicular structures, termed collectively the endoplasmic reticulum (ER). This reticulum consists of "rough"- and "smooth"-surfaced elements, depending on the presence or absence of bound ribosomes.

Numerous studies on the biochemical counterpart of this endoplasmic reticulum - the microsomes - have taught that a number of enzymes are present in or on these subcellular particles. Therefore it must be assumed that the endoplasmic reticulum within the living cell performs a multitude of functions in for example protein-synthesis (strictly speaking, this is primarily a function of the ribosomes!), electron- and ion-transport, detoxications etc. But as Dallner (1963) had pointed out, "the exact localization of these functions within the morphological elements of the microsomes has not yet been clarified, nor is the possible interdependence between structure and function elucidated". Indeed, a thorough knowledge of these aspects is indispensable in order to arrive at a more definite understanding of the functions of the endoplasmic reticulum within the living cell. Moreover, it will be necessary to define precisely the appearance and the disposition of the ER in the intact cell and to analyse the range of variations that are demonstrated by this reticular system in situ under normal physiological conditions as well as under the impact of a variety of imposed variables.

We have seen above (chapter 5) that there are a number of indications present in literature that point to a special interrelationship between smooth-surfaced vesicle-like structures (taken to be part of the smooth endoplasmic reticulum!) and the metabolism of glycogen. However, from the survey given it is also evident that there is no agreement as regards the functional meaning of the observations made. There is even no uniformity in the morphological descriptions of the cellular events that accompany the processes of glycogen accumulation and depletion.

Owing to the fact that selective changes in the density of the various types of microsomal vesicles could be introduced (by adding Cs^+ and Mg^{++} as components of the fractionation medium), Dallner (1963) succeeded in subfractionating the microsome-fraction into three distinct classes of vesicles. The 10.000 x g supernatant of a rat liver homogenate was subjected to successive centrifugations at 250.000 x g on a discontinous sucrose gradient in the presence of first Cs^+-ions and then Mg^{++}-ions. Three microsomal subfractions were obtained: one of them binding both Cs^+ and Mg^{++} and consisting exclusively of rough-surfaced vesicles; a second fraction, binding Mg^{++} and not Cs^+, which contained particle-free smooth-surfaced vesicles, and a third fraction binding neither Cs^+ nor Mg^{++}, also containing smooth-surfaced vesicles, which, however, are distinctly smaller (50-100 mμ). The curious fact emerged that the first two fractions contained an almost identical equipment of enzymes, like glucose-6-phosphatase, nucleoside diphosphatase, pyridine nucleotide-specific cytochrome c reductases and diaphorases. Whereas nucleoside triphosphatase was equally active in all three subfractions, the enzyme DT-diaphorase (a reduced pyridine nucleotide-oxidizing flavoprotein, which reacts with both NAD and NADP) was present exclusively in the Mg^{++}-free smooth vesicles. Dallner concludes that the rough and the Mg^{++}-binding smooth vesicles are derived from the rough and the smooth-surfaced portions of the endoplasmic reticulum in the intact cell, whereas the smooth vesicles that do not bind Mg^{++} apparently are not derived from the same source. It is supposed that these vesicles derive from the glycogen areas of the liver cell.

With the intention to define exactly the morphological evidence for an involvement of the endoplasmic reticulum either in the deposition of glycogen or in its depletion, a description will be given in this chapter of the ultra-structural differentiation of the rat liver cells during the interval of fetal development which covers both the prenatal phase of glycogen accumulation and the postnatal period of glycogen depletion. We will restrict our descriptions mainly to an analysis of the above mentioned relationship. For further aspects of liver histogenesis, such as the problem of the derivation of the hepatic parenchyma cells and the hematopoietic cells; the differentiation of the vascular endothelium and the bile duct epithelium; the development of the bile capillaries and the spaces of Disse, the reader is referred to the studies of Elias (1955), Wilson et al. (1963) and Peters et al. (1963). Electronmicroscopic descriptions of the cytological

differentiation of the hematopoietic elements are given by Grasso et al. (1962) and Sorenson (1963).

8-2. EXPERIMENTAL PROCEDURE

8-2-1. Animals

See 6-2-1; only Wistar animals were studied.

8-2-2. Electronmicroscopic techniques

See 2-2-2/3.

8-3. CYTOLOGICAL CHANGES IN THE FETAL AND NEONATAL LIVER CELLS OF THE RAT

8-3-1. Lightmicroscopic observations

The micrographs fig.26 up to and including fig.29 illustrate the processes of prenatal glycogen accumulation and postnatal glycogen depletion, as these can be observed in sections from lightmicroscopy. The micrographs are taken from 1 μ thick PAS-stained Epon sections.

Whereas during the early stages of liver development discrimination between the presumptive hepatocytes and the hematopoietic elements is hardly possible, the identification of both cell types causes little trouble at the 17th gestational day: fig.26. A reliable criterion is that the liver cells contain almost without exception one or more fat droplets (particularly easy to observe in osmium-fixed thin sections) whereas the blood cells do not.

Moreover, the nuclei of the liver cells are transparent and measure 6-9 μ in diameter, whereas those of the bloodforming cells (erythroid cells) are smaller and distinctly darker. It can be seen from this micrograph that the latter cell-types at this stage of development still outnumber the hepatic cells.

All the various stages from hemocytoblasts to reticulocytes and erythrocytes are present; the granulocytes and megakaryocytes (cf. fig.4) are relatively scarce troughout. None of the liver cells at this stage of development demonstrated PAS-positivity but some of the granulocytes showed a faint reaction. Amylase or salivary digestion control-experiments to prove that glycogen is present, were unsuccessful.

Sections, taken from fetal liver at the 19th day of pregnancy (fig.27) show that all liver cells now contain considerable amounts of glycogen, mostly located in the peripheral parts of the cytoplasm. The

majority of hepatocytes have acquired polygonal outlines and this, according to Peters et al. (1963) can be taken to indicate that the cells have lost mobility. A number of sinusoids are pictured in this micrograph, but the extra-vascular situation of most of the erythroid elements is also evident.

The increasing amounts of glycogen that are stored by the liver cells make them grow considerably; see fig.28, which is a picture taken from a fetal liver one day before birth.

The huge quantities of glycogen push aside the mitochondria and ergastoplasmic membranes. The crowded cellular elements, however, always remain in immediate contact with the nucleus. Twenty-four hours after birth (fig.29) the liver cells have lost virtually all of their glycogen and the cells now contain numerous lipid droplets, thus demonstrating the abrupt change from an intra-uterine carbohydrate diet to the postnatal fatty milk diet. This fall in glycogen content runs parallel with a distinct decrease in cellular size.

8-3-2. Electronmicroscopic observations

At the seventeenth gestational day, the RER-elements of the liver cells are not (yet) regularly disposed in parallel cisternae - which is the characteristic configuration of this reticular system in the adult hepatic parenchymal cell - but are present as a number of particle-studded membranes, which enclose vesicular, tubular or flattened spaces in the cell (fig.30). These lie scattered throughout the cell cytoplasm and no variation is observable in the relative size of these ergastoplasmic vesicles according to their location within the cell. When the surface of these RER-vesicles is sectioned tangentially, so that the distribution of the ribosomes on their surfaces can be seen in full-face view, a number of the particles appear to be arranged in the form of rosettes, chains or spirals. Some apparently free ribosomes are present, too.

The second form of the endoplasmic reticulum, the smooth-surfaced variant, which is regularly observable in the adult liver cells, among other things in association with deposits of glycogen (Porter, 1961), is completely lacking at this development stage. Apart from the smooth membranes of the Golgi complex (to be described below), there is a small number of rounded, minute vesicles with smooth contours, to be found below the cell surface membrane (fig.30). In all probability, these vesicles represent pinocytotic vacuoles.

With the electron microscope, too, no lead-stainable glycogen particles could be detected in the hepatic cells of this development stage.

This statement is based on a careful analysis of a large number of sections of this development stage. In order to facilitate the judging of the lead-staining results (cf. chapter 2), the sections of fetal liver were several times processed together with some sections (on the same grid) of an adult rat liver, in which glycogen was known to be present.

It has been reported (G r a u m a n , 1960) that especially the preservation of embryonal glycogen sometimes causes trouble.
However, it seems highly unlikely that the absence of glycogen particles at day 17 is due to inadequate preservation, since glycogen particles are readily found (though in small quantities) on the 18th day of fetal development.
In our opinion, therefore, this evidence can be taken to prove that with regard to glycogen accumulation the developing hepatocytes actually go through a critical period between the 17th and 18th day of fetal life.
The mitochondria at this stage of development are relatively small and few in number, but otherwise have the normal ultrastructural appearance. They are irregularly shaped and have a moderate electrondense matrix, in which the cristae demonstrate the pattern of distribution characteristic of the adult liver mitochondria.

Although the Golgi complexes are readily recognizable as such, they have not yet developed the characteristic pattern of organization as is found in the adult liver cells. Some paired smooth membranes are present that are distended at their periphery to form relatively large vacuoles. Furthermore, a number of small vesicles are seen in the surroundings of the Golgi apparatus. The dilated vacuoles sometimes contain small particles of slightly electrondense material, the nature of which is still largely unknown.
It has been suggested by K a r r e r and C o x (1960a/b) that in the embryonic chick liver cells the Golgi complex plays a role in glycogen deposition. This assumption was based, among other things, on the parallelism observed in the development of the Golgi complexes and the glycogen-containing cell-areas; on the occasional contiguity of the Golgi complexes to the glycogen areas as well as on the observation that the Golgi regions were PAS-positive (K a r r e r and C o x , 1960a). However, upon closer examination it appeared that the Golgi regions did not show a positive PAS-reaction. (K a r r e r and C o x , 1960b; cf. chapter 2, par.4). This led the authors to suggest that the granular content of the Golgi vacuoles represents an accumulation of glycogen-precursors or of enzymes necessary for glycogen synthesis. No further arguments were advanced for this supposition.
In our material from the fetal rat liver, we found that the slightly electrondense particles in the Golgi-vacuoles were present two to

three days before glycogen deposition begins. Moreover, we did not come across any particular morphological relationship between the Golgi regions and those cell areas where the first glycogen particles emerge. Therefore, our findings are in agreement with those of Peters et al. (1963) in that no indications were found to point to a direct role of the Golgi complexes in either glycogen synthesis or depletion.

At the eighteenth day of intra-uterine growth (fig.31) no clear-cut changes in the pattern of organization of the endoplasmic reticulum have occurred, when compared with the preceding day. The system retains its vesiculated aspects of somewhat swollen, sac-like structures, bordered by ribosome-studded membranes. Only the RER-cisternae which lie aligned along a mitochondrial surface, partly encircling the mitochondria, seem to acquire a less tortuous outline. Here too, no relation is observable between the relative size of the ergastoplasmic vesicles and their location within the cell.

The mitochondrial profiles also have not changed to any appreciable extent.

It has been mentioned already that at this developmental stage the glycogen particles can be detected for the first time, albeit that the frequency in which they occur in sections of this stage is still rather low.

It is important to emphasize that this first appearance of the glycogen deposits may always be seen to occur in the near vicinity of rough-surfaced elements of the endoplasmic reticulum. This point is illustrated in fig.31 and, particularly well, in fig.32.

In the centre of fig.31 (18 days' old fetus) a small number of lead-stained, highly electrondense glycogen particles lie amidst a number of vesicular structures, which without doubt represent elements of the rough endoplasmic reticulum. Virtually all the membraneous profiles that lie focussed around this centre of initial glycogen formation are stuck with ribosomes, a number of which are observable in the characteristic linear or spiral configurations. Nevertheless, the arrangement of the ribosomes on the ER-membranes is not as regular as one is used to encounter at later stages of liver development (cf. fig.20a, 21). Most of the membranes have small smooth-faced parts.

The contiguity between RER-structures and areas of initial glycogen deposition is also illustrated in fig.32, which is taken from a fetal liver at the nineteenth day of gestation. It can be seen from this micrograph that the RER-cisternae are still rather distended. On occasion, however, groups of flattened vesicles appear, in which

the membranes achieve a more or less parallel arrangement. A number of endoplasmic reticulum vesicles lie intermingled with the glycogen particles. These glycogen deposits clearly display the alpha-particle type of configuration.

It may also be observed from fig.32 that part of the ER-membranes, especially those which lie in between the glycogen granules, are free from ribonucleoprotein-particles. Especially these smooth membranes are characterized by irregularities in the contours of their limiting membranes. Several of these smooth membranes show continuity with the membranes that "radiate" out of the glycogen area and that are still studded with ribosomes. This transition between the smooth elements and the adjacent rough profiles indicates that both types of vesicles represent manifestations of one single system.

In fig.33, also taken from a 19 days' old fetal liver, a somewhat larger glycogen area is pictured. Here, too, some smooth-surfaced vesicles are present amidst the glycogen particles. Others are (still) clearly belonging to the rough variant of the ER, although they lie almost completely entangled in between the glycogen granules. The arrows point towards some solitary glycogen particles, which are surrounded either by ribosome-dotted cisternae or by apparently free, multiple-arranged, ribosomal structures.

Since in the liver cells, studied at the pre-glycogen stage, the smooth or particle-free form of the endoplasmic reticulum is an insignificant element of hepatocyte fine structure, the question arises where these smooth profiles do proliferate or develop from. According to Millonig and Porter (1960) they appear as the result of a proliferation, largely from "borders" of rough cisternae. This conclusion is apparently based on the observed continuity between the two forms of the system. However, it remains questionable whether the first appearance of these smooth membranes (occurring concomitantly with the first appearance of glycogen granules) must indeed be understood as resulting from a process of de novo synthesis of smooth membranes; in other words whether they can be taken as resulting from a true outflow of smooth membranes from rough-surfaced areas.

In this connection it seems worthwhile to reveal a finding of Peters et al. (1963). They studied the ultrastructural development of the mouse liver and observed that during the prenatal period the ergastoplasmic cisternae, adjacent to glycogen accumulations, showed bulbous terminal swellings. The membranes were either partially or completely deficient in ribosomes. This observation led them to assume that the cisternal swelling and the appearance of vesicular terminal forms (preceding and accompanying the accumulation of glycogen) represented a "special type of ergastoplasmic differentiation", which was related to glycogen synthesis. It was, however, not

reported how this relationship ought to be understood.

In our sections from the fetal rat liver we have encountered trough-out - with the exception of the last two days - an ergastoplasmic system that was rather distended and often took the appearance of membrane-bound cavities.

Nevertheless, we have made comparable observations on "terminal saccate swellings": see fig.33 (asterisks), 34 and 35. However, we do not think that this terminal swelling itself has special importance in the process of glycogen synthesis, nor do we think that, as a result, it would deserve to be qualified as a "special ergastoplasmic differentiation".

In our opinion the emphasis has to be put on the fact that these terminal profiles are partially or completely deficient in ribosomes. In this respect, the observation of Peters et al. (1963) does not differ from a finding reported earlier by Drochmans (1960), but which has had little attention since no interpretation was presented at that time. Drochmans described in the glycogen-loaded liver cells of adult rats the presence of "un type particulier de vésicule appar-"tenant au reticulum endoplasmique. Cette vésicule aplatie (sic!) en-"toure les mitochondries: la paroi en contact avec la mitochondrie "est garnie de grains de Palade, alorsque l'autre paroi en contact "avec le glycogène est systematiquement pourvue de grains de "Palade."

We have repeatedly been able to establish comparable relationships in adult hepatocytes. As such a disposition is not at all suggestive of "a process of outflow" of smooth membranes, the more obvious interpretation is to assume a transformation of rough membranes into smooth ones in consequence of a process in which the adhering ribonucleoprotein particles are set free. Of course it is very difficult to derive conclusive arguments in favour of this supposition from electronmicrographs, because it is hazardous to interpret the essentially static pictures in a dynamic way. Nevertheless, the fact that quite a great number of straight, curved or looped chains of ribosomes are found alongside of the RER-cisternae, - even when these RER-cisternae apparently are not sectioned tangentially: see fig.32 and also fig.37 - is very suggestive of a process of release of these polyribosomal arrangements from the endoplasmic reticulum membranes.

More solid ground, however, may be derived from a finding that is illustrated in the figures 34 and 35. These micrographs were taken from an 18 days' old rat fetus. Both sections were stained with uranyl-acetate. We have seen above (chapter 2, paragraph 7) that ribonucleoprotein structures have special affinity for uranyl.

In fig.34 parts of two adjacent liver cells are pictured. In each of the two cells a small glycogen area is present (Gl). The particles in these two glycogen areas, however, display a distinctly different

electron opacity. The glycogen granules of the hepatocyte on the right - except for a few small particles at the periphery of the area - apparently had a low affinity for uranyl ions since they demonstrate only a faint electrondensity. It may be observed that there are no vesicular structures present in between the polysaccharide particles of this glycogen area.

The particles in the glycogen area of the liver cell on the left, on the other hand, demonstrate a high electrondensity. It is worth noticing that these glycogen deposits are present in between vesicular structures.

It is deducible from this distinct difference in electron opacity, or to put it more exactly, from the obviously different affinity for uranyl, that the chemical composition of the particles in the two groupings is not identical.

A similar arrangement of vesicular profiles and "uranyl-positive granules" is pictured at higher magnification in fig.35. It may be seen that the highly electrondense particles are present in a closely-meshed network of haphazardly distributed membraneous profiles of the smooth type. It may also be observed that there is an overlap in size and shape of the smallest glycogen units and the ribosomes present in the surroundings of this area of initial glycogen deposition. This manifestation of the smooth reticular system closely resembles the "tri-dimensional lattice of small tubules and vesicles" (Porter, 1961), described as occurring in the liver cells of rats that were fed after a period of fasting (Fawcett, 1955) and in the paraboloid of the turtle (Yamada, 1961).

Once again it is to be observed from fig.35 that the ER-vesicles of the liver-cell cytoplasm outside of the glycogen area, are studded with ribosomes. However, those vesicular walls that adjoin the glycogen area, are smooth.

It is important to emphasize that these uranyl-positive particles - present in an "essentially vesicle-locked situation" (Porter, 1961) - also possess strong affinity for lead-ions from basic lead-solutions: see fig.36, taken from a two months' old calf fetus.

Another point that deserves some attention is the fact that when the glycogen-areas are growing in size, the uranyl-positive particles are only to be encountered at the periphery of the area, adjacent to the ER-membranes (see fig.35 and also fig.21).

It is useful to point out that this is not the first description of uranyl-positive glycogen-rosettes. Biava (1963) reported that "usually the complex glycogen particles in liver cells are not stainable with uranyl-acetate except impredictably in some pathological livers". This author comments on this observation that the reasons for this particular reactivity are not apparent.

It is the present writer's opinion that the observations of Björkman (1964)

can be taken to be concordant with our observations, in spite of this author describing the uranyl-positive particles as "aggregated ribosomes". Björkman encountered these aggregates (distinct from the well-known chains or loops of ribosomes) in the livers from dog, sheep, rat, mouse and rabbit under normal as well as under certain pathological conditions. This author, too, noticed that the aggregates were readily stainable with uranyl and somewhat less so with lead. They occurred in cell areas where the endoplasmic reticulum was mainly smooth. Adjacent to these aggregates the usual polyribosomal arrangements were present.

The most obvious explanation to understand the particular reactivity of these initial glycogen particles is to assume that glycogen is deposited in intimate association with the ribosomes.

The appearance of a smooth-membraned system concomitant with the formation of the first glycogen granules, might then be understood by assuming that the ribosomes either before or during this process of glycogen deposition (this cannot be settled with certainty with the aid of electronmicroscopic observations)- are detached from the originally rough ER-membranes.

Within the scope of this concept it does not seem necessary to attribute a particular role to the smooth membranes in the subsequent events of glycogen accumulation. It is, indeed, well-known, that the number of smooth profiles present in the glycogen areas, decreases as the glycogen areas increase in size. One might take the blurred tortuous outline of the membranes as indicative of a process of desintegration: see fig.32, 33 and 36.

The evidence presented above is once more supported by the picture 36 and 37, taken from a two months' old fetal calf.

Fig.36 shows a rather extensive area of initial glycogen formation. It may be observed that an unusual high number of loops and spirals of ribosomes are present in the vicinity of this glycogen area (asterisks). Bottom left is pictured a diffusely organized Golgi region, containing one inclusion-body with highly electrondense material (lysosome). In this Golgi region no glycogen particles are present. The membraneous elements in between the glycogen particles are rounded, swollen and their electron opacity has hardly increased under the impact of an otherwise successful lead-staining.

The arrows point towards arrays of newly formed glycogen particles whose disposition still represents the original arrangement of the RER-cisternae. In the ergastoplasmic region, middle left on the picture, very small but strongly stained particles are detectable alongside the RER-membranes. Their electron opacity is distinctly higher than those of the ribosome-spirals.

Fig.37 represents another section of the same tissue block. In this

particular region the glycogen particles are still scarce and lie among the RER-membranes without apparent order. The latter are irregularly studded with ribosomes and on many places arrangements of ribosomal chains are seen that suggest their release from the membranes.

With regard to the problem specified above, the ultrastructural development of the liver cells during the last two days of gestation offers few important data. The transition of the irregularly arranged micro-vesicular form of the ER into the usual parallel cisternae continues. In accordance with the findings of Dvorack (1964) we noticed an elongation of the mitochondria (figs.20, 21). The increasing amounts of glycogen push the cellular organelles aside into restricted areas, usually around the nucleus, while leaving intact a number of "protoplasmic bridges" of packed mitochondria and RER-cisternae that reach from the perinuclear regions to the plasma membrane. The ER-cisternae partly or completely enwrap the mitochondria (figs.19, 20).
It is striking that the glycogen particles never contact the plasma membrane immediately, but always leave a small rim of protoplasm.

After the maternal blood supply has ceased at birth, the liver of the newborn animal is in charge of maintaining the blood glucose level. A number of changes are induced in the liver cell ultrastructure.
The most prominent alterations are: 1. a dilatation of the endoplasmic reticulum cisternae; 2. a dictinct swelling together with a rounding of the mitochondria and 3. a progressive shrinkage of cell size concomitant with the disappearance of glycogen.
The earliest and most persistent postnatal change in hepatocyte fine structure is a dilatation of the ER. It may be observed from fig.38 (2 hours post partum) that the cisternal lumina are already prominently widened. Moreover, the well-ordered parallel-orientated groups of cisternal profiles, as encountered during the last two days before birth, have now changed into irregularly arranged groove-like profiles, which sometimes seem to be liable to a process of disruption. Nevertheless, most of the membranes keep enwrapping the mitochondria and this close association between endoplasmic cisternae and mitochondria seems to be maintained with special care during the period of rapid glycogen depletion, cf. fig.39.
Coinciding with this change of the ER, a swelling and rounding of the mitochondria could clearly be established. The stretched polymorphous mitochondria of the prenatal period are no longer encountered at 2 hours postpartum. This process of mitochondrial swelling seems to start from the central parts of the mitochondrial matrix as judged from focal decreases in electrondensity. Subsequently there

is a gradual extension to the peripheral parts. Our micrographs of this early postnatal period are strongly indicative of another change in mitochondrial fine structure; viz. a process of mitochondrial division. Several regularly encountered facts point in this direction. Figure 39 illustrates this. It should be noticed that the particular animal from which this micrograph was taken (17 hours after birth) has been prevented from sucking, but completely comparable findings were made on sections from animals that did suck. These animals, however, seemed to be less suited for studying these mitochondrial alterations, as large amounts of fat accumulate in the liver cells soon after sucking. In this case mitochondria may be observed in intimate association with the fat droplets, which often induces defective outer membranes.

The facts mentioned below point to mitochondriogenesis through a process of division of the existing structures. Figure 39 demonstrates two mitochondria that have a crista which interconnects the opposite interior walls (asterisks). Cristae in liver mitochondria very rarely demonstrate this pattern of organization. Moreover, one readily lights upon the point of connection between these (interconnecting) cristae and the interior membrane of the mitochondrial wall, which also seldom occurs in liver mitochondria. Thirdly, numerous mitochondria may be observed from which part of the outer membranes are missing (arrow in fig.39). These observations in our opinion are best explained by assuming a process of mitochondrial division.

We have not been able to confirm the observations of Peters and co-workers (1963), who reported that the glycogen areas in the newborn mouse were invaded by smooth-membraned tubular forms of the endoplasmic reticulum during the process of glycogen depletion. According to them the quantity of this "tubular" endoplasmic reticulum varies inversely with the amount of glycogen. We have seen in chapter 6 that the lowest levels are attained within 12 hours after birth. Therefore we have focussed our attention on this early postnatal period. If one studies the liver cells at a somewhat later stage, - as Peters and collaborators did (24 hours postpartum) - one should be aware of the possibility that a number of liver cells again can be active in synthesizing glycogen. Dawkins (1963) showed that blood-glucose had risen again as early as 6 hours after birth. In our sections from 1 day old newborn animals we have encountered arrangements of smooth-membraned profiles in continuity with rough cisternae, lying in between glycogen granules. Such pictures did not differ from the one presented in figure 41 (3 days' old newborn) and, in our opinion, should be taken to reproduce relationships that are completely comparable to those found in the fetuses.

8-4. DISCUSSION

In liver cells, in which glycogen is rapidly metabolized, a characteristic structural association of smooth surfaced endoplasmic reticulum membranes and glycogen deposits may readily be encountered. Such a close topographical association is very suggestive of a functional involvement of the agranular reticulum in the events of glycogenesis and glycogenolysis and this has been claimed on many occasions. However, a number of recent studies on the intra-cellular localization of the enzymes, most directly involved in glycogen-synthesis and glycogenolysis, have shown that these enzymes either are bound to the glycogen particles or are free in the cytoplasm.

For example, Sie et al. (1964) could show that the liver cytoplasmic glycogen particles were complex "metabolically active glycogen multi-enzyme granules", consisting of a combination of glycogen with UDPG-glycogen-synthetase, α-glucan phosphorylase and phosphorylase phosphatase. This association of the polysaccharide particles and the enzymes most likely is not the result of a random physical binding since an independent temporal behaviour of two of these enzymes (UDPG-glycogen-synthetase and α-glucan phosphorylase) during glycogen depletion could be established.

As, moreover, in a number of tissues other than the liver the glycogen particles do not show a close association with smooth reticulum membranes, an involvement of the agranular reticulum in glycogen metabolism has been questioned by others.

The liver plays a unique role in one of the most finely regulated homeostatic mechanisms: the maintenance of normal levels of sugar in the blood. This has led to the supposition that the association of endoplasmic reticulum structures and glycogen particles in the liver cells must be interpreted to represent - at a morphological level - a mechanism of release of glucose into the blood (Siekevitz, 1959; Porter, 1961; Biava, 1963; Rosen, 1964).

As a matter of fact glucose-6-phosphatase, which plays an important role in hepatic glucose release, is concentrated in the liver and the kidney. Other tissues possess no activity or very little. This enzyme is known to be firmly bound to the endoplasmic reticulum (Cahill et al. 1959). This latter point is demonstrated once more in figure 40. This micrograph was taken from adult hepatic tissue (rat) and has been treated according to the prescriptions of Tice and Barnett (1962) for demonstrating glucose-6-phosphatase activity at the ultrastructural level (frozen sections of hydroxyadipaldehyde-fixed tissue). It can be seen that the lead reaction product follows the characteristic arrangements of the ergastoplasmic membranes. The nuclear membrane (not pictured) also showed positive reaction. The mitochondria are essentially free from the reaction product. It may be

observed that some reaction product is present in the smooth-surfaced vesicles that are lying in the glycogen zone at the bottom left of the picture.

Rosen (1964) studied the fine structural localization of glucose-6-phosphatase in the neonatal mouse livers and reported that "the "activity of the glucose-6-phosphatase splitting enzyme in the tubular "ER of the glycogen areas supports the morphological evidence pre-"sented by Peters et al. (1963) that the tubular ER is not related "to glycogenesis but rather to glycogenolysis through some associa-"tion with glucose-6-phosphatase activity".

However, if this interpretation were a valid one - that is to say: if the glycogen-endoplasmic reticulum association was to be understood as functional in the release of glucose through the mediation of glucose-6-phosphatase - then one might expect such agglomerations of smooth membranes and glycogen particles to be absent in the fetal liver since very low or zero values were found for glucose-6-phosphatase during intra-uterine liver development (chapter 7). But as was shown above, the characteristic topographical association of smooth vesicular profiles and glycogen particles is easily to be observed during the period of initial glycogen formation in the fetal liver. Therefore this interpretation does not seem valid either.

From our studies it follows that during prenatal glycogen accumulation, the initial glycogen granules emerged in the ergastoplasmic areas of the cell. It was found also that the first appearance of the smooth tubular profiles was concomitant with the first appearance of the glycogen deposits. Moreover, it could be established that the initial glycogen particles demonstrate a peculiar affinity for uranyl, whereas the fully outgrown glycogen granules of the liver do not. Finally it was noted that the number of smooth profiles present in between the glycogen granules decreased as the glycogen areas increased in size.

For the present the following hypothesis is presented to explain the phenomena observed: the initial glycogen granules are formed on or closely near the ribosomes which either preceding to or during this process become detached from the rough endoplasmic reticulum membranes. The ribosomes in question are enveloped in the growing glycogen granules, which accordingly manifest a reactivity with uranyl ions. In consequence of this release of the ribosomes, the rough endoplasmic reticulum membranes are left as smooth-membraned profiles. During the subsequent event of glycogen storage these smooth profiles are no longer functional and desintegrate.

In course of time the glycogen granules loose their affinity for uranyl so that it is to be assumed that the ribosomal constituents are

broken down or lost otherwise.

In view of the finding, reported in chapter 7, that the enzyme UDPG-glycogen synthetase is the major rate-limiting step in prenatal glycogen accumulation, it is tempting to consider the ribosomes in question as being actively concerned in the synthesis of this enzyme-protein. But this supposition requires more conclusive evidence.

The occurence of two "types" of glycogen granules, that is to say of uranyl-positive and uranyl-negative particles (i.e. granules that do and do not contain ribosomal material), might have interesting relationships to the problem of free versus bound glycogen fractions (see chapter I, section 2). If indeed the uranyl-positive glycogen granules contain considerable amounts of ribonucleoprotein material, then one might expect them to be insoluble in TCA. As it concerns initially formed and, as we have seen, small particles (see fig.35) which are in actual growth, many of the studies concerning the metabolic heterogeneity of cellular glycogen (tracer experiments with glucose-^{14}C etc. as well as the findings of differential incorporation of active glucose into high and low molecular weight glycogens cf. chapter 1-2) might be better understood. But it will be evident that further studies, following the way indicated by these observations, are required to settle this question.

The postnatal changes in hepatic ultrastructure give little cause for comment. No ingrowth of smooth membranes into the glycogen areas diminishing in size could be observed. The dilatation of the ER-cisternae already present probably represents the augmented output of glucose, but morphological observations cannot give sufficient security for the exactitude of this supposition.

The swelling of the mitochondria might bear relationships to the presumed activation of phosphorylase after birth. The activation of this enzyme is related to the availability of ATP, which not only phosphorylates dephosphophosphorylase but also provides 3'-5' cyclic AMP as a co-factor for the phosphorylase reaction (Nigam, 1962).

In view of the available evidence for the presence of contractile proteins in the mitochondria (which contract in the presence of ATP), one might relate the swelling of the mitochondria to the activation of phosphorylase by assuming an increased release of ATP from the mitochondria.

SUMMARY

The investigation described in part I of this thesis, deals among other things with the possibilities of a selective staining of glycogen in electronmicroscopic specimens. A consideration of these methodological problems was requisite to the investigation, reported in part II, into the functional significance, if any, of the endoplasmic reticulum of liver cells in glycogenesis and/or glycogenolysis.

As an introduction to part I a brief discussion of the molecular structure of glycogen is given, based on the data available in the literature. Chapter 1 also contains a summary of recent studies regarding the metabolic heterogeneity of tissue glycogen (free or lyo-fraction and bound or desmo-fraction).

In chapter 2 the usefulness is studied of a number of methods, proposed in the literature, for the identification of glycogen at the sub-cellular level. As glycogen demonstrates no affinity to osmiumtetroxyde, which is the most commonly employed fixative in electron-microscopy, the possibility exists of a misinterpretation with regard to the presence of glycogen in particle form or otherwise. It is found that staining of thin sections with highly alkaline lead-containing solutions yields results that are consistently reliable. A gain in knowledge regarding the mechanism of staining of glycogen with lead, made it possible to develop a method for differentiating glycogen-particles and RNA-containing structures.

In chapter 3 the chemical mechanism of the glycogen-lead interaction is analysed. From our studies it follows that lead is bound to the polysaccharide-component of the glycogen particles and not to the proteins that in all probability are adhered to the glycogen granules. Lead-solutions prepared according to the prescriptions of Millonig (1961) or Reynolds (1963) appear to contain the lead as a negatively charged complex-ion. A number of indications make it highly probable that the glycogen-lead interaction should be considered as resulting from H-bond formation between the polysaccharide and the lead ions.

The main purpose of the studies described in chapter 4 was to define the effects of the most commonly employed glycogen extraction-fluids (TCA and conc.KOH) on the structure of particulate glycogen, as it could be isolated from livers of rats. From turbidity measurements, ultra-centrifuge analyses and electron-microscopic studies it follows that a treatment with TCA causes a rapid degradation of the glycogen particles. KOH-solutions, on the other hand, only demonstrate a distinct effect if the treatments are carried out at high temperature.

From the ultra-centrifuge data no arguments could be derived in

favour of the existence of so-called γ-elements that - according to Drochmans (1963) - constitute the smallest subunits in the complexly organized α-structures. Nor are there, in our opinion, sufficient arguments for considering as β-sub-units the fragments that are released from these complex structures under the impact of acids.

As an introduction to part II of this thesis a survey is given on the cellular metabolism of glycogen (chapter 5). Section 2 of chapter 5 presents the pertinent data indicating an involvement of the endoplasmic reticulum in glycogen metabolism.

The pronounced changes in liver-glycogen contents occuring during the perinatal period were taken to offer a possibility for studying separately the cellular processes in glycogen storage and glycogen depletion. As the activities of a number of enzymes that are known to be microsome-bounded (e.g. those enzymes which are involved in glucuronide-formation or in detoxification), are low or even completely absent during intra-uterine existence, no interference in this respect is to be expected.

Chapter 6 presents quantitative data on the changes in glycogen-content of the developing rat liver from the 15th day of gestation until the 5th day after birth. Although a small quantity of glycogen is present prior to the 17th gestational day, the accumulation starts suddenly between the 17th and 18th day of intra-uterine development. At birth the glycogen content has increased to about 100 mg/g wet liver weight; within 12 hours after birth it decreases again to about 20 mg/g wet liver weight.

The fetal liver being an active site of hematopoiesis, quantitative data were collected regarding the shift in the ration hepatocytes: hematopoietic elements.

As homozygous Gunn-strain rats (with a recessive hereditary defect of glucuronyltransferase) were reported to have very low levels of liver-glycogen (Halac and Stuart, 1960), the experiments were extended to these animals. However, no results were obtained that deviated from those gained when studying normal Wistar-rats. It appears that homozygous embryos of the Gunn rats accumulate normal amounts of liver glycogen. On grounds mentioned in chapter 6 it is assumed that fetal liver glycogen is very stable.

Changes in the activities of a number of enzymes concerned in glycogen metabolism - viz. phosphoglucomutase, UDPG-pyrophosphorylase, UDPG-glycogen-synthetase, α-glucan-phosphorylase, glucose-6-phosphatase and branching enzyme - are studied in chapter 7. The results show that liver glycogen synthesis in the prenatal period follows the UDPG-pathway. It would appear that the activity of the enzyme UDPG-glycogen-synthetase is the major rate-limiting

step in prenatal glycogen storage.

Concurrent with a sharp decrease in glycogen content immediately after birth, there is a strong increase in the activity of glucose-6-phosphatase. Recent studies of Dawkins (1963), however, make it unlikely that there is a direct relationship between these two events.

Chapter 8 presents a description of the ultrastructural changes in the liver cell during the perinatal period, with special regard to those changes that occur in appearance and disposition of the endoplasmic reticulum. The initial glycogen granules are not generated in or near the Golgi-zones, as has been claimed by Karrer and Cox (1960), but appear in the ergastoplasmic areas of the liver cell; in fact, immediately alongside the membranes of the rough, ribosome-studded, ER-membranes.

The newly-formed glycogen particles not only show strong affinity to lead but are also stainable with uranyl. This is taken to indicate that ribosomal material is (still) present in or on these newly-formed glycogen deposits. The fact that the ER-membranes are encountered smooth after the initial glycogen deposits are formed, supports this assumption. Guided by the results of our enzyme-estimations (chapter 7) the ribosomes in question are supposed to be involved in the synthesis of the enzyme UDPG-glycogen-synthetase. The formation of "uranyl-positive" glycogen granules during the process of glycogen synthesis is brought up in connection with the problem of metabolically heterogeneous fractions of glycogen. As the particle-free reticulum (left behind as a smooth-surfaced system after the release of the ribosomes) is no longer detactable when the glycogen-areas increase in size, it is concluded that these smooth profiles themselves are not functional in the processes of glycogen synthesis or accumulation.

The changes occurring immediately after birth consist of a distinct dilatation of the rough-surfaced ER-cisternae as well as of a swelling of the mitochondria, which seem to divide. A proliferation of smooth membranes, penetrating the rapidly diminishing glycogen areas, could not be ascertained. This leads to the conclusion that the smooth-surfaced ER is not involved either in the degradation of glycogen.

SAMENVATTING

Het onderzoek dat in deel I van dit proefschrift wordt beschreven betreft onder meer de vraag naar de mogelijkheden van het selectief aantonen van glycogeen in electronenmicroscopische preparaten. Bestudering van deze methodologische kwestie was noodzakelijk ten behoeve van het in deel II beschreven onderzoek. Hierin wordt nagegaan welke functionele betekenis toekomt aan het endoplasmatisch reticulum van levercellen bij de glycogenese en/of glycogenolyse.

Deel I wordt ingeleid door een aan de hand van de literatuur samengesteld overzicht over de moleculaire structuur van glycogeen (hoofdstuk 1). Hieraan is toegevoegd een samenvatting van recente gegevens omtrent het vraagstuk van de metabolische heterogeniteit van weefselglycogeen (vrije of lyo-fractie naast gebonden of desmo-fractie).

In hoofdstuk 2 wordt de bruikbaarheid van een aantal in de literatuur voorgestelde methoden ter identificatie van glycogeen op subcellulair niveau onderzocht. Omdat glycogeen geen affiniteit bezit voor het meest gebruikte fixatie-middel in de electronenmicroscopie, het osmiumtetroxyde, bestaat het gevaar dat verkeerde conclusies worden getrokken over het al dan niet in partikel-vorm aanwezig zijn van het glycogeen. De "kleuring" van coupes met lood-bevattende oplossingen van hoge pH geeft - mits op de juiste wijze uitgevoerd - reproduceerbare en goede resultaten. Op grond van verkregen inzichten in het mechanisme van de "kleuring" van glycogeen met lood, kon tevens een methode ontwikkeld worden waardoor het mogelijk werd te differentiëren tussen glycogeen-partikels en RNA-bevattende structuren.

In hoofdstuk 3 wordt het chemisme van de binding glycogeen-lood nader onderzocht. Dit onderzoek wijst uit dat de polysaccharide-component van het partikel-glycogeen - en niet het vermoedelijk tevens aan de glycogeen-partikels gebonden eiwit - de binding met lood aangaat.

In de volgens de recepten van Millonig (1961) en Reynolds (1963) samengestelde lood-oplossingen is het metaal aanwezig als een negatief geladen complex-ion. Een aantal aanwijzingen maken het waarschijnlijk, dat de interactie tussen glycogeen en lood berust op waterstofbrugvorming tussen het polysaccharide en de lood-ionen.

Het in hoofdstuk 4 beschreven onderzoek heeft in de eerste plaats tot doel na te gaan welke invloed de meest gebruikte glycogeen-extractiemedia (TCA en KOH) hebben op de structuur van uit de lever van ratten geïsoleerd "particulate" glycogeen. Uit turbiditeits-metingen, ultracentrifuge-analyses en electronenmicroscopisch onderzoek blijkt, dat behandeling met TCA snel voert tot afbraak der structuren. Behandeling met geconcentreerde loog-oplossingen heeft

alleen een duidelijk effect, wanneer hoge temperaturen worden toegepast. Ultracentrifuge-onderzoek levert voorts geen argumenten op ten gunste van het bestaan van zogenaamde γ-elementen, die volgens Drochmans (1963) als kleinste subeenheden deel uitmaken van de complexe α-structuren. Ook zijn er onzes inziens onvoldoende argumenten aanwezig om de brokstukken, welke onder inwerking van zuren worden afgesplitst, te beschouwen als β-subeenheden.

Als inleiding tot deel II van dit proefschrift wordt in hoofdstuk 5 een overzicht gegeven van recente onderzoekingen over de cellulaire stofwisseling van glycogeen. Paragraaf 2 van dit hoofdstuk bevat een overzicht van de in de literatuur vermelde, maar sterk uiteenlopend geïnterpreteerde, aanwijzingen voor een functioneel verband tussen het endoplasmatisch reticulum en de glycogeen-stofwisseling.

De zeer sterke wijziging van het glycogeen gehalte welke gedurende de perinatale periode in de zich ontwikkelende lever van de rat optreden, maken het mogelijk de cellulaire processen bij glycogeenstapeling en glycogeen-depletie afzonderlijk te onderzoeken. Bovendien is er het voordeel dat de activiteiten van een aantal microsomaal gebonden enzymen gedurende de prenatale periode bijzonder laag zijn of geheel ontbreken, zoals die van de enzymen welke een rol spelen bij de glucuronidering en de detoxicatie.

Hoofdstuk 6 vermeldt de quantitatieve gegevens over de veranderingen in het glycogeen gehalte in de zich ontwikkelende rattelever vanaf de 15e zwangerschapsdag tot 5 dagen na de geboorte. Hoewel een geringe hoeveelheid aanwezig is voor de 17e ontwikkelingsdag, begint de stapeling van glycogeen abrupt tussen de 17e en 18e zwangerschapsdag.

Bij de geboorte is dit gehalte opgelopen tot ca. 100 mg/g nat levergewicht; binnen 12 uur na de geboorte daalt het echter weer tot ca. 20 mg/g nat levergewicht.

Omdat in de foetale lever een sterke haemopoiesis plaatsvindt, worden quantitatieve gegevens verzameld over de wijzigingen in de verhouding van hepatocyten tot haemopoietische elementen. Omdat op grond van gegevens uit de literatuur bij homozygote Gunn-ratten (met een recessief erfelijk tekort aan glucuronyltransferase) bijzonder weinig glycogeen in de lever werd verwacht, worden deze proefdieren ook bij de onderzoekingen betrokken. Onze bepalingen laten echter geen afwijking van het normale beeld zien.

Ook de homozygote embryonen van de Gunn-rat stapelen normale hoeveelheden glycogeen.

Op ter plaatse genoemde gronden kan worden aangenomen dat het foetale glycogeen bijzonder stabiel is.

De veranderingen in de activiteiten van een aantal enzymen van de "glycogeen-cyclus" - te weten: phosphoglucomutase, UDPG-pyrophosphorylase, UDPG-glycogeen-synthetase, α-glucan-phosphorylase,

branching-enzym en glucose-6-phosphatase - worden beschreven in hoofdstuk 7. De verkregen resultaten wijzen uit, dat ook tijdens de prenatale periode de glycogeen-opbouw verloopt door tussenkomst van het enzym UDPG-glycogeen-synthetase. Bovendien blijkt dat dit enzym waarschijnlijk de belangrijkste "rate-limiting" factor vormt bij de glycogeen stapeling. Tegelijk met de scherpe daling van het glycogeengehalte onmiddellijk na de geboorte is een sterke stijging meetbaar in de activiteit van glucose-6-phosphatase. Op grond van recente onderzoekingen van Dawkins (1963) is het echter onwaarschijnlijk dat hier van een directe relatie sprake is.

In hoofdstuk 8 wordt de submicroscopisch-cytologische differentiatie van de levercel beschreven, met name voor wat betreft de veranderingen welke optreden in vorm en rangschikking van het endoplasmatisch reticulum. De waarnemingen wijzen uit, dat de eerste glycogeen-granula niet ontstaan in of nabij de Golgi complexen, maar in het ergastoplasma-gebied van de cel, en wel onmiddellijk langs de membranen van het ruwe, met ribosomen bezette, endoplasmatisch reticulum. De eerst gevormde glycogeen-partikels zijn niet alleen sterk kleurbaar met lood, maar hebben ook hoge affiniteit tot uranyl. Dit wordt geïnterpreteerd als wijzende op de aanwezigheid van ribosomaal materiaal in deze eerste glycogeen-depots. Het feit dat de membranen na de eerste glycogeen-afzettingen gladwandig bevonden worden maakt dit temeer waarschijnlijk. Op grond van hetgeen in hoofdstuk 7 werd gevonden, wordt verondersteld dat de betreffende ribosomen actief betrokken zijn bij de synthese van het enzym UDPG-glycogeen-synthetase. De aanwezigheid van "uranyl-positieve" glycogeen-granula tijdens de glycogeen-synthese wordt in verband gebracht met het probleem van metabolisch heterogene glycogeenfracties (hoofdstuk 1).

Omdat het als gladwandig membraansysteem achtergebleven reticulum bij de uitgroei der glycogeen-velden niet langer waarneembaar is, wordt geconcludeerd, dat aan het gladwandige ER zelf geen functie meer toekomt bij het verdere proces van de glycogeen stapeling.

De veranderingen welke onmiddellijk na de geboorte optreden bestaan in duidelijke zwellingen van het ruwwandige ER en van de mitochondrien welke laatste zich bovendien waarschijnlijk delen. Een proliferatie van gladwandige membranen in de in omvang snel afnemende glycogeen-velden kan niet worden vastgesteld. Op grond hiervan wordt geconcludeerd dat het gladwandige endoplasmatisch reticulum niet onmiddellijk betrokken is bij de afbraak van de glycogeen partikels.

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PARTICULATE GLYCOGEN

A correlated electronmicroscopical and
biochemical study

A M. STADHOUDERS

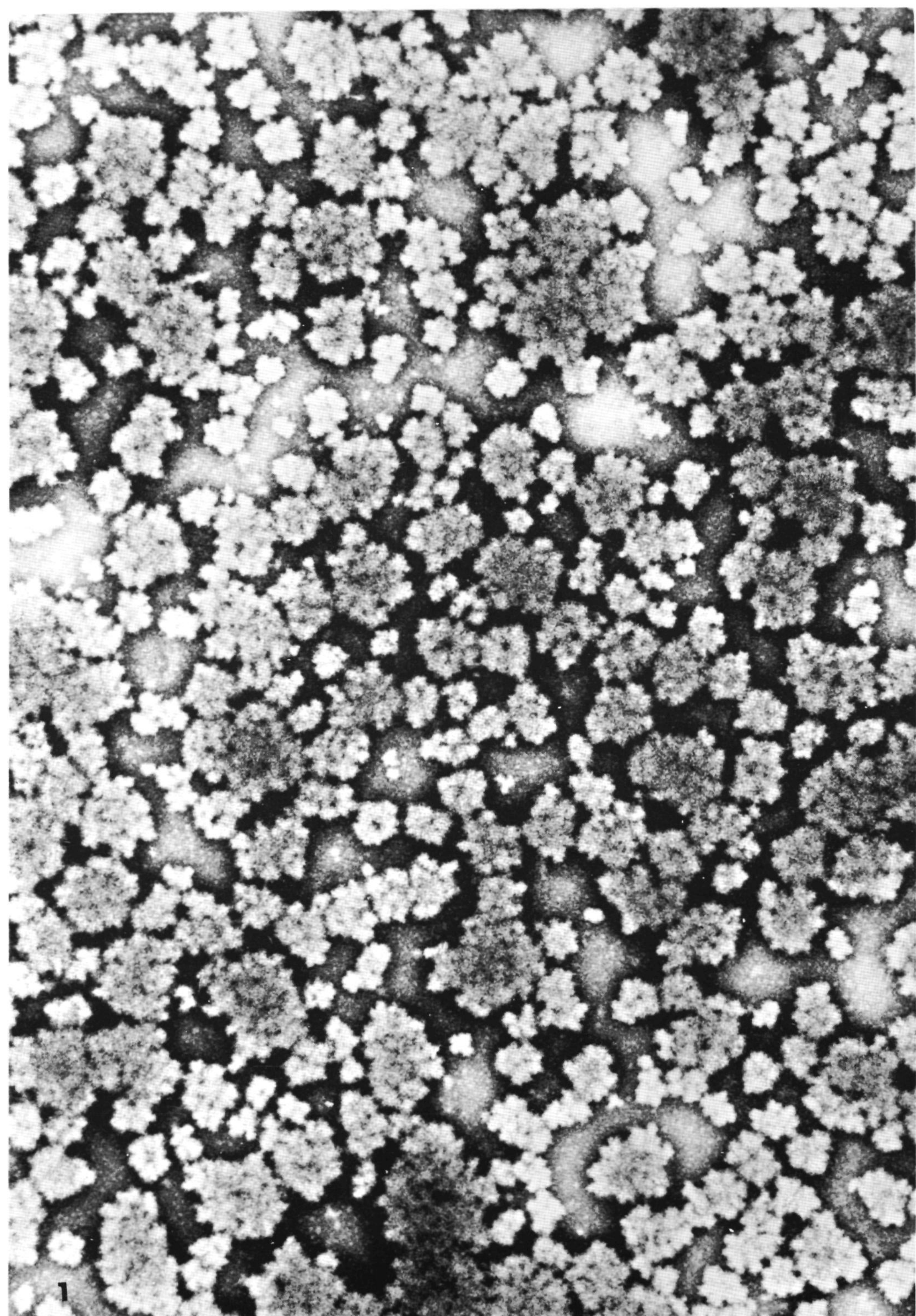
ILLUSTRATIONS

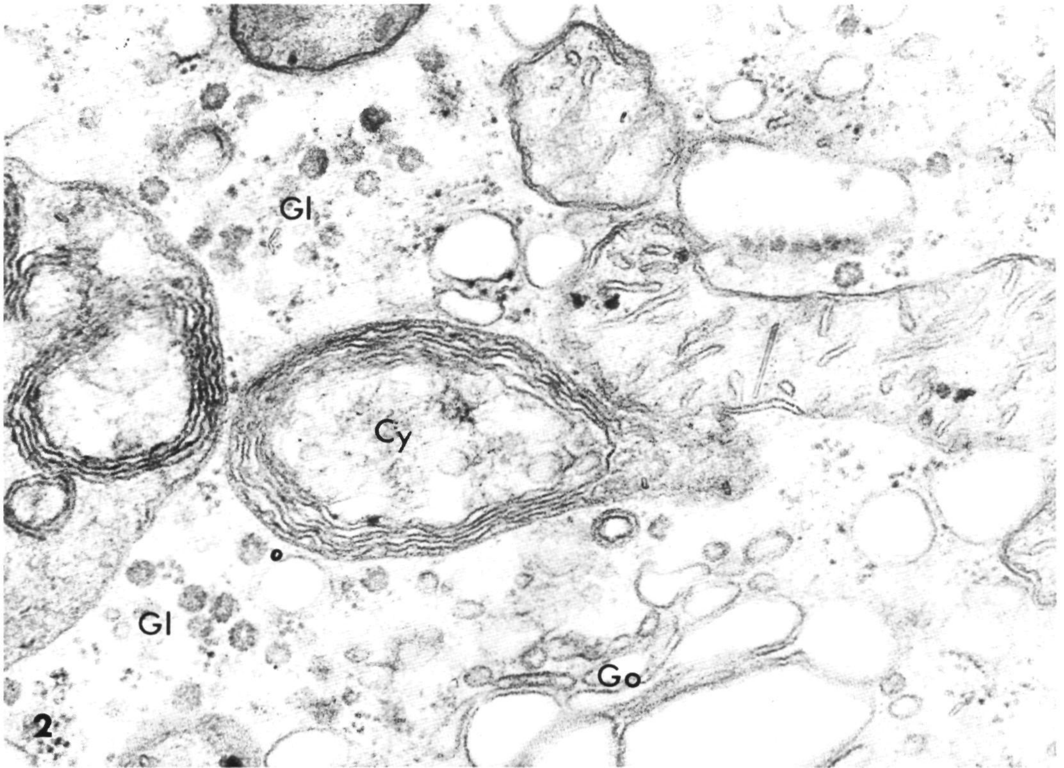
LIST OF ABBREVIATIONS

BC	Bile canaliculus
BM	Basement membrane
Ce	Centriole
Cy	Cytosome
D	Desmosome
E	Excretory granule
ER	Endoplasmic reticulum
Gl	Glycogen
Go	Golgi complex
Gr	Granule
Hep	Hepatocyte
M	Mitochondrion
$\underline{M}$	M line
Mb	Microbody
Mv	Microvillus
MVB	Multivesicular body
Meg	Megakaryocyte
N	Nucleus
PM	Plasma membrane
R	Ribosome
RER	Rough endoplasmic reticulum
SER	Smooth endoplasmic reticulum
SR	Sarcoplasmic reticulum
T	Tonofilament
TW	Terminal web
$\underline{Z}$	Z line

TECHNICAL DATA

Ep	Epon
Glu	Glutaraldehyde
LH	Lead hydroxide
Meth	Methacrylate
Os	Osmiumtetroxide
PAS	Periodic acid Schiff
UA	Uranyl acetate

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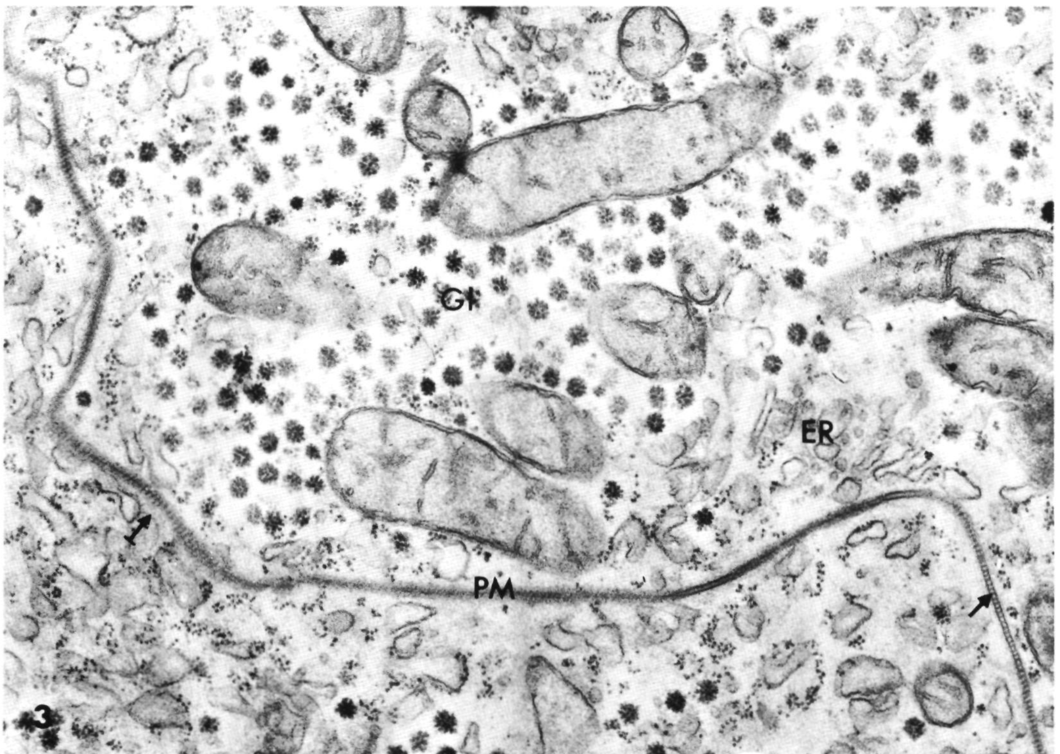
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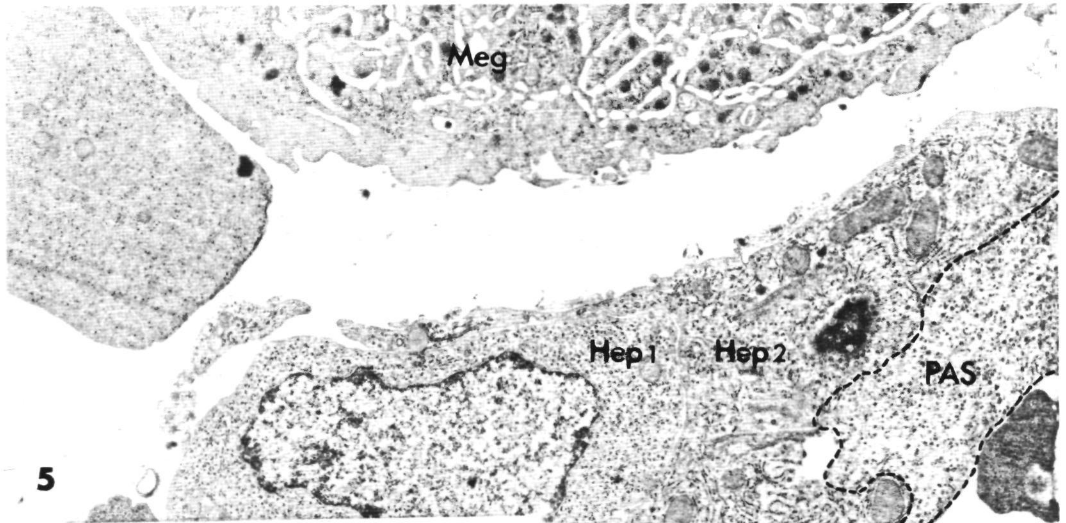
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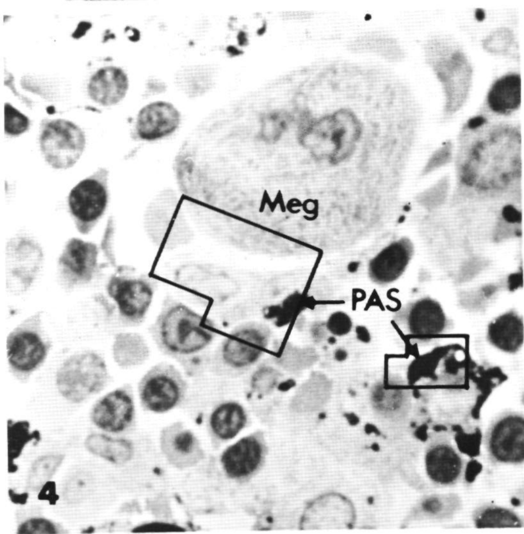
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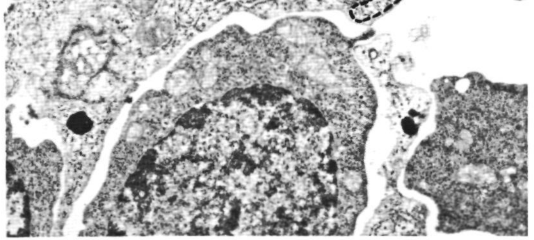

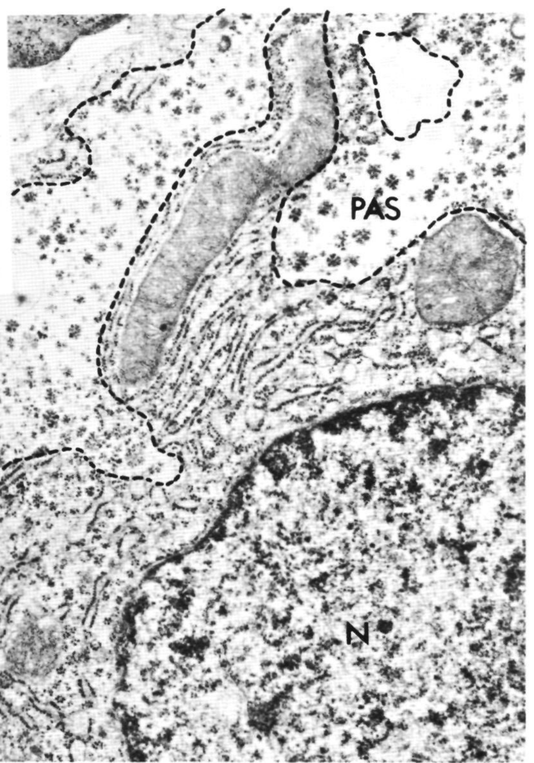
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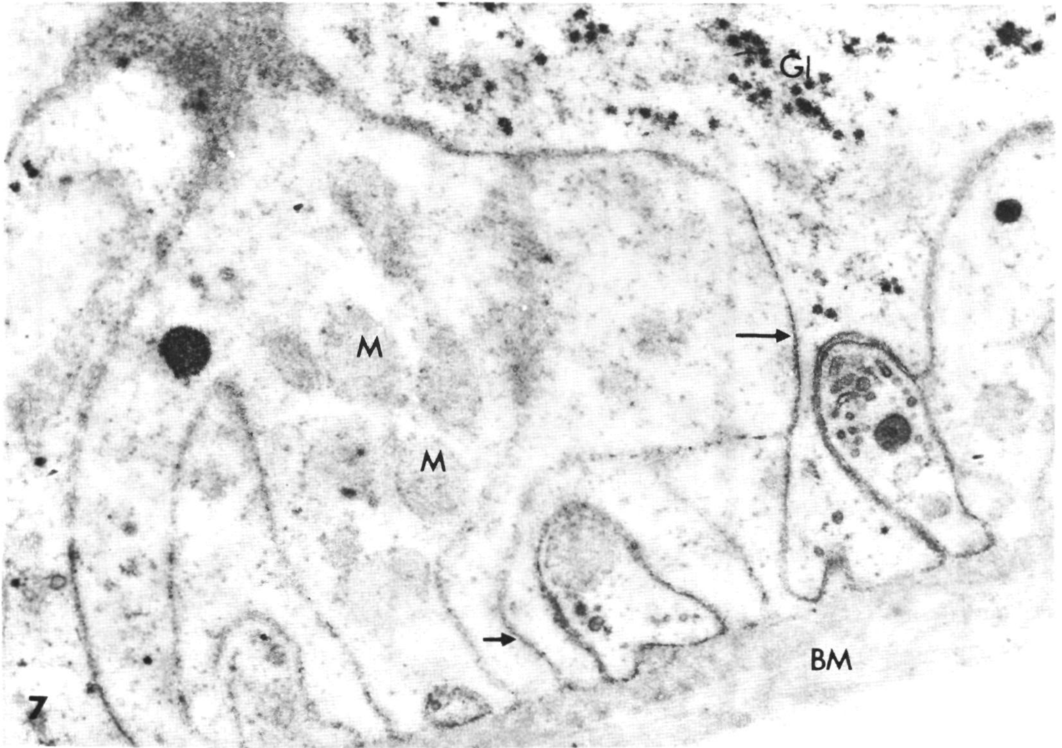
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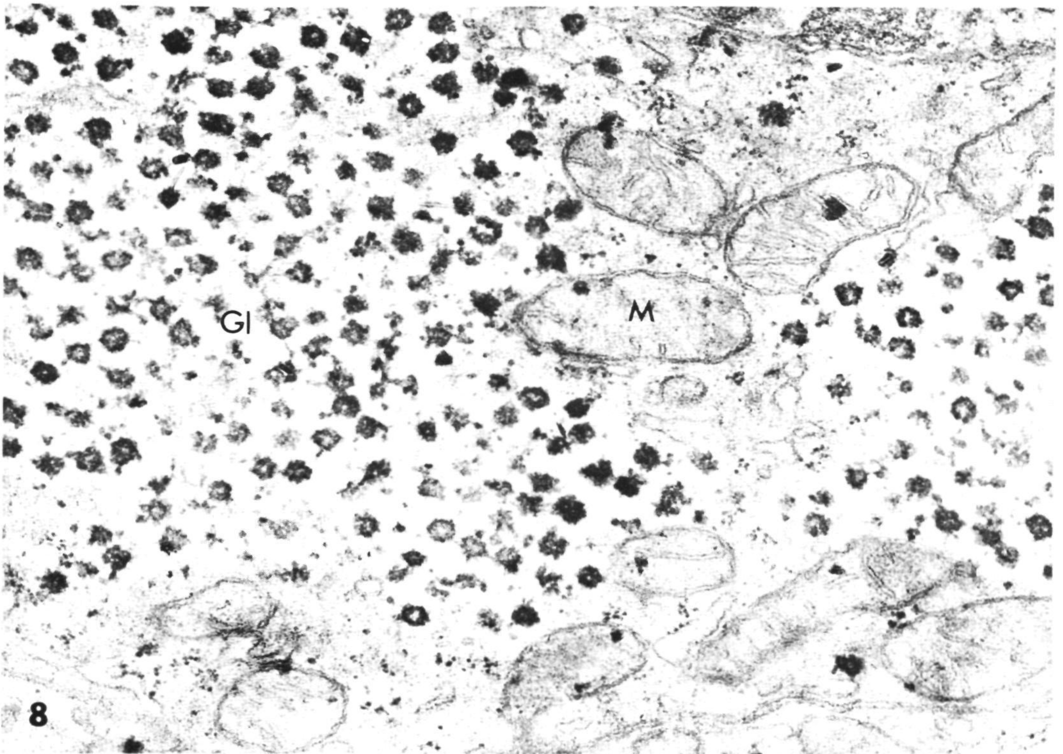
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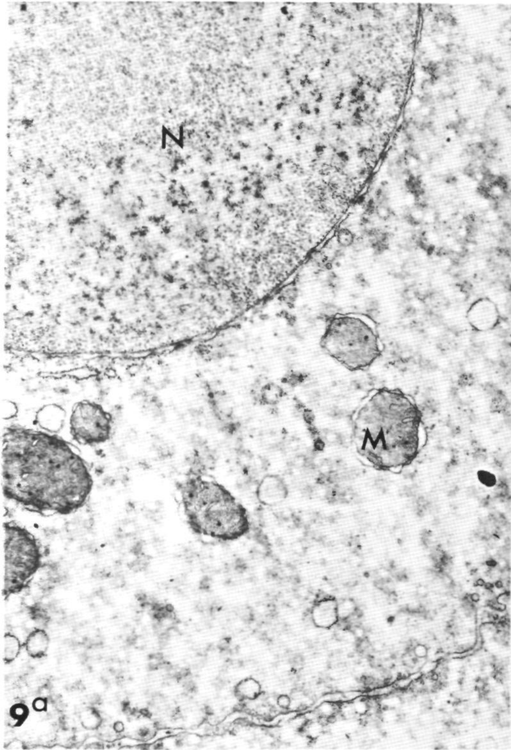
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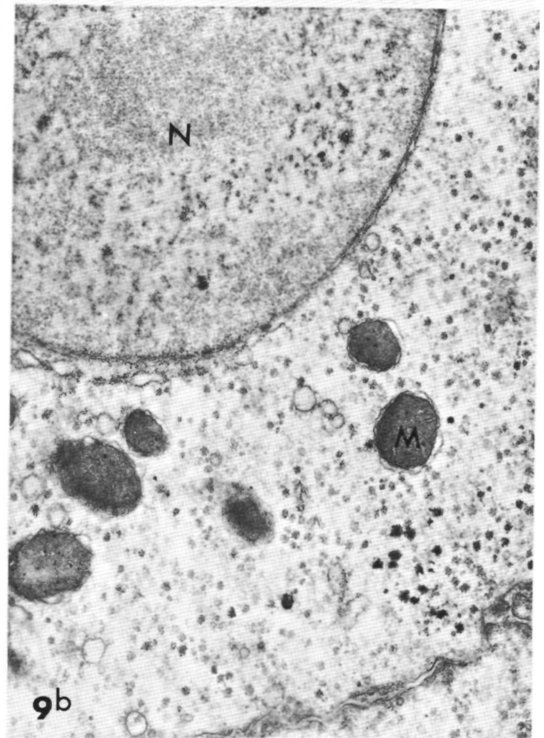
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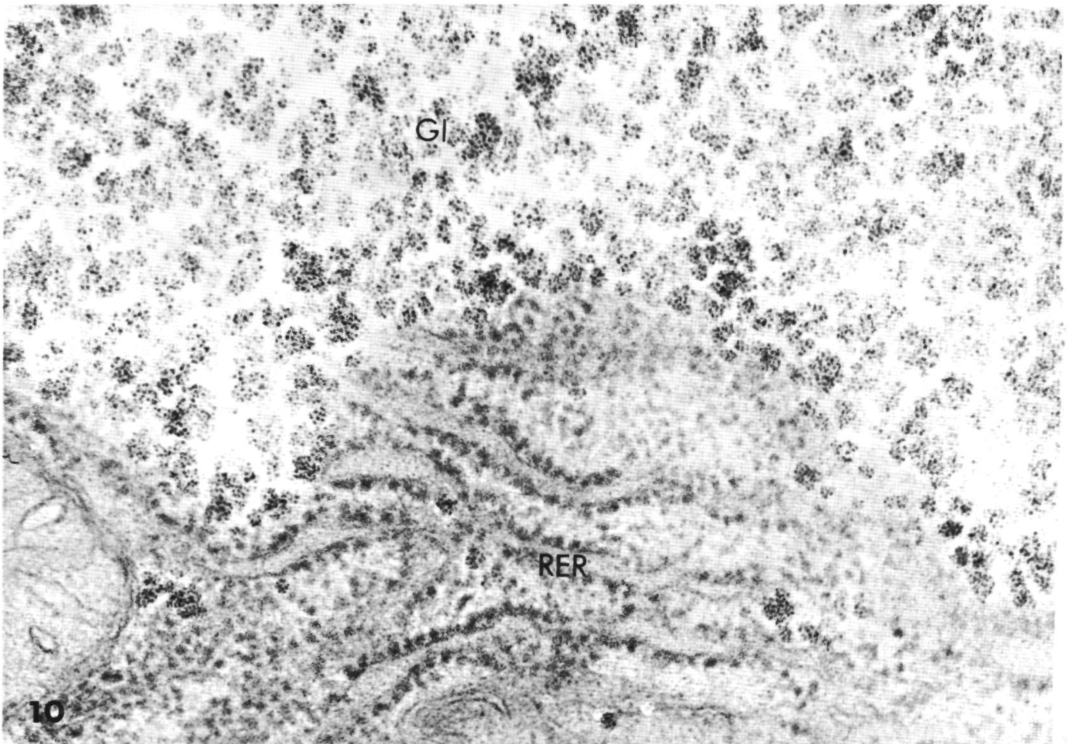
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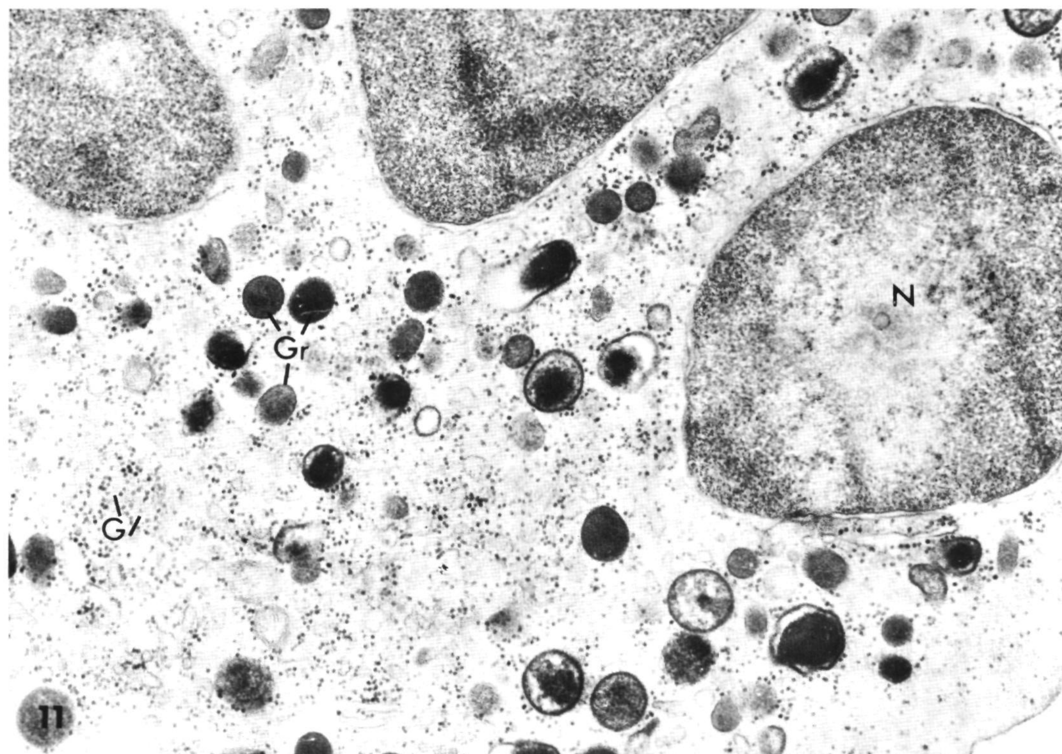
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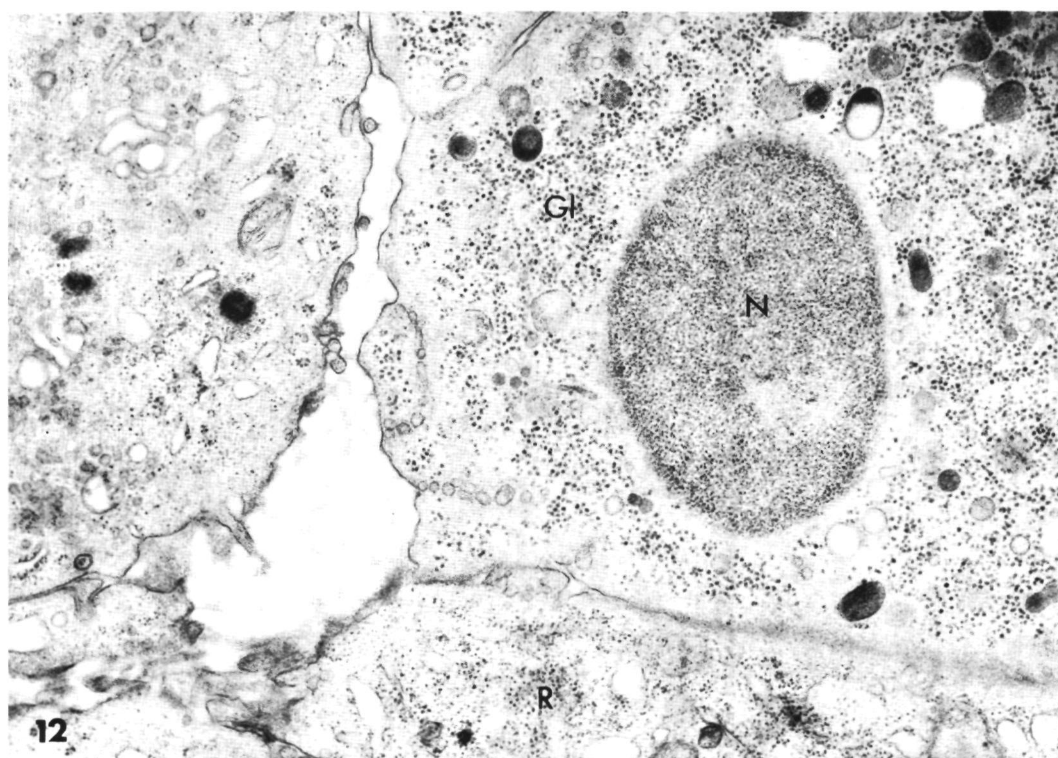
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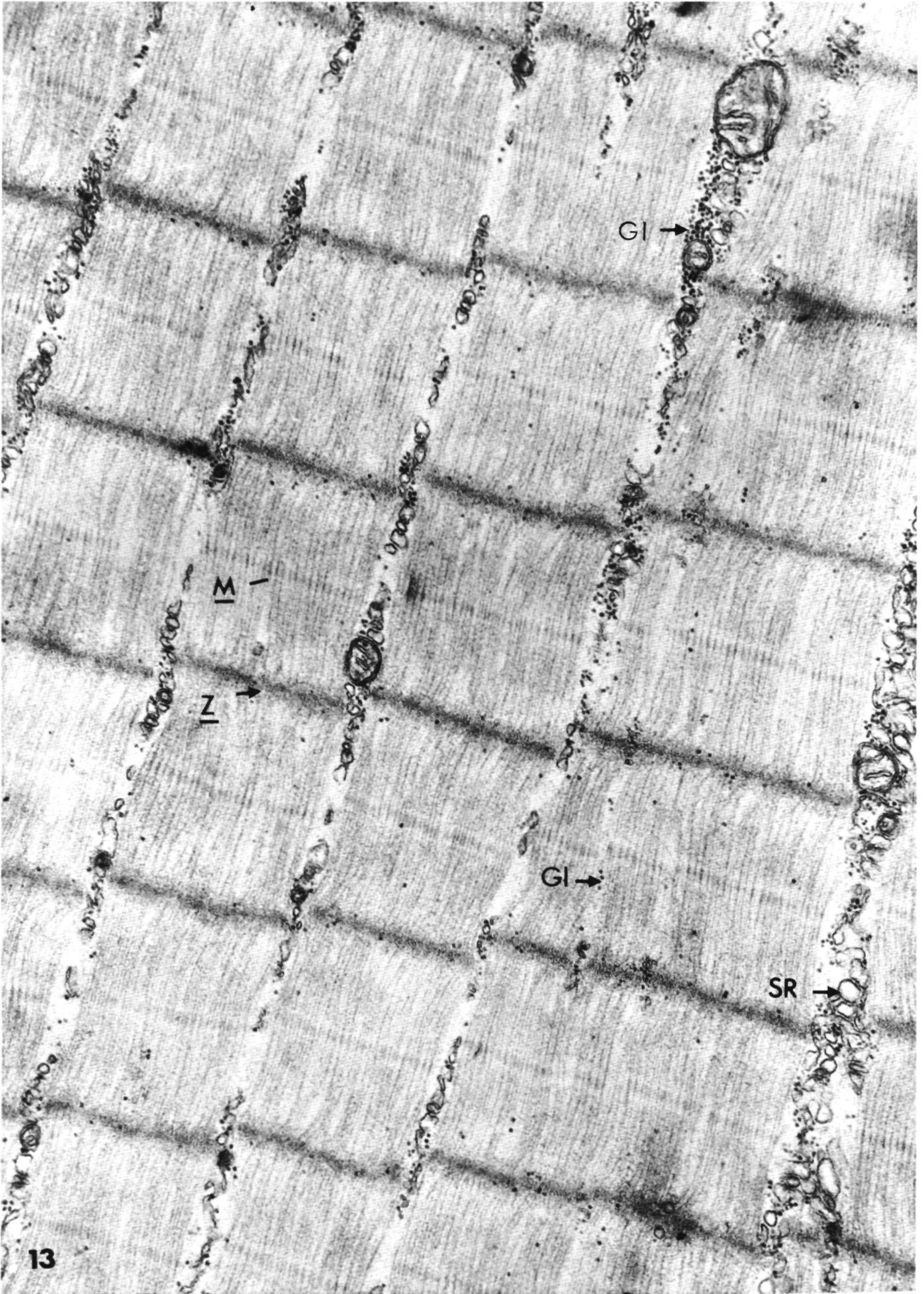
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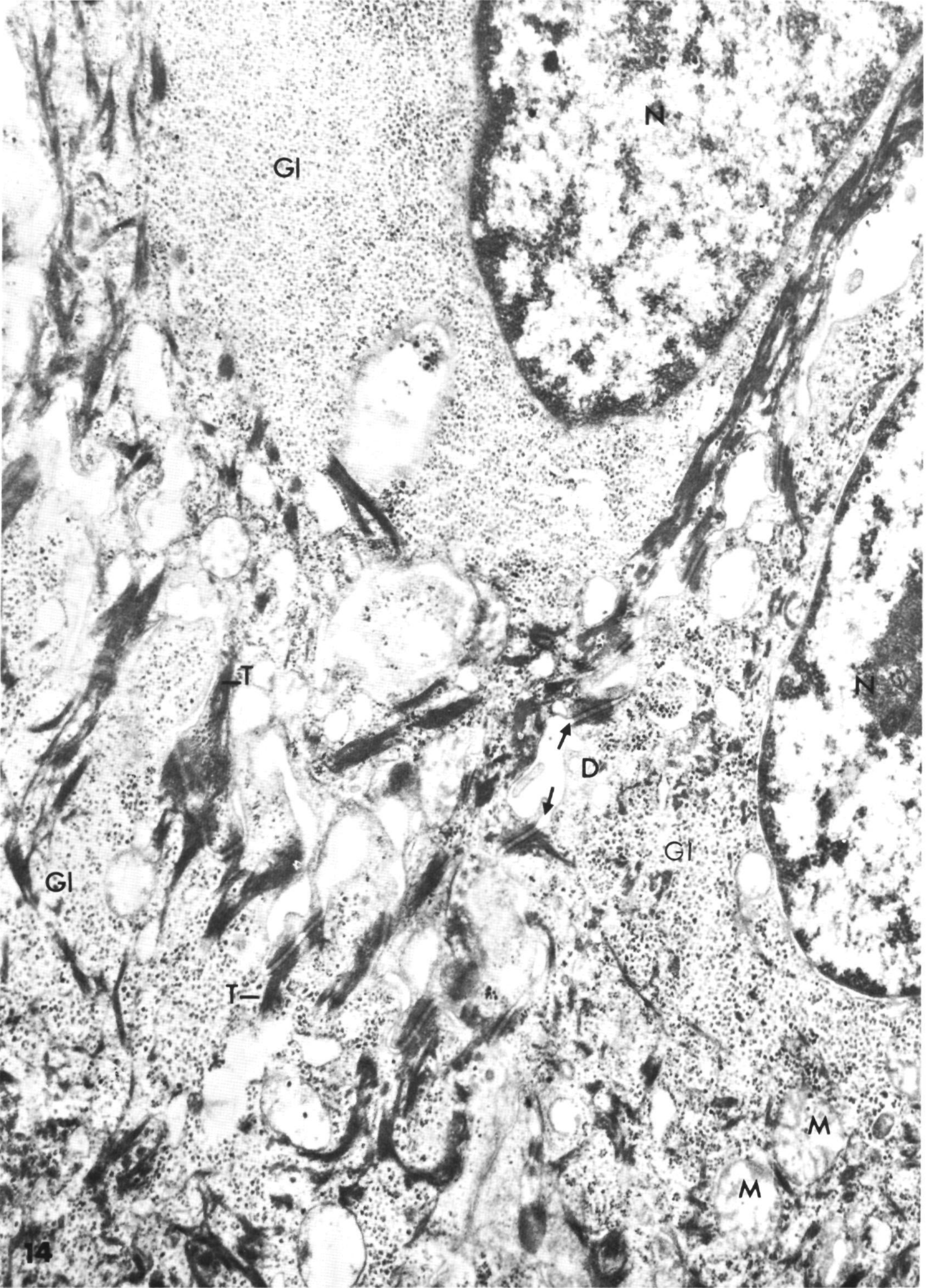
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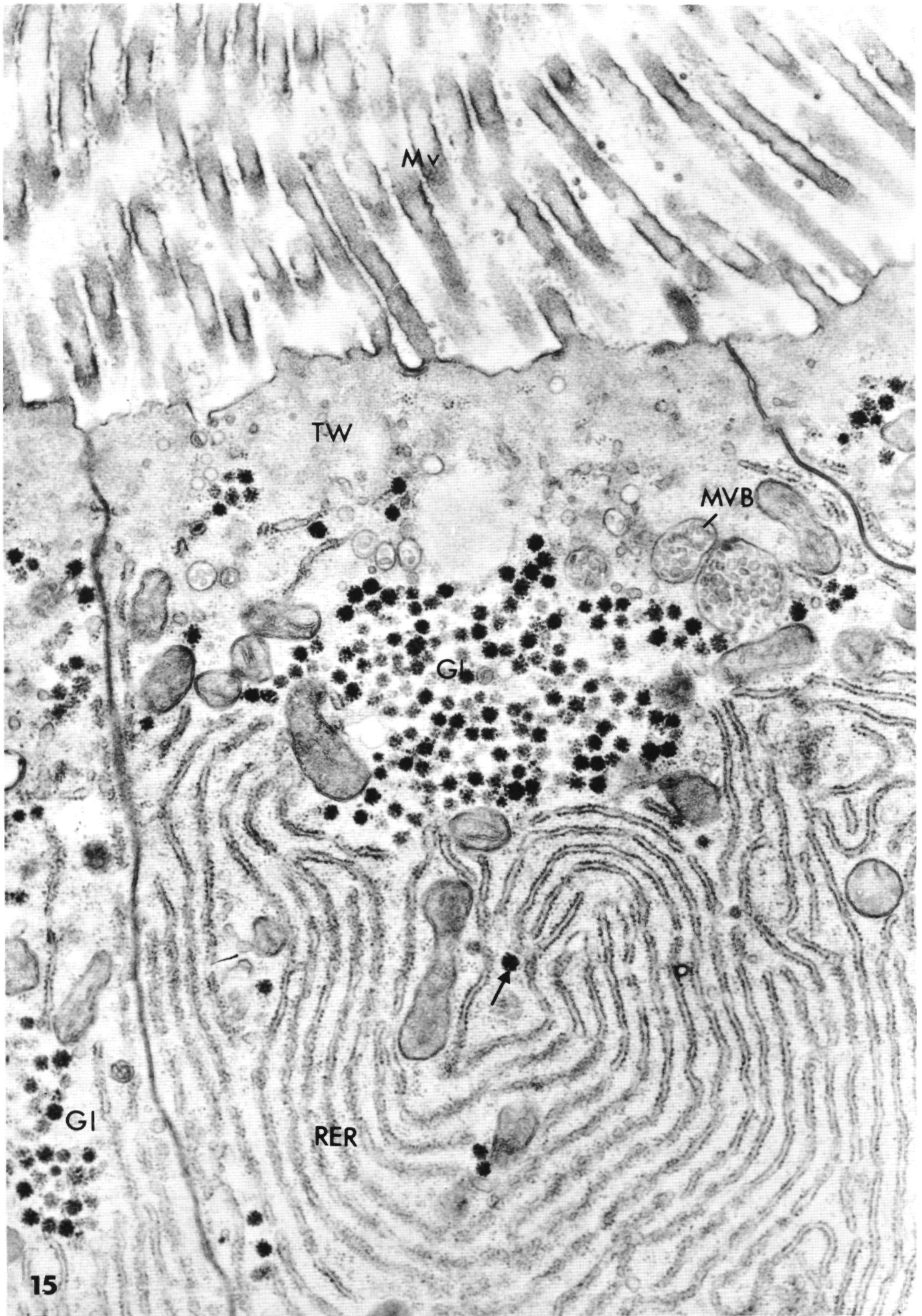
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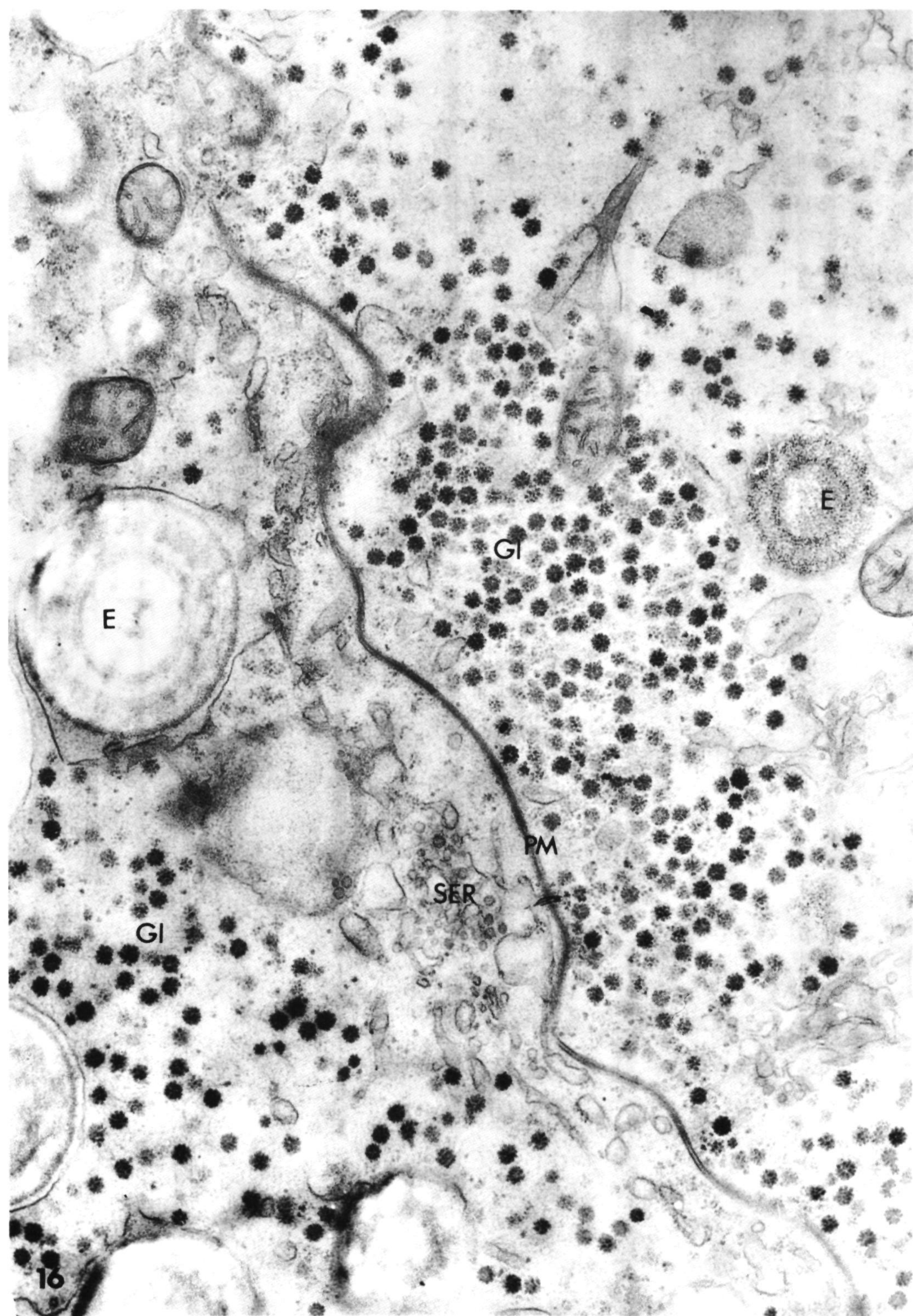
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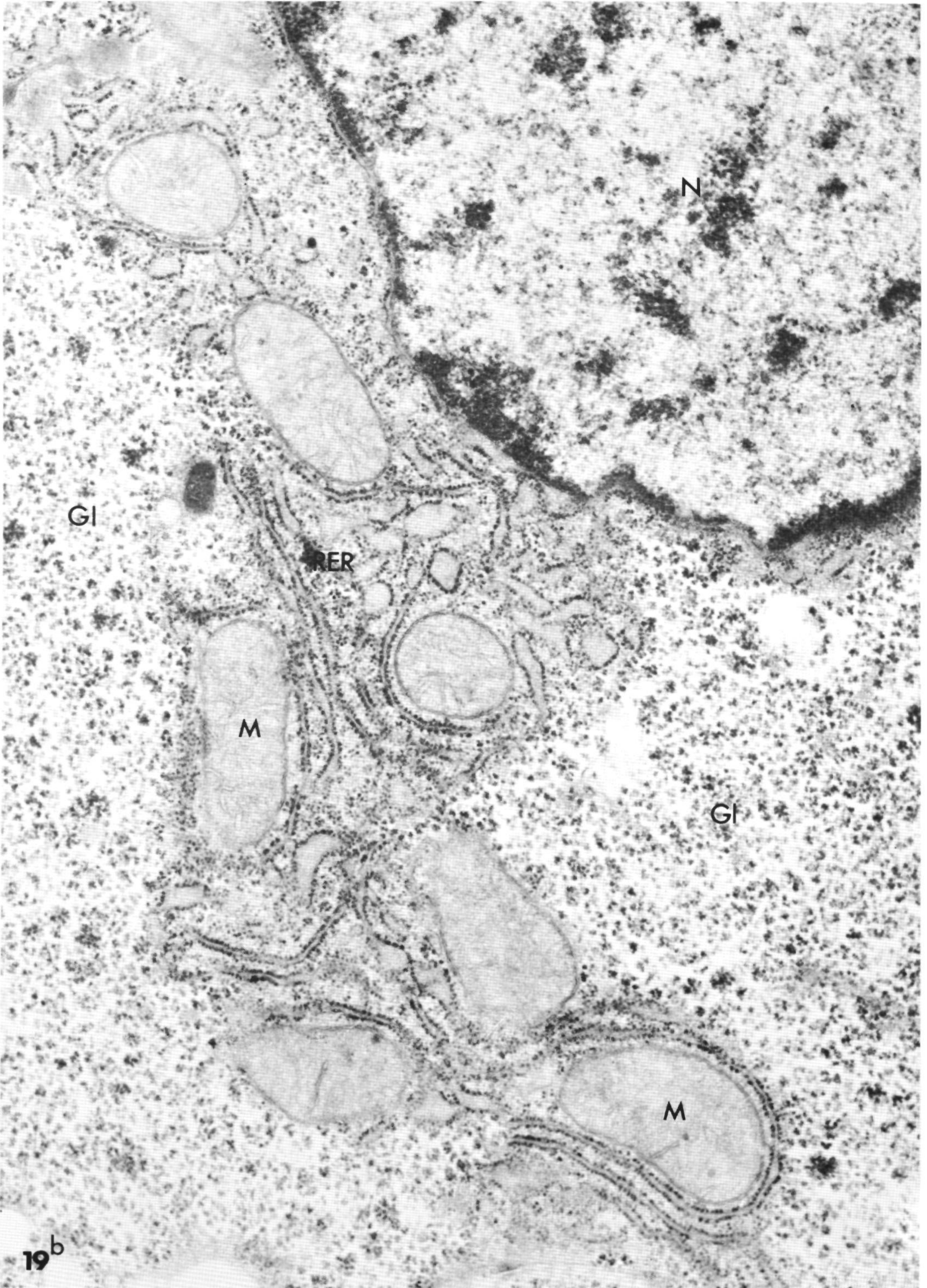
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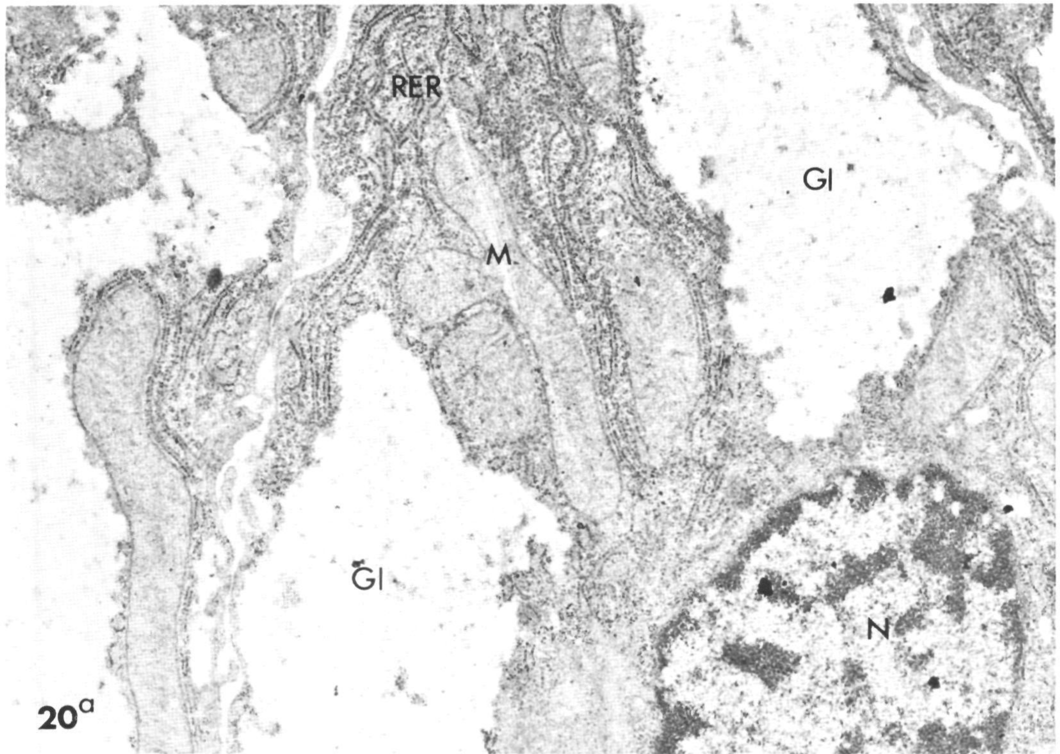
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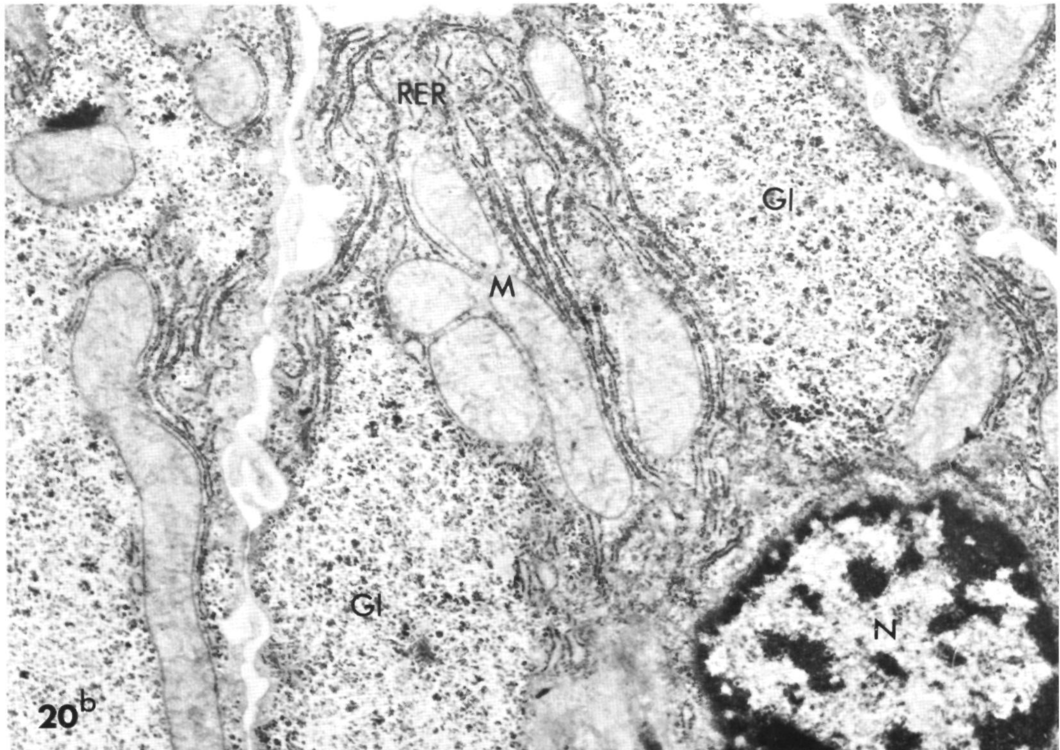
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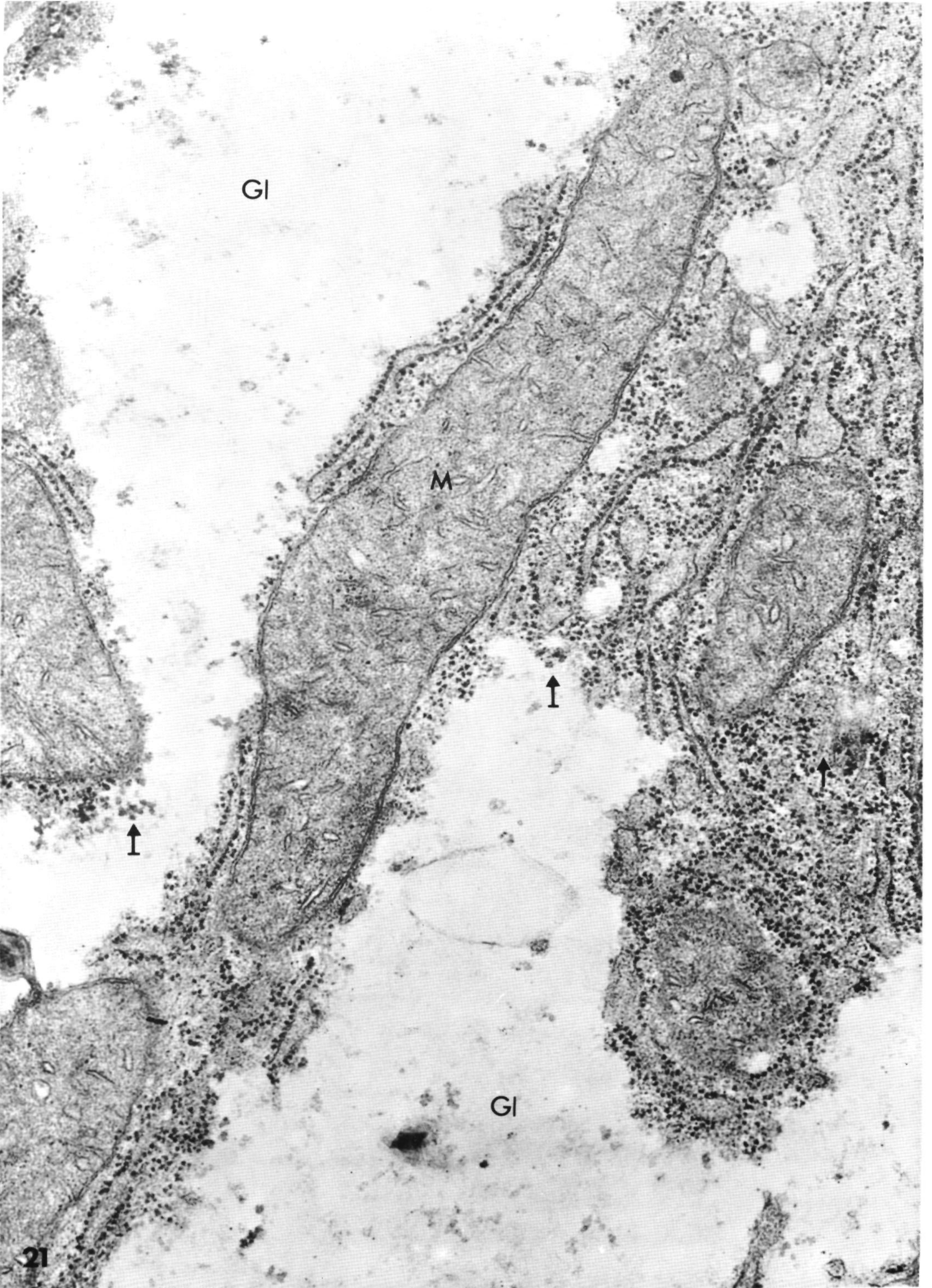
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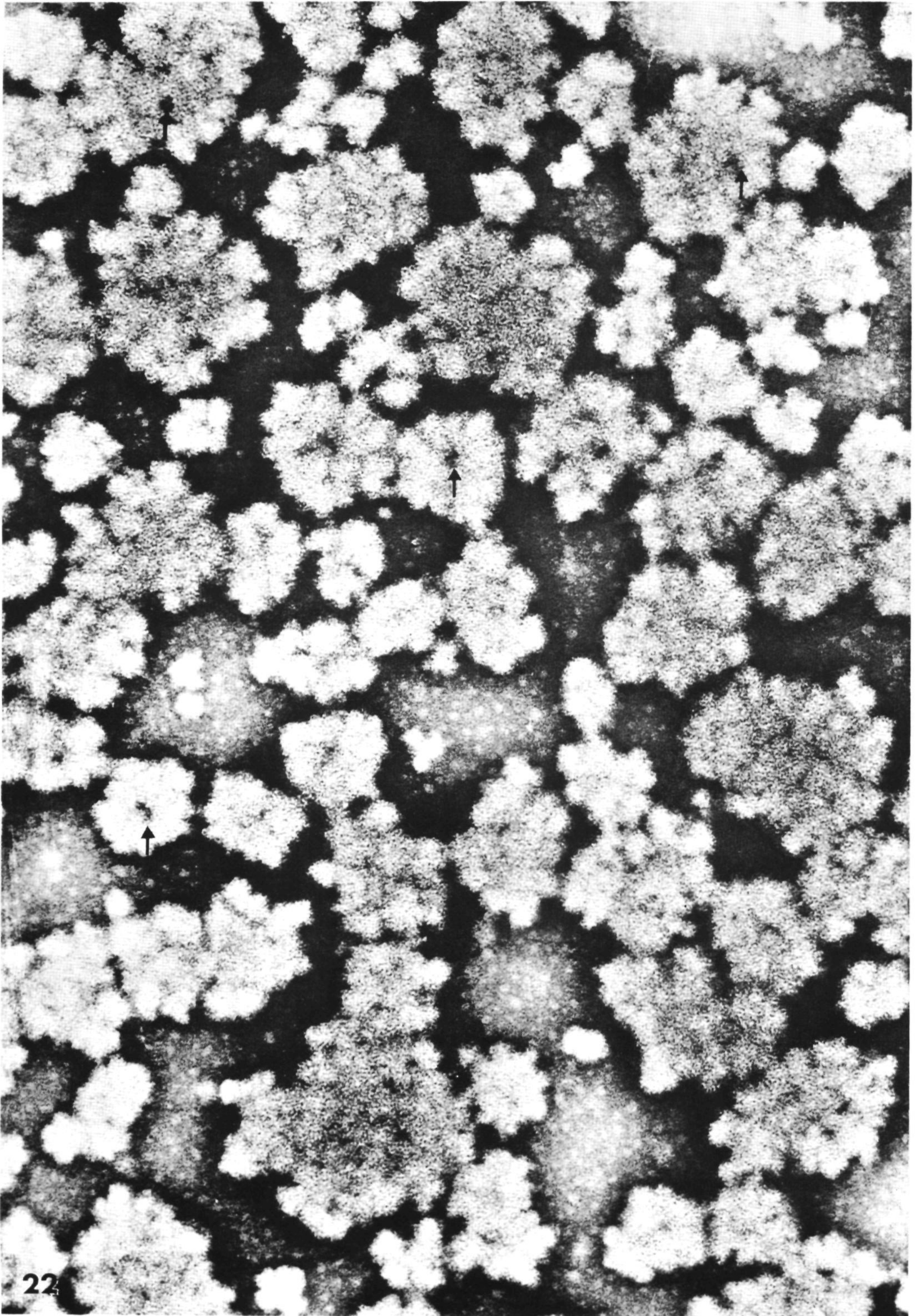
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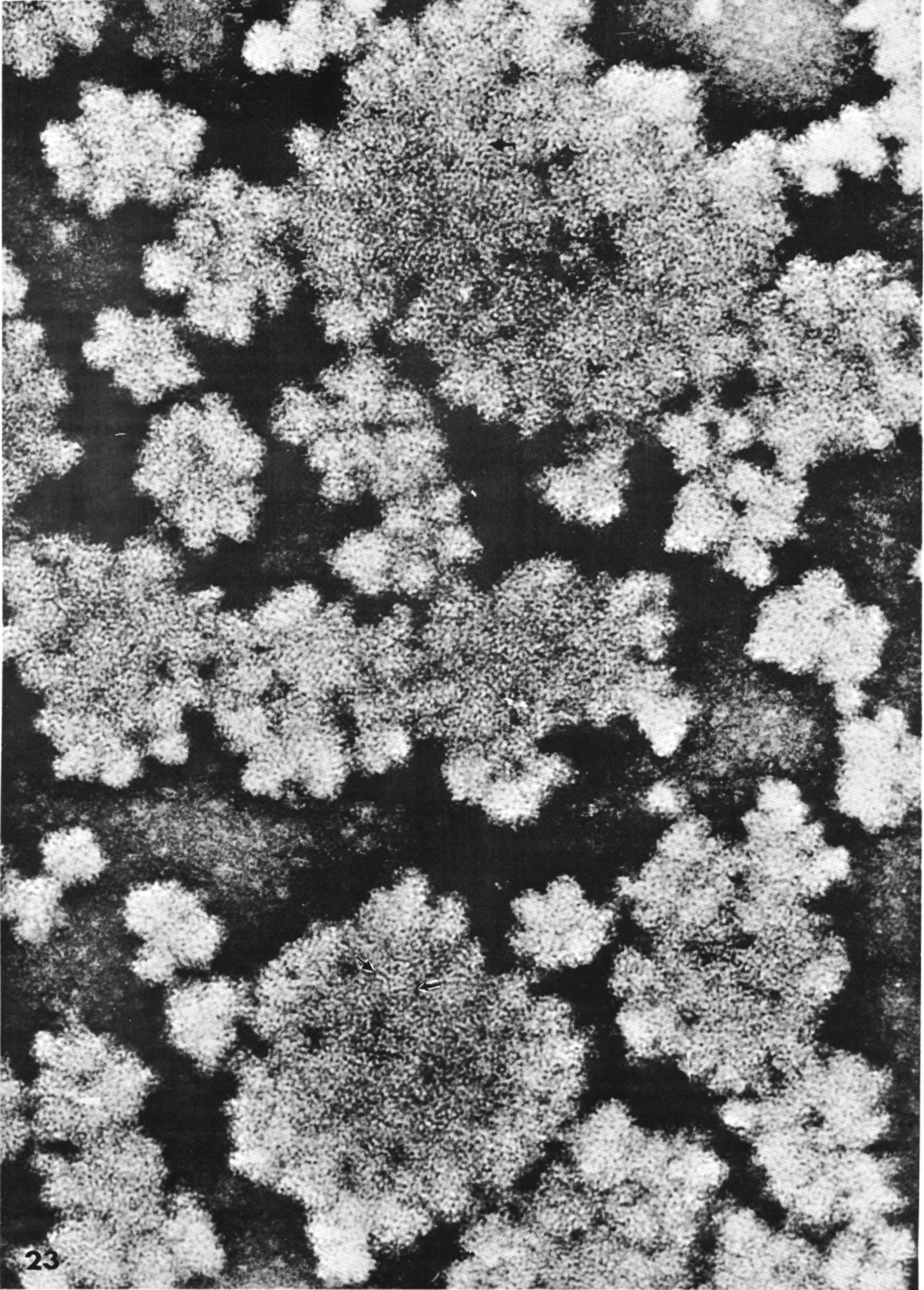
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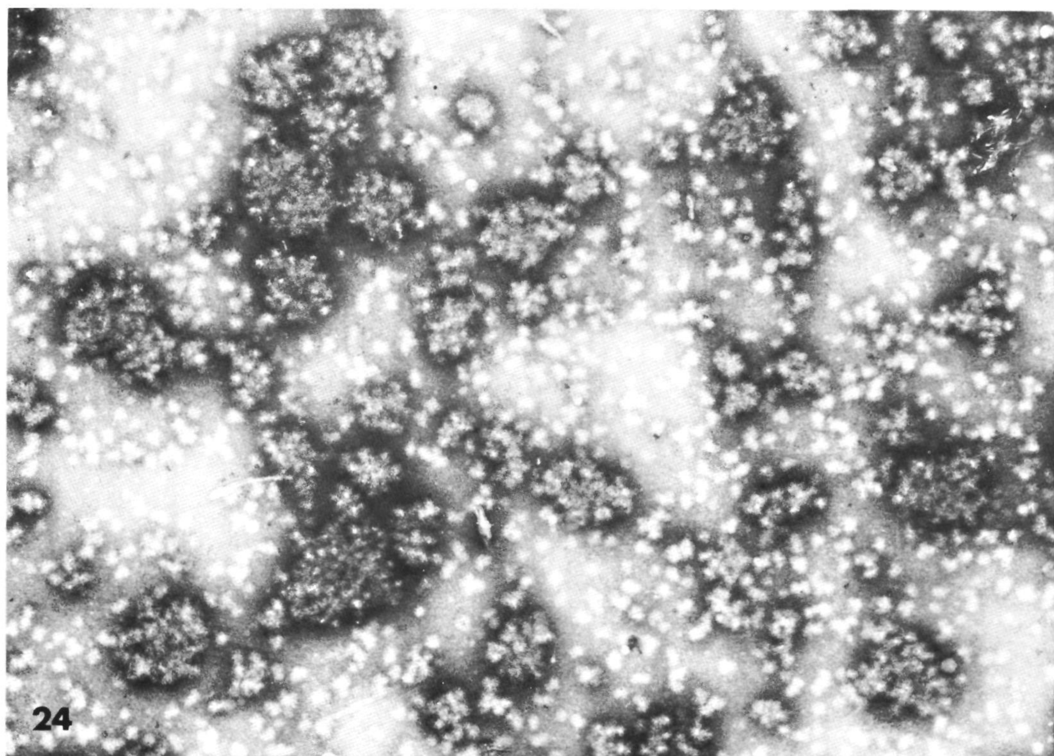
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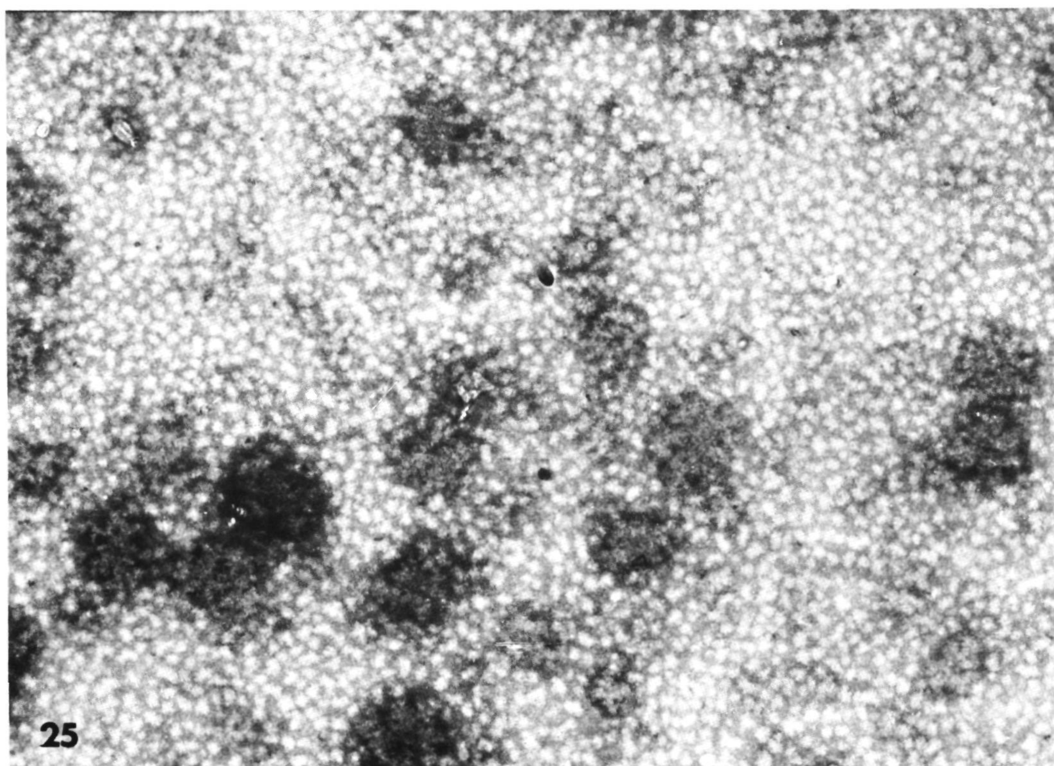
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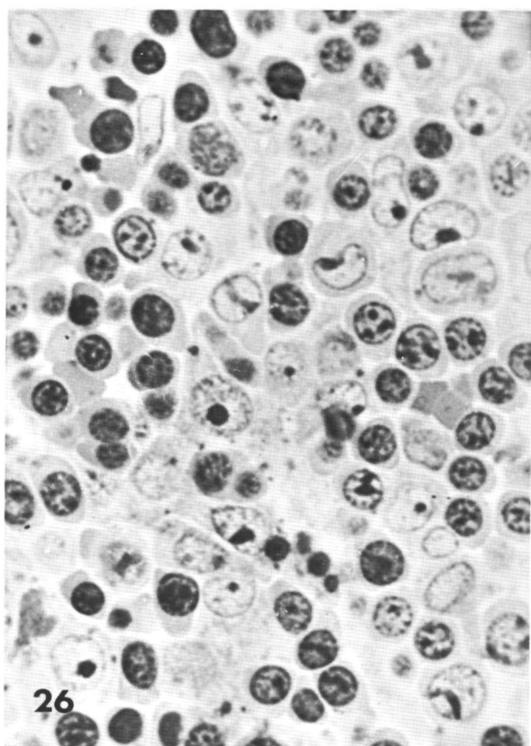
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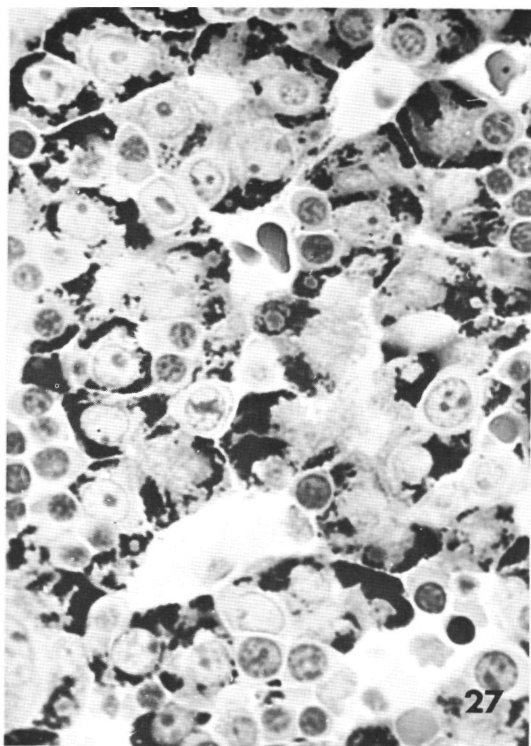
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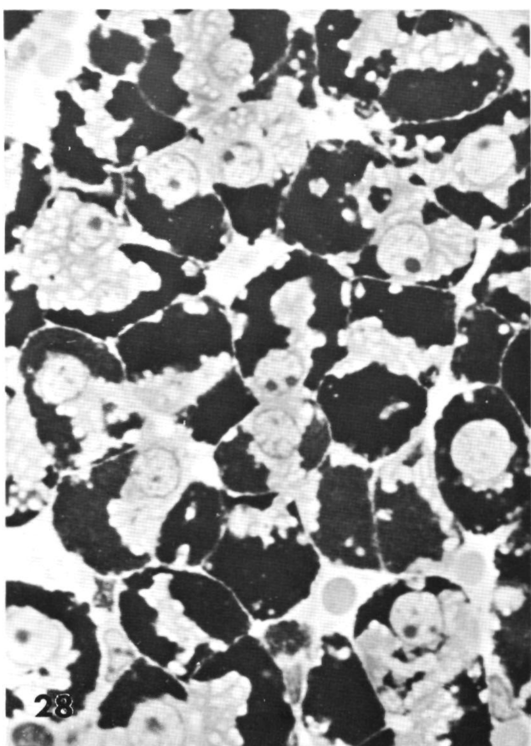
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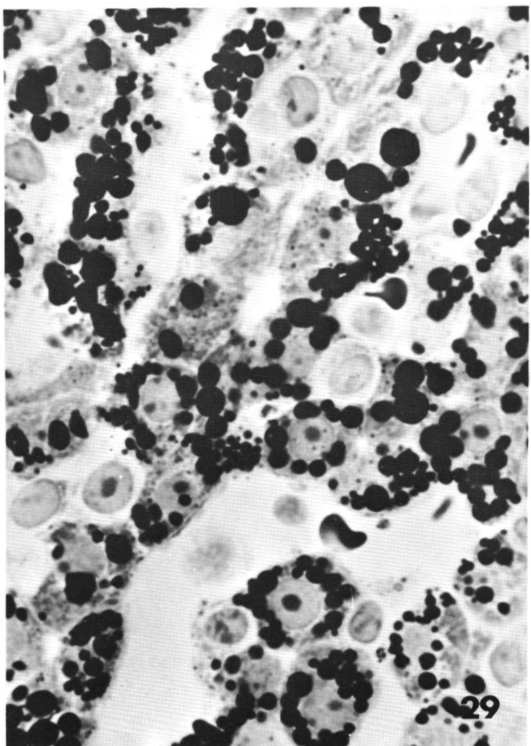
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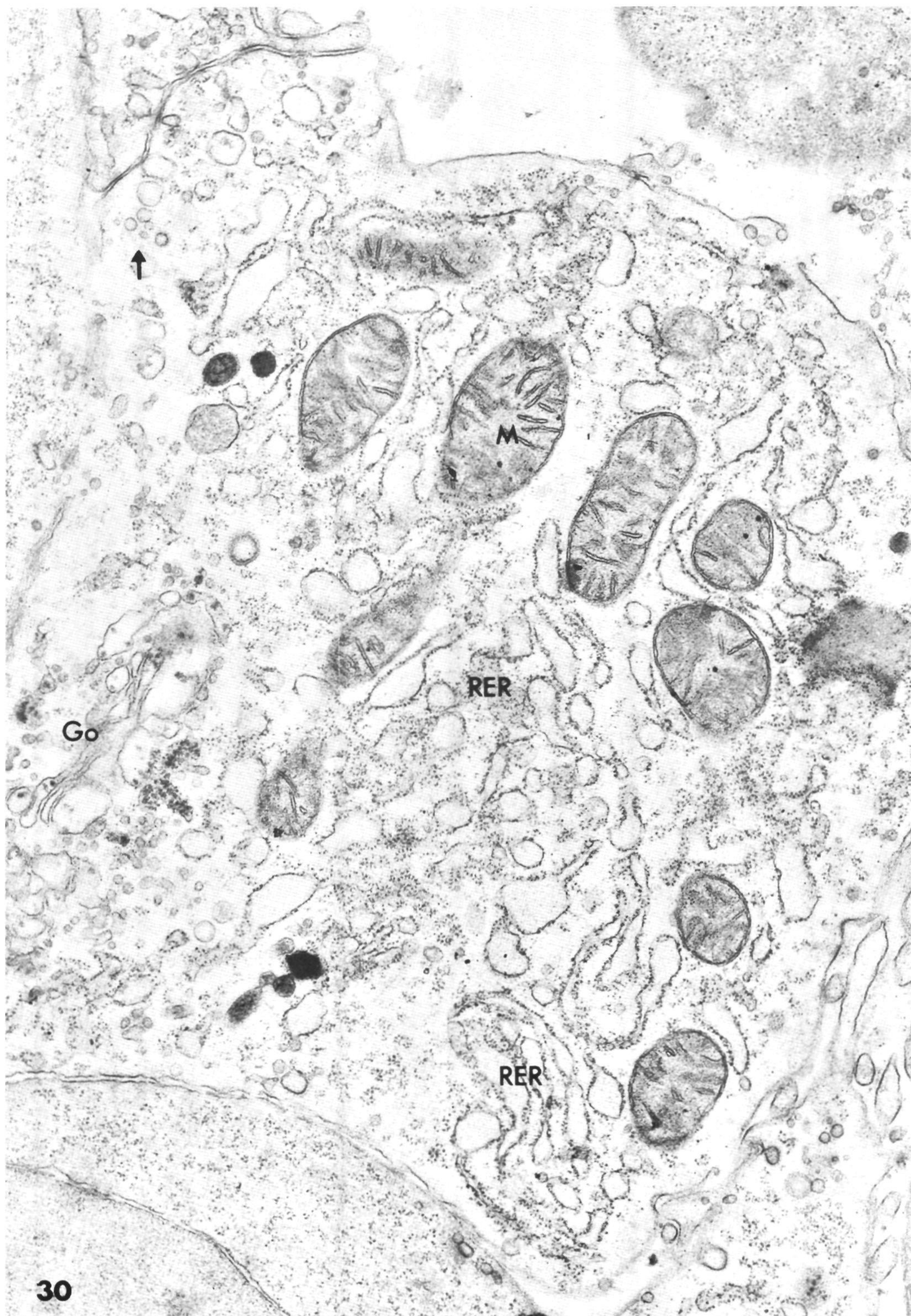
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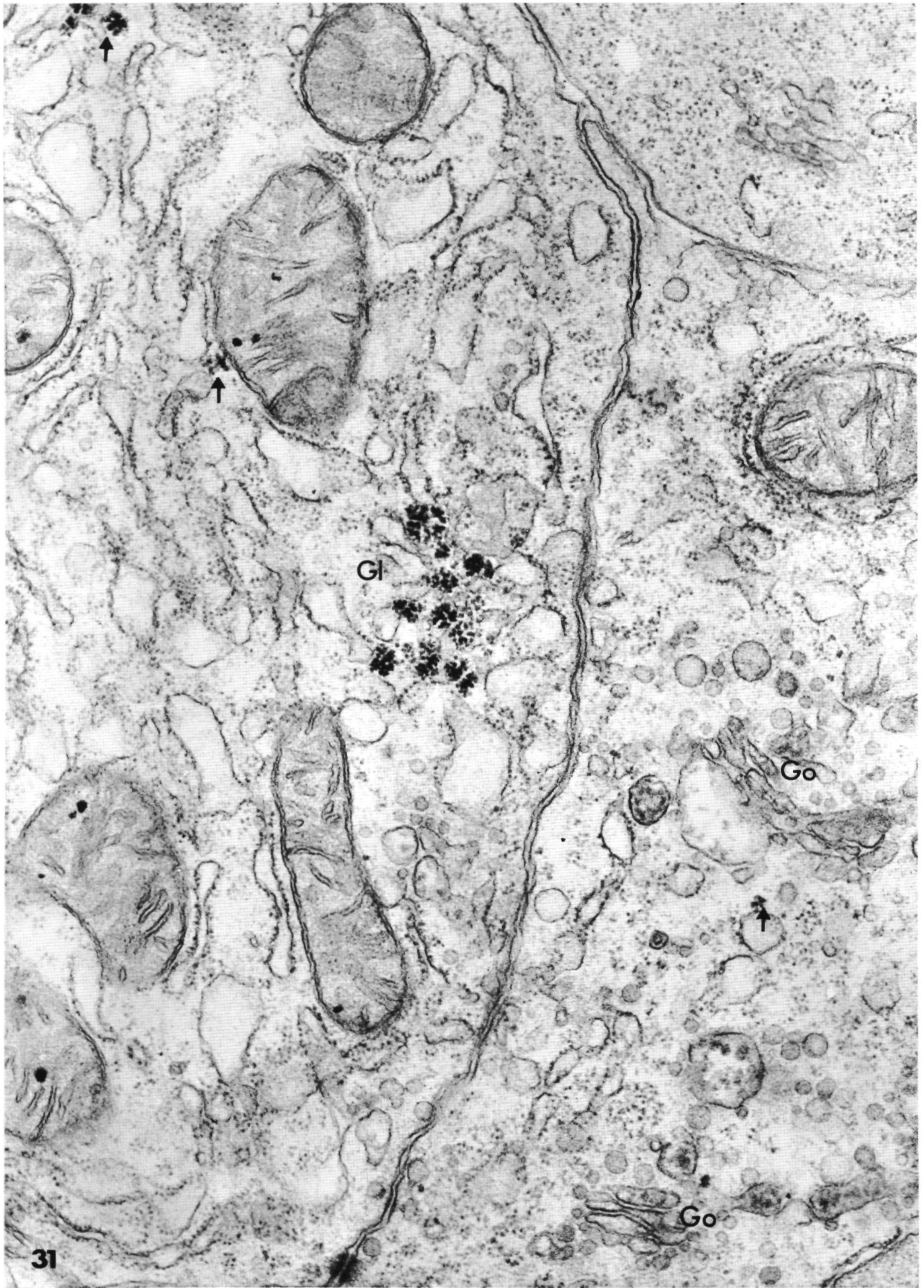
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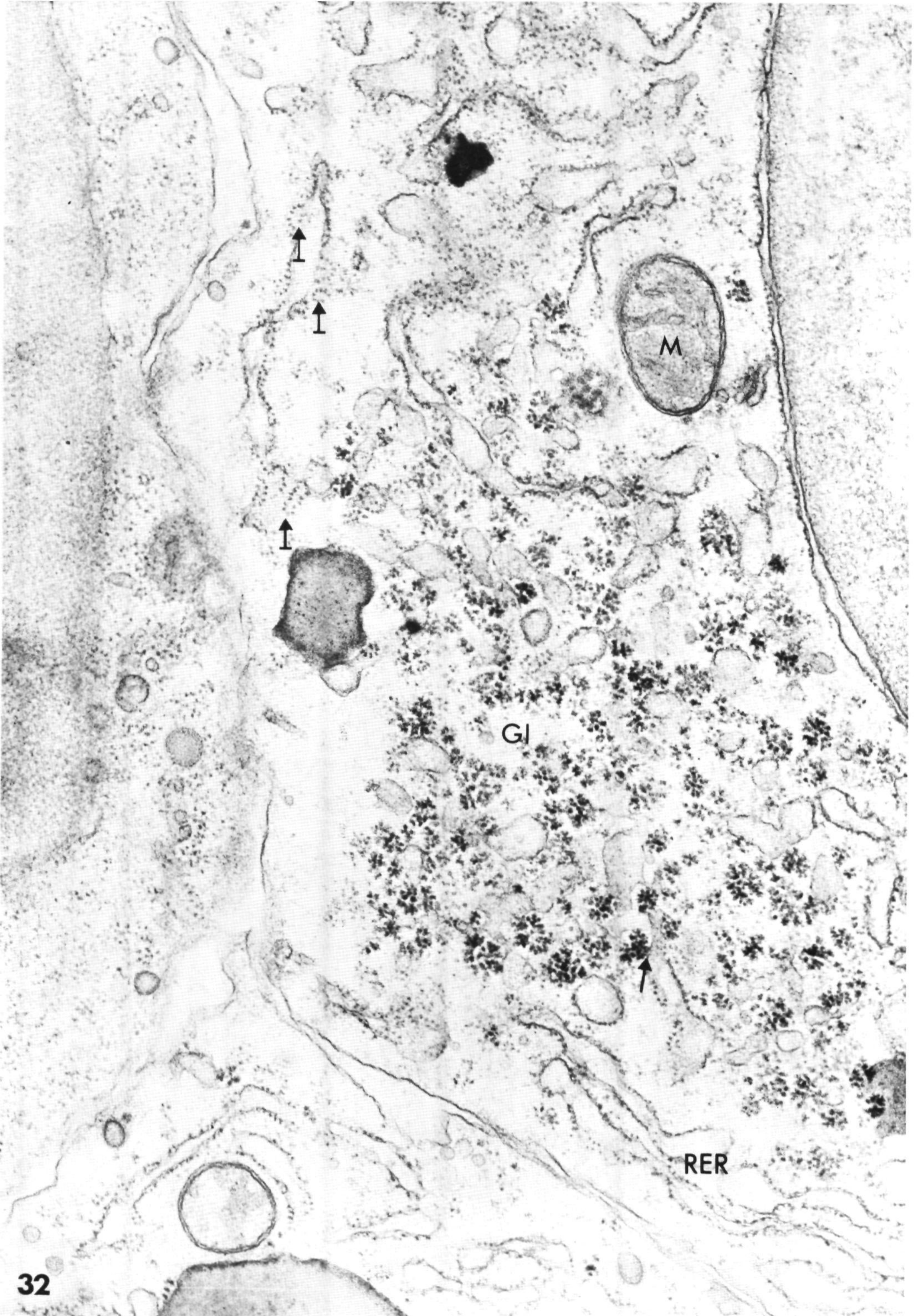
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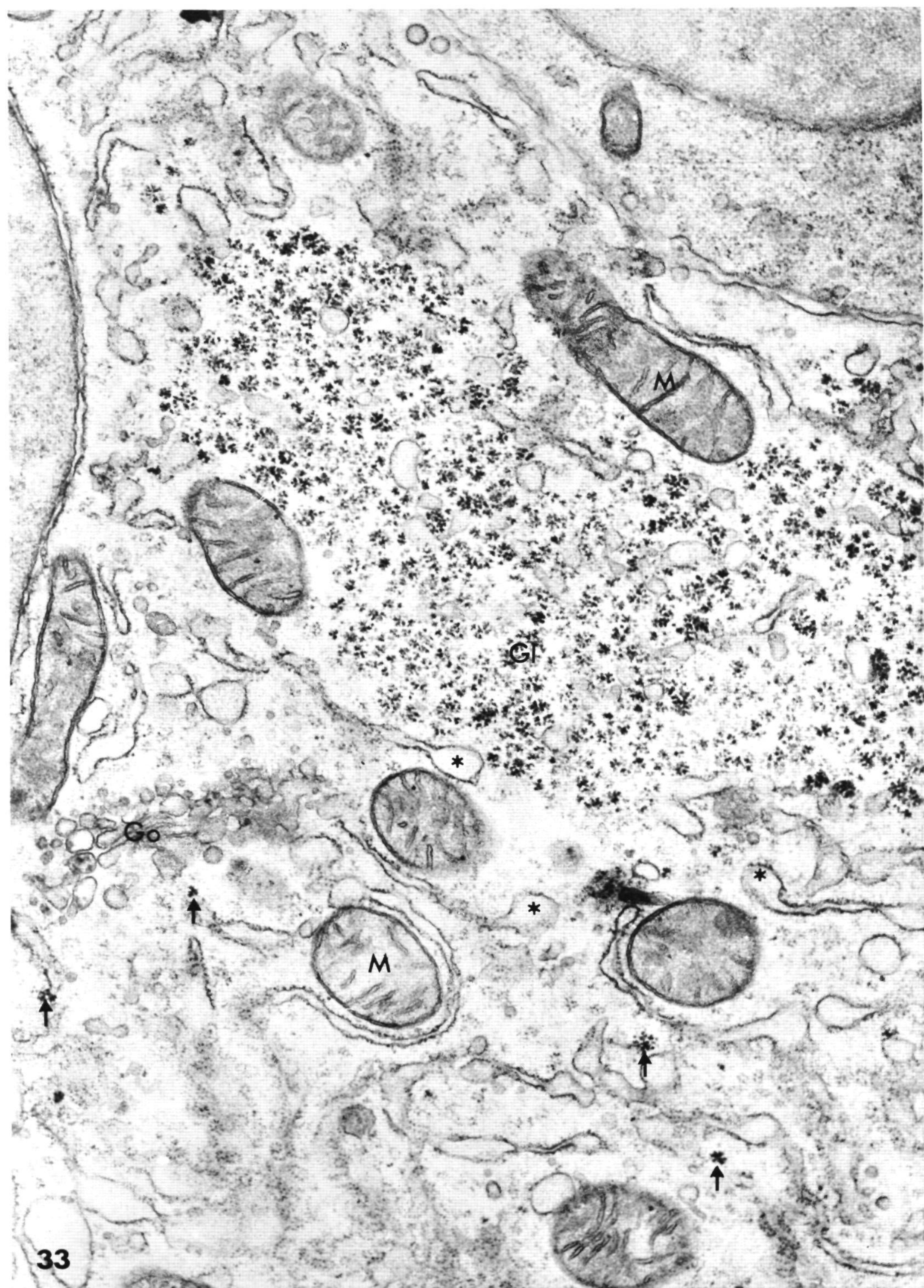
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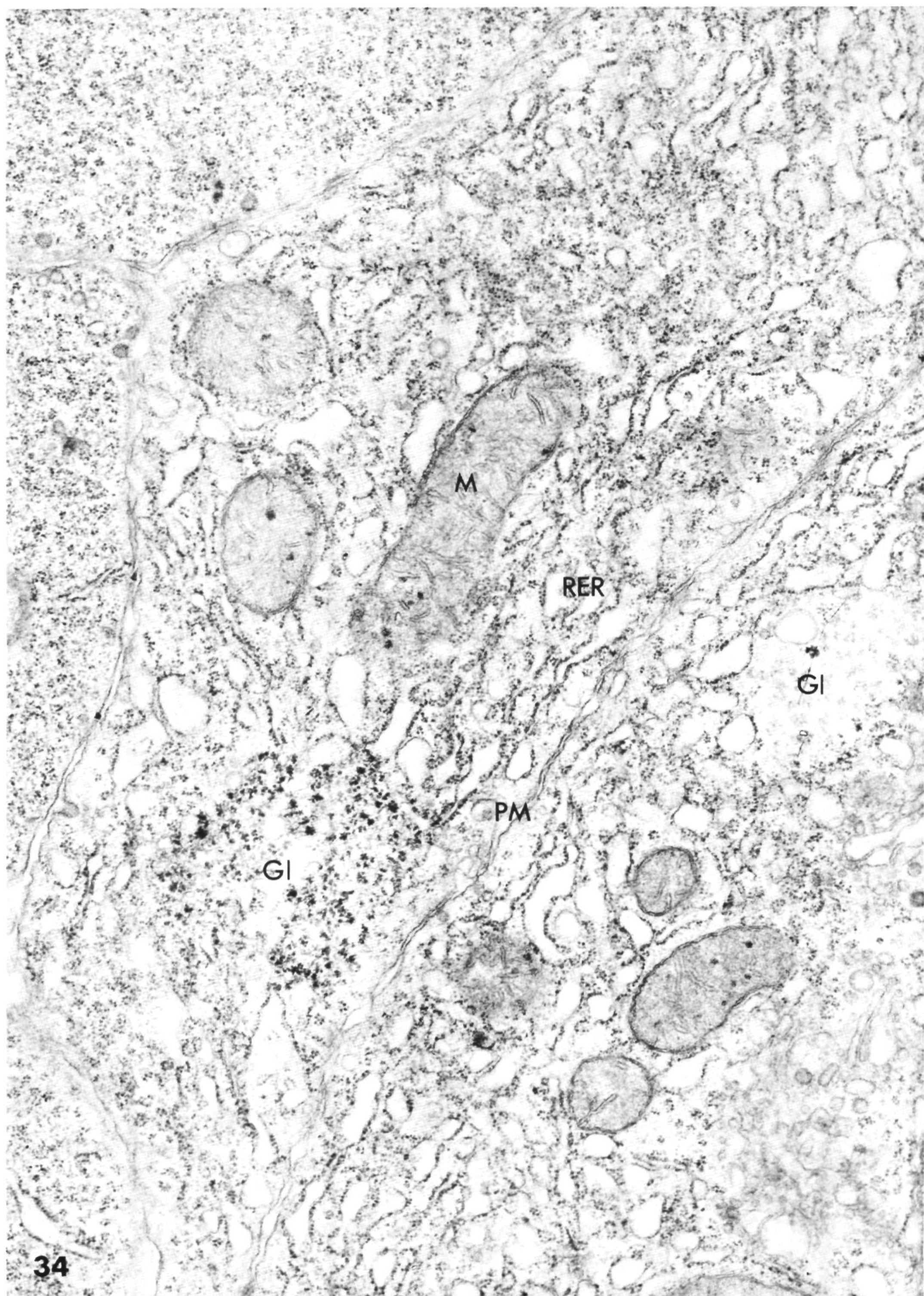
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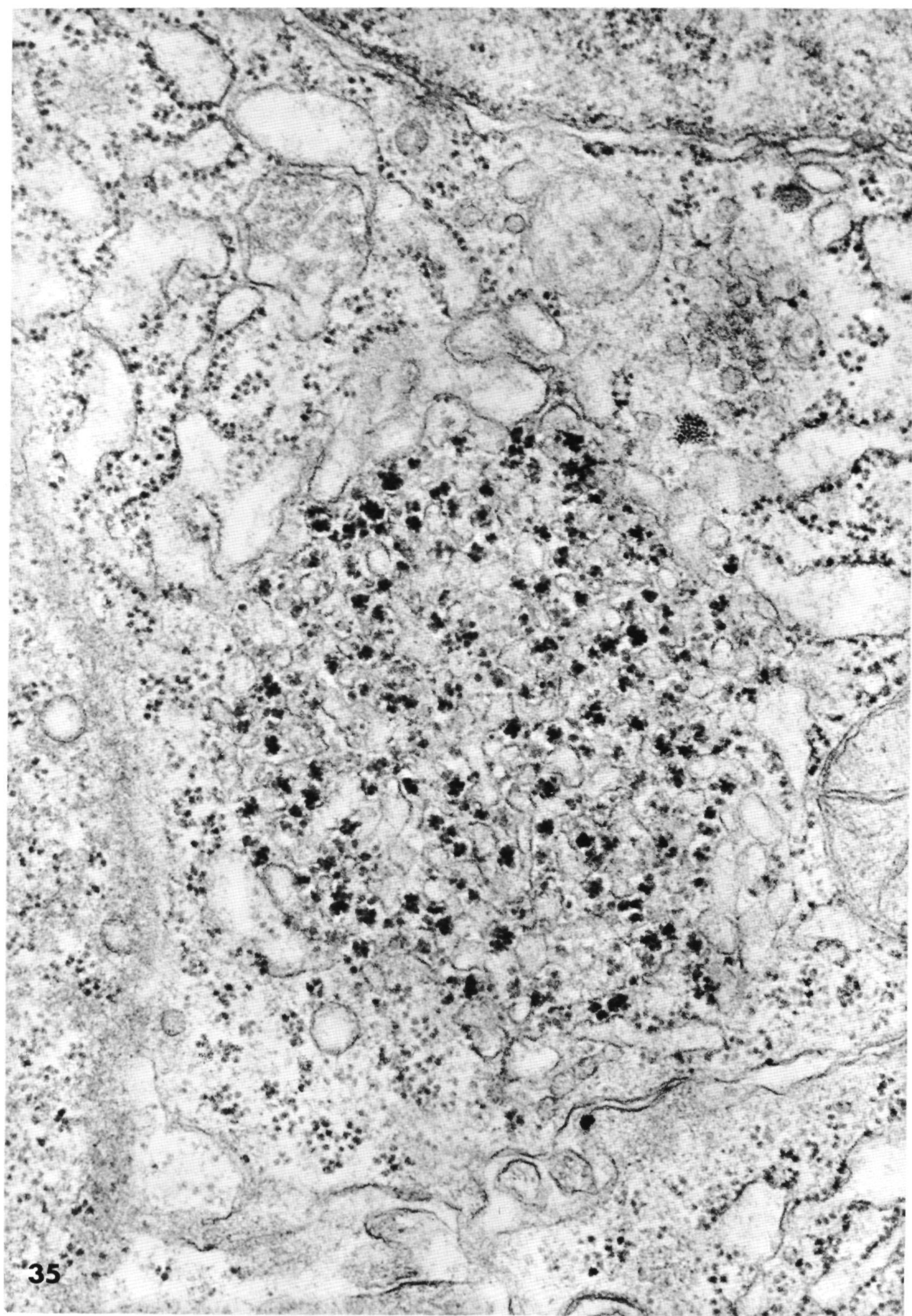
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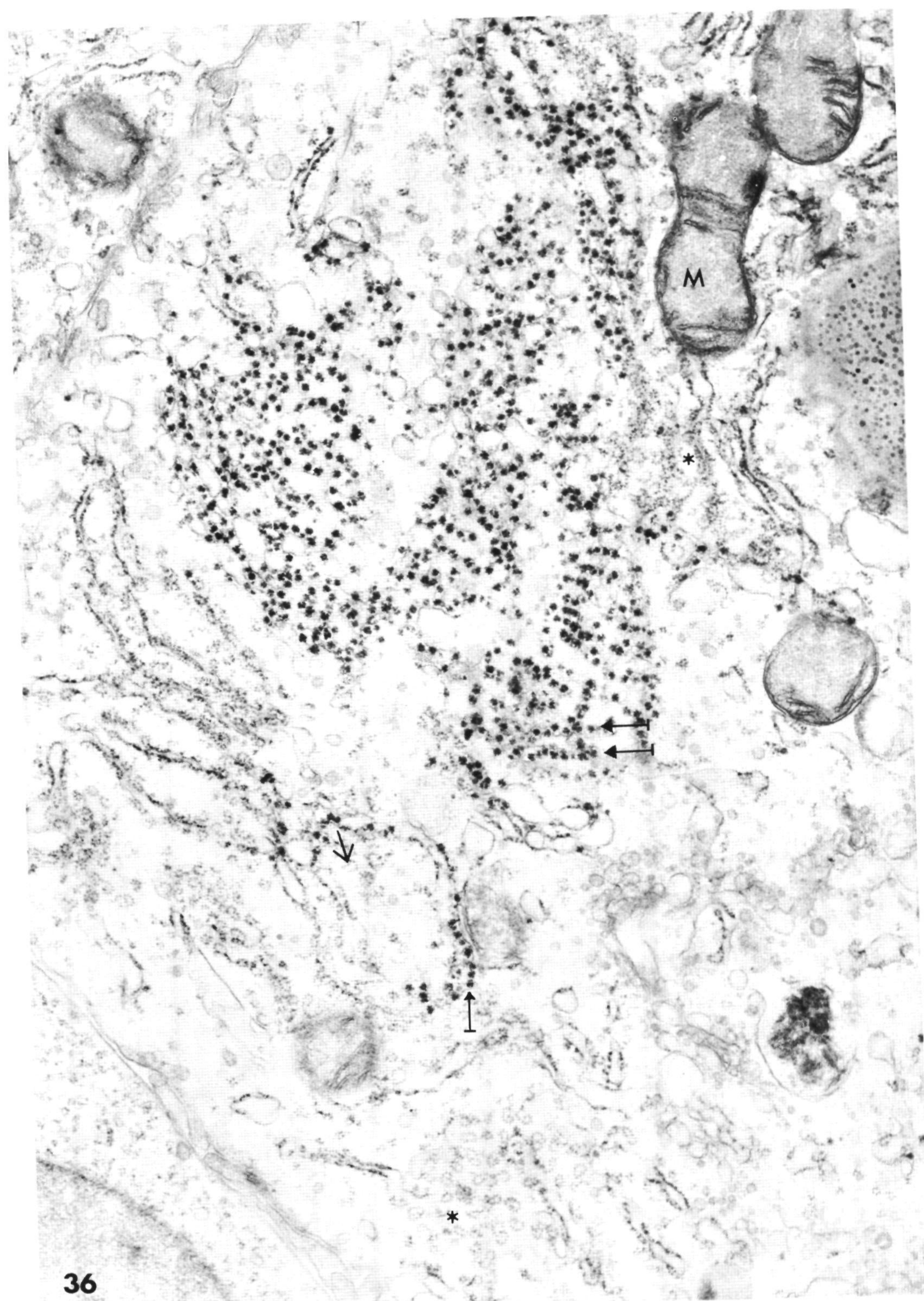
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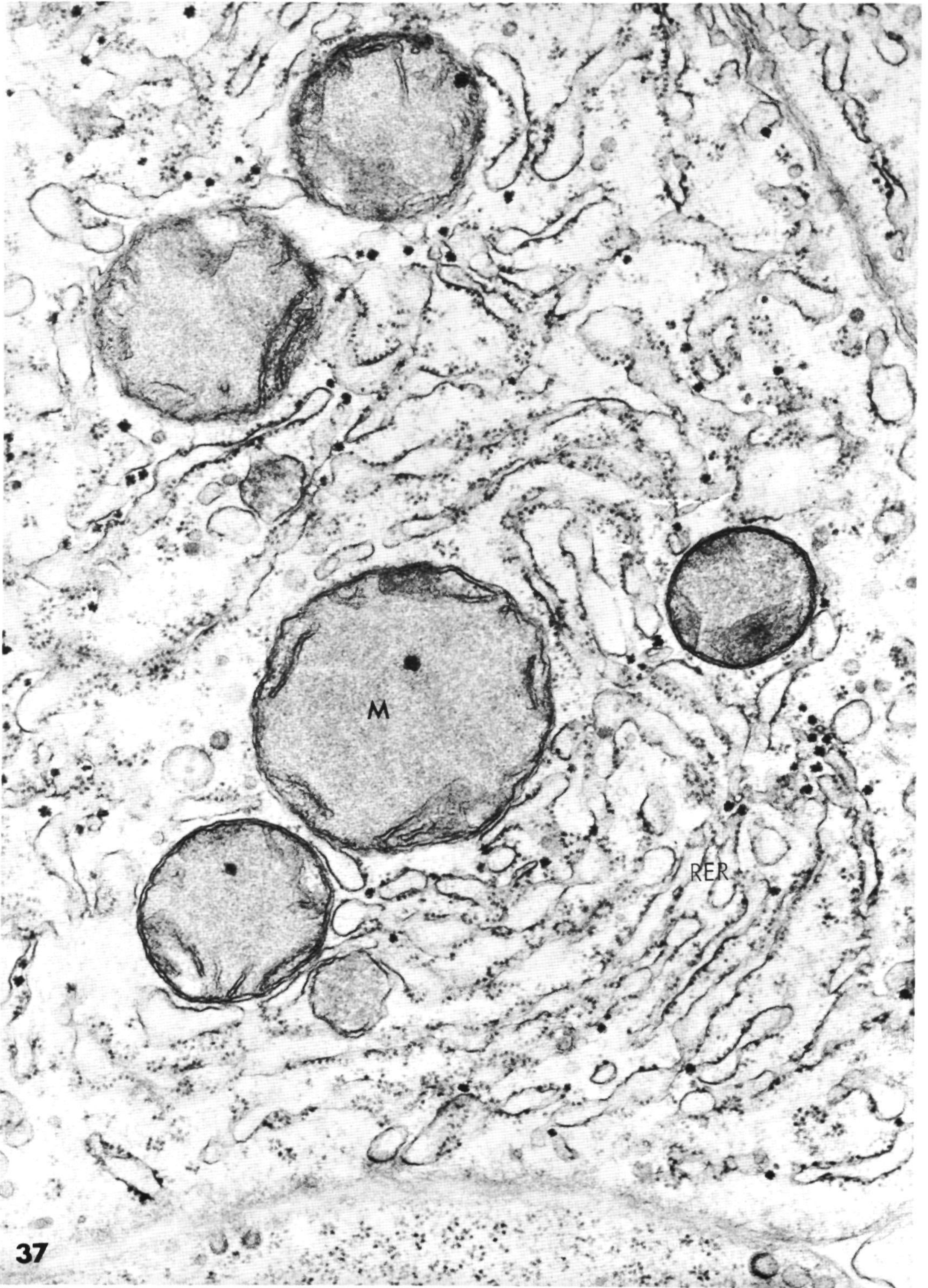
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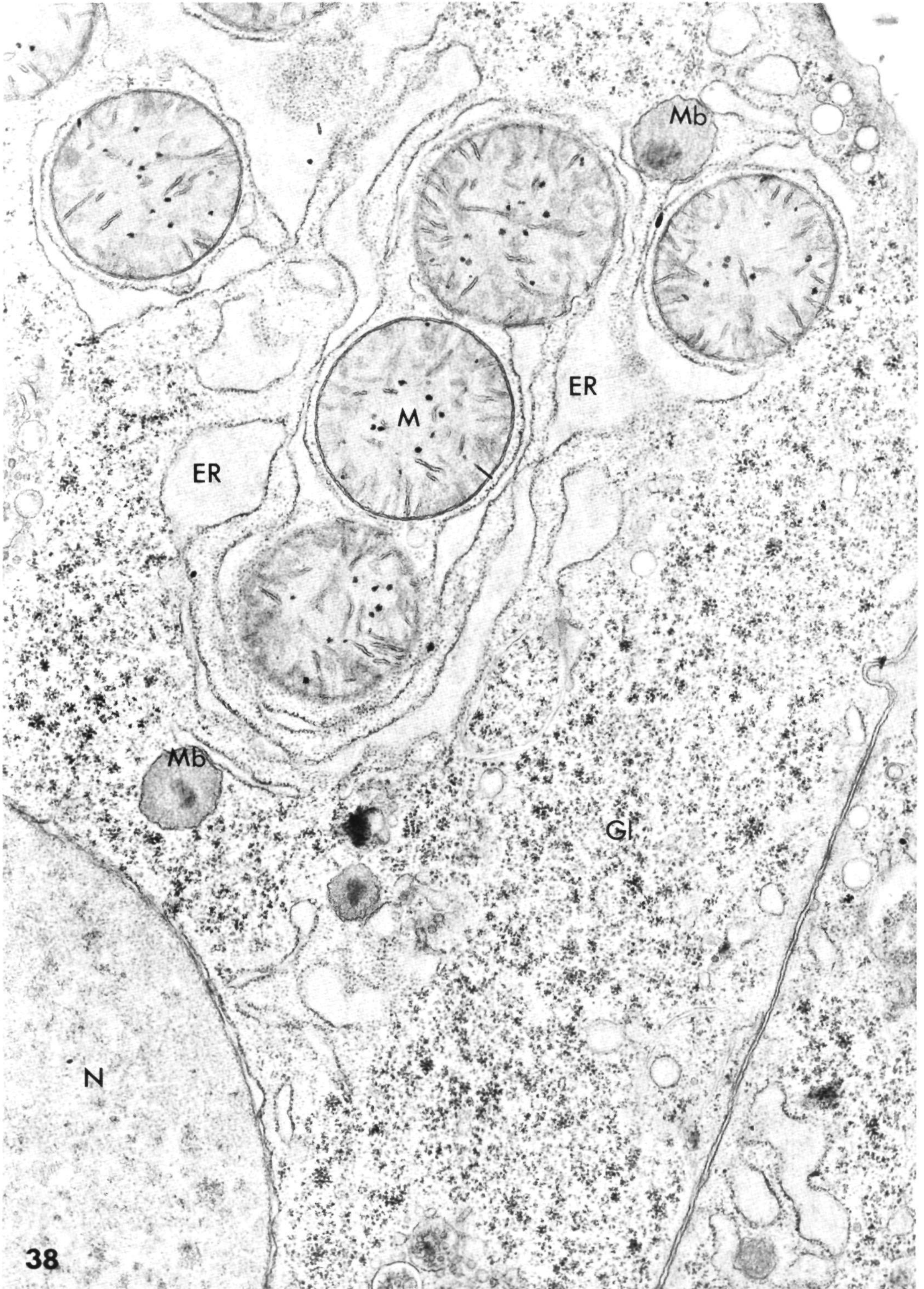
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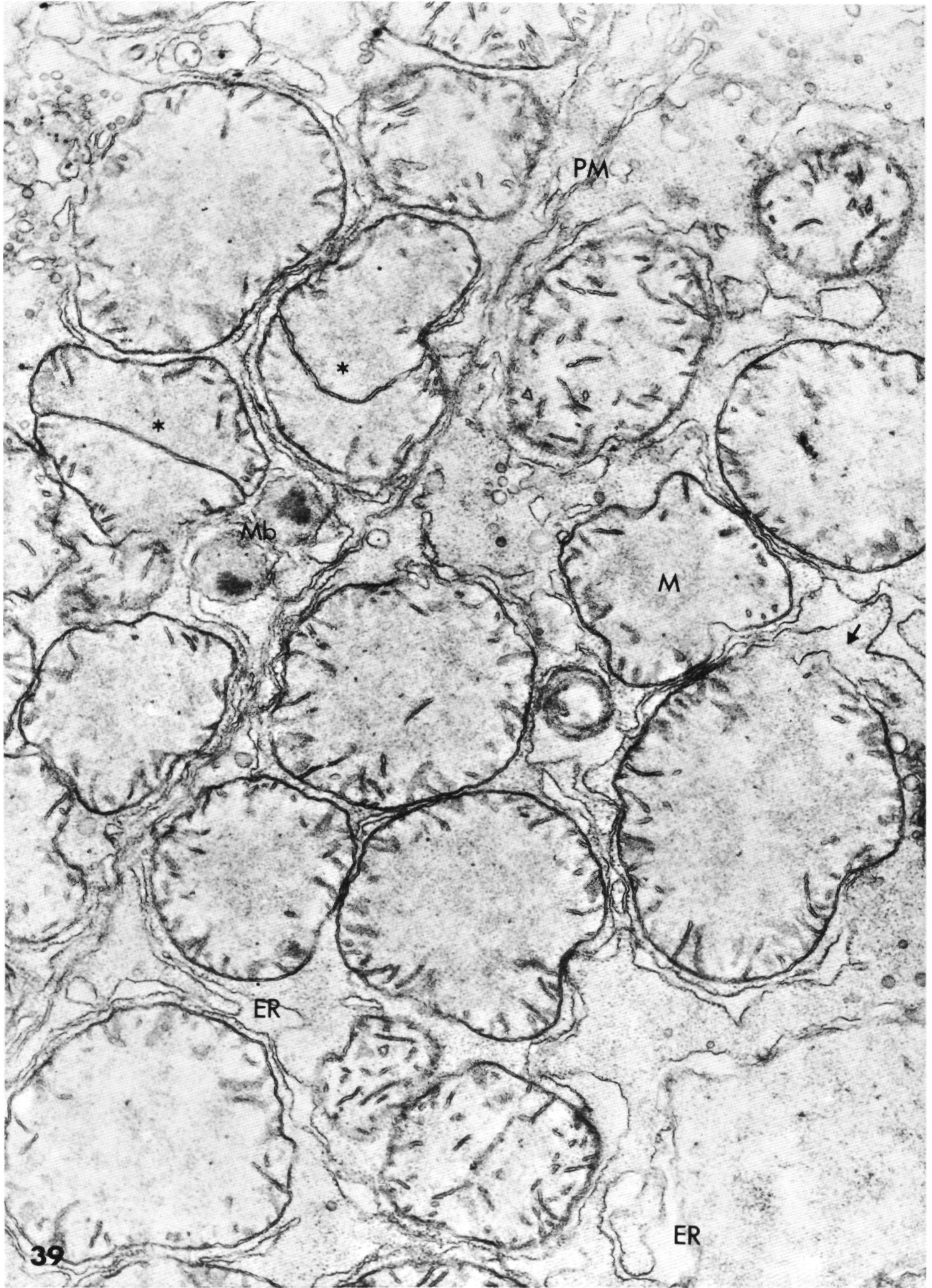
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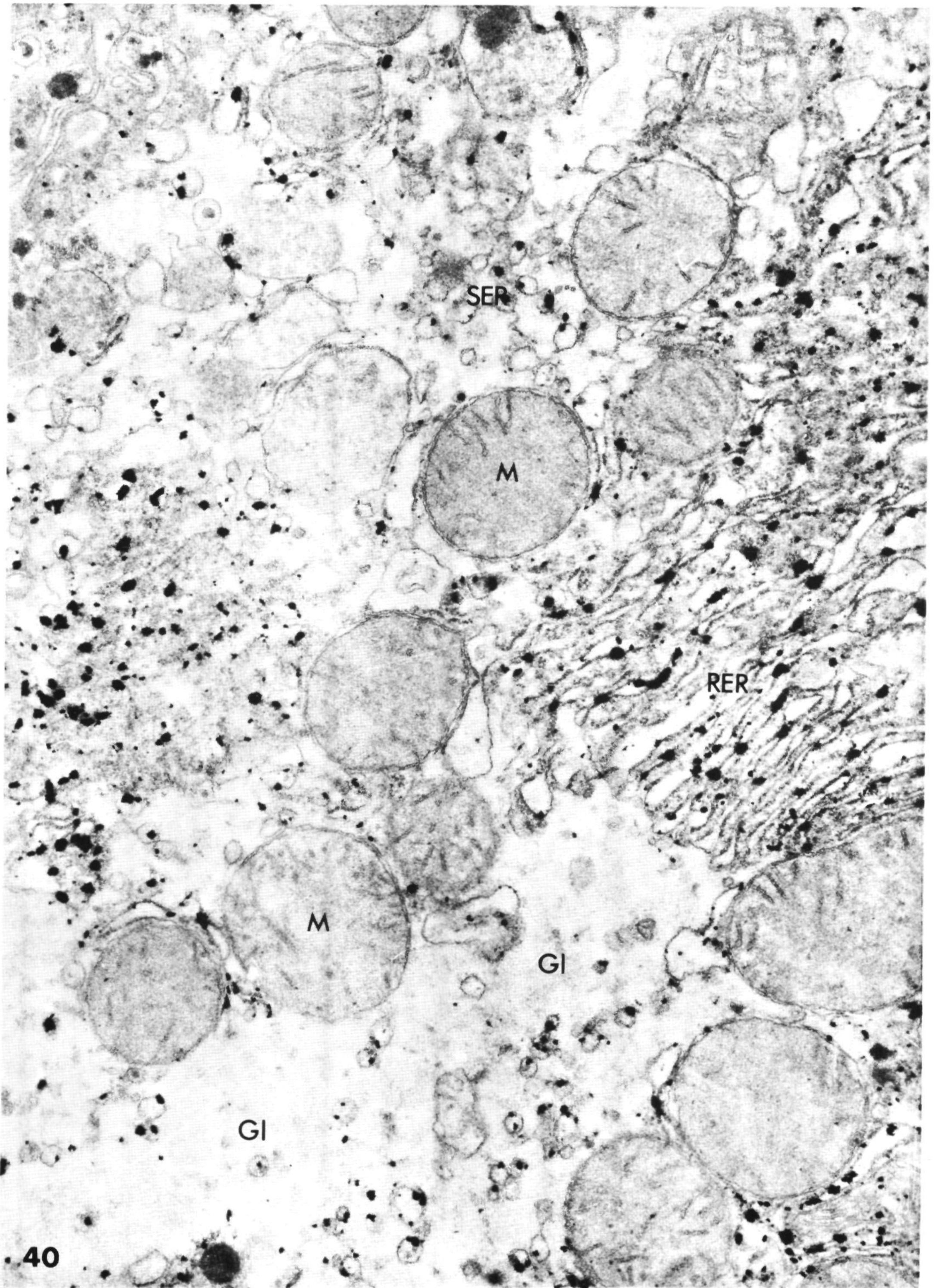
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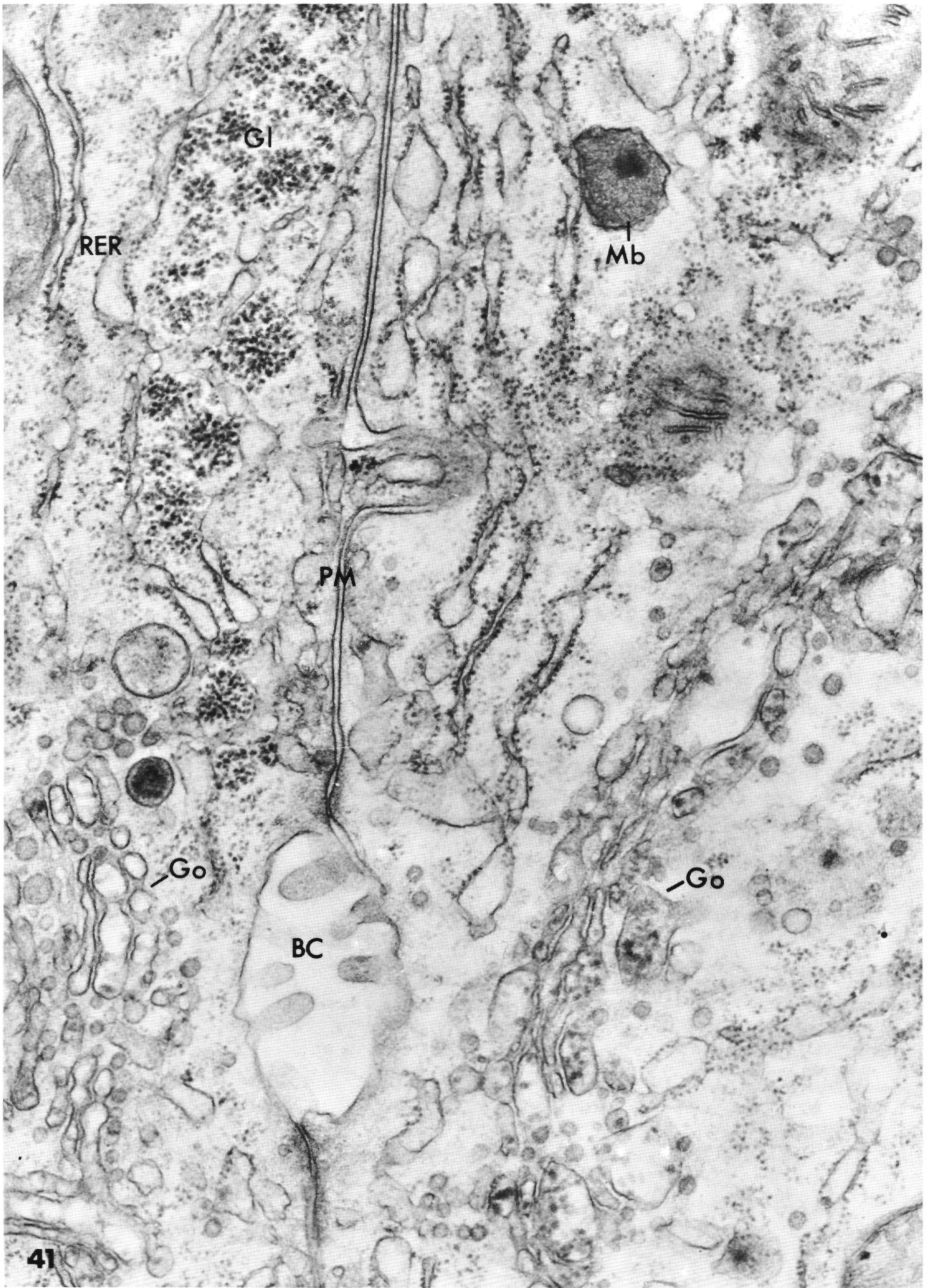
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FIGURE 15 UP TO AND INCLUDING FIGURE 18. Examples of tissue- or celltypes in which glycogen is encountered in alpha-particle form

FIGURE 15. This electronmicrograph shows the complexly organized form of glycogen deposition, as it is encountered in the malpighian tubular epithelium of Periplaneta americana. The glycogen particles demonstrate a distinct tendency to gather in restricted cell areas. In the vicinity of these areas a number of smooth vesicles is present. The particles clearly demonstrate a star-like appearance and show a remarkable uniformity in size (100-130 mμ). The differences in electron-opacity between the various particles are presumably to be related to the amount of polysaccharide that remains in the section after the particles have been in cut. Parts of two cells from the transitional zone between the upper and lower (distal and proximal) halves of a tubule are pictured. In these cells numerous concentrically arranged excretory granules are present. Os, Ep, LH-Millonig. Magnification x 19.800

FIGURE 16. Columnar epithelial cell of the mid-intestine of Periplaneta americana. Glycogen granules of the alpha-particle type are present, some of which (———→) lie dispersed in between the membranes of the rough endoplasmic reticulum. Here, too, the particles show a distinct tendency to crowd together. Though lying tightly-packed, they nevertheless retain their individuality. The free surface of the cell is provided with long slender microvilli (Mv). The cytoplasm in these structures has a slightly fibrillar aspect and is continuous with the ectoplasmic- or terminal web (TW)-zone, which is also traversed by a meshwork of fine filaments. The multivesicular bodies presumably are aggregates of small pinocytotic vesicles, which originate between the bases of the microvilli.

Os, Ep, LH-Millonig. Magnification x 25.5000

FIGURE 17. In the proximal halves of the malpighian tubules of Periplaneta, solitary dark cells are regularly encountered among the functionally excretory cells, which show a normal electron opacity. The glycogen particles in these dark cells, though belonging unmistakably to the complex alpha-type, are significantly smaller in diameter (50-80 mμ) than those of the electron-transparent cells. Note the presence of a centriole and the characteristic arrangement of the basal cellmembrane infoldings.

Os, Ep, LH-Millonig Magnification x 6500

FIGURE 18. Electronmicrograph showing the complex alpha-particles from the epithelium of the malpighian tubulus of Periplaneta at high magnification. An unequivocal interpretation of the fine structural organization of these star-like particles is hard to give. In many instances rod-like elements radiating out of the structures may be discerned (———→). These rods sometimes run parallel to each other on many occasions, however, they can be observed to join in the more central parts of the particles, thus indicating the presence of branched, Y-like structures. In the central area of the particles, circular "sub-units" are often discernible. In the encircled particles it may be seen that these circular elements are connected with one another as well as with the peripheral rod-like structures by tiny connecting-rods that are stainable with lead. It is conspicuous that the central parts of the α-particles are in many cases more easily transmitted by the electron-beam than are the peripheral parts Os, Ep, LH-Millonig Magnification x 90 000

FIGURES 19 a/b AND 20a/b. Two sets of adjacent sections of fetal rat liver (21 days' old embryo) to demonstrate the possibilities for differentiating between ribonucleoprotein-containing particles and glycogen particles. The transparent plasma-regions in the one figure are seen to contain glycogen-granules in the adjacent section.

FIGURE 19a/20a Glut, post-fix. Os, Ep, UA, lead-acetate pH 7.3

FIGURE 19b/20b Glut, post-fix. Os, Ep, UA, LH-Reynolds

Magnification fig.19 x 28.800 fig.20 x 14.000

FIGURE 21. Livercell of a 21 days' old rat embryo. The ribosomes demonstrate strong electron-density. At the periphery of the RER-areas, clusters of small particles (———→) are present, which distinguish themselves from the ribosomes with regard both to their size and their affinity to uranyl. These particles are supposed to participate in glycogen (enzyme formation) synthesis.

Glut, post-fix, Os, Ep, UA/lead-acetate pH 7.3 Magnification x 37.500

FIGURE 22. Particulate glycogen from rat liver, negatively stained with PTA, pH 7.4. The mamillated outline of the complex alpha-particles is very suggestive of the presence of "sub-units". The negative-staining material appears to penetrate more readily into the central parts of the structures, in a number of them a central "hole" is apparent (———→). Magnification x 153.000

FIGURE 23. Negatively stained particulate glycogen at high magnification. At (———→) small filament-like structures are discernable (Y-elements of Drochmans), but their regular occurence is not evident.

Magnification x 238.000

FIGURES 24 AND 25. Degradation of particulate glycogen under the impact of acids.

FIGURE 24. This sample of rat liver particulate glycogen was treated for 2 hrs with 2.5% PTA, pH 3.0. Small fragments of glycogen (beta-particles) have been released from the alpha-structures.

Magnification x 69.500

FIGURE 25. Negatively stained sample of particulate glycogen that has been treated for 5 hrs with 2.5% PTA pH 1.7. Numerous beta-particles are present. The alpha-particels which are left over, have an appearance that is reminiscent of a desintegration of these structures. Magnification x 69.000

FIGURES 26, 27, 28 AND 29. These lightmicrographs illustrate the changes in glycogen-content of the developing liver during the perinatal period. PAS-staining, 1μ thick Epon-sections.

FIGURE 26. Section of the liver of a 17 days' old embryo. At this developmental stage numerous blood-forming elements are present. The livercells can be recognized by their having a transparent nucleus as well as fat-droplets. None of the hepatocytes demonstrate PAS-positivity. Magnification x 925

FIGURE 27. Section of a 19 days' old embryo. In all livercells glycogen is present. The hepatocytes acquire a polygonal contour. The number of blood-forming cells has decreased. Magnification x 825

FIGURE 28. At the 21st day of gestation the livercells have accumulated large amounts of glycogen. The nuclei, mitochondria and basophilic substance are pushed aside in restricted cell-areas and the cells show a considerable increase in size. Magnification x 825

FIGURE 29 At one day after birth the livercells hardly contain any glycogen They are now loaded with fat-droplets. During glycogen-depletion the livercells decrease in size again. Magnification x 825

FIGURE 30. Hepatic cell from a 17-days' old rat fetus. No glycogen deposits are encountered at this stage of development. A Golgi complex is present middle-left. In the Golgi-vacuoles small, moderately electrondense particles are present. The mitochondria have a normal ultrastructural appearance. The endoplasmic reticulum profiles are particle-loaded. No smooth ER is present. The arrow points towards pinocytotic vesicles with smooth contours. Bottom left a late erythroblast is present.
Os, Lp, LH-Millonig. Magnification x 34.000

FIGURE 31. Electronmicrograph of a liver cell from a 18 days' old fetus. Stage of beginning glycogen-formation. A few glycogen particles are present in the centre of the picture. These particles lie in the midst of a number of vesicles which clearly belong to the RER. The arrows point towards some solitary glycogen particles. The fine structure of the mitochondria has not changed to any appreciable extent when compared with the preceding day. Os, Lp, LH-Millonig. Magnification x 34.000

FIGURE 32. At the 19th day of intra-uterine life the glycogen-areas have grown considerably in size. Intermingled with the glycogen particles a number of smooth endoplasmic reticulum vesicles are present. In several places, however, these are continuous with the ribosome-studded ER-membranes outside the glycogen area. The fact that several spirals, rosettes or chains of ribosomes (⟼) are found next to the RER-cisternae, even when these RER-cisternae apparently are not sectioned tangentially, is very suggestive of a process of release of these (poly-) ribosomal arrangements from the endoplasmic reticulum membranes. Os, Lp, LH-Millonig. Magnification x 34.000

FIGURE 33. This electronmicrograph, too, is taken from a 19 days' old fetus. The ergastoplasmic cisternae adjacent to the glycogen area show bulbous terminal swelling (*). The arrows point towards solitary glycogen particles. These, too, are always encountered in the immediate vicinity of the RER-elements. Os, Lp, LH-Millonig. Magnification x 30.500

FIGURE 34. Uranyl-acetate stained section of a hepatic cell from an 18 days' old fetus. Parts of two liver cells are pictured. The glycogen particles in the glycogen area of the hepatic cell on the right (no ER-membranes in between the particles) show only a low affinity to uranyl, except for some particles at the periphery of the glycogen area. On the other hand, the particles in the glycogen-area of the hepatic cell on the left (in which area the glycogen particles are still enclosed within vesicular elements) demonstrate strong electrondensity. This affinity to uranyl of the "initial glycogen particles" is taken to indicate that these structures also contain ribosomal material.
Os, Lp, UA. Magnification x 23.750

FIGURE 35. Close-meshed network of haphazardly distributed smooth profiles, enclosing "uranyl-positive" glycogen particles (liver of 18 days' old rat fetus). The glycogen granules appear in the form of small rosettes or solitary particles. There is an overlap in size and shape of the ribosomes and the smallest glycogen units. Those membranes of the endoplasmic reticulum vesicles that lie adjacent to the region of initial glycogen formation are denuded of ribosomes.
Os, Lp, UA. Magnification x 62.000

FIGURES 36 AND 37. These electronmicrographs demonstrate the relationship between the granular endoplasmic reticulum (RER) and the initial glycogen formation as this can be observed in the fetal calf liver.
FIGURE 36. Hepatic cell of a 2 month' old fetal calf. Large numbers of spirals or chains of ribosomes(*) often apparently detached from the ER-membranes, surround the area of initial glycogen deposition. The membrane-profiles, lying between the glycogen particles are swollen and smooth. The arrows point towards glycogen arrays, which still indicate the original disposition, sometimes parallel, of the RER-cisternae. Os, Lp, LH-Reynolds. Magnification x 24.000
FIGURE 37. Section from the same tissue block as fig. 36. In this particular livercell-region the glycogen particles are still scarce and lie without an apparent order among the elements of a membrane-system, which undoubtly is of the granular type. In many places polyribosomal arrangements, reminiscent of a release from the membranes, are encountered.
Os, Lp, LH-Reynolds. Magnification x 35.000

FIGURES 38 AND 39. Changes in the ultrastructure of the rat hepatocytes after birth.
FIGURE 38. Hepatic cell of a neonatal rat, 2 hours postpartum. The most prominent alterations are: 1) a progressive shrinkage of cell-size coinciding with the disappearance of glycogen, 2) a swelling of the ER-lumina; 3) a swelling, together with a rounding, of the mitochondria. At 2 hours postpartum large quantities of glycogen are still present. There is no invasion of tubular profiles of the smooth endoplasmic reticulum into the glycogen areas. Os, Lp, LH-Millonig. Magnification x 22.100
FIGURE 39. Pronounced swelling of the mitochondria in the hepatic cells of a neonatal rat at 17 hours postpartum. The swelling is attended by a "dilution" of the mitochondrial matrix, as follows from the decrease in electrondensity of this mitochondrial space. This particular animal has been prevented from sucking, but the process of mitochondrial swelling is equally pronounced in those animals that did suck. Two mitochondria can be seen to have a crista which interconnects the opposite interior walls (asterisk). Frequently mitochondria are encountered from which part of the outer membrane seems to be missing (arrow). Os, Lp, LH-Millonig. Magnification x 26.300

FIGURE 40. Fine structural localization of glucose-6-phosphatase activity. Tissue (liver adult Wistar rat) processed in accordance with the prescriptions of TICE and BARRNETT (1962). It can be seen that the lead-deposits are present mainly in the granular elements of the endoplasmic reticulum. Some smooth vesicles, present in the glycogen area, also show a positive reaction. The mitochondria as well as the Golgi membranes (top left) are essentially free from lead precipitates.
Glut, Lp. Magnification x 23.100

FIGURE 41. Electronmicrograph of parts of two livercells from a 3 days' old newborn rat. Note the *presence of strongly developed Golgi-complexes on both sides of the bile canaliculus. In the cell at* the left complexly organized glycogen granules of considerable size are present in between rough surfaced membranes of the endoplasmic reticulum. Os, Lp, LH-Millonig. Magnification x 35.500

LEGENDS TO THE FIGURES

FIGURE 1. Electronmicrograph of particulate glycogen, negatively stained with phosphotungstic acid, pH 7.4. Due to differential sedimentation losses during the extraction procedure, the lower molecular weight fractions are not present. The predominant type is the complex alpha-particle type, measuring 50-200 mμ. The particles are relatively stable between pH 5 and pH 8. Cf.fig. 22 for a higher magnification. Magnification x 73.000

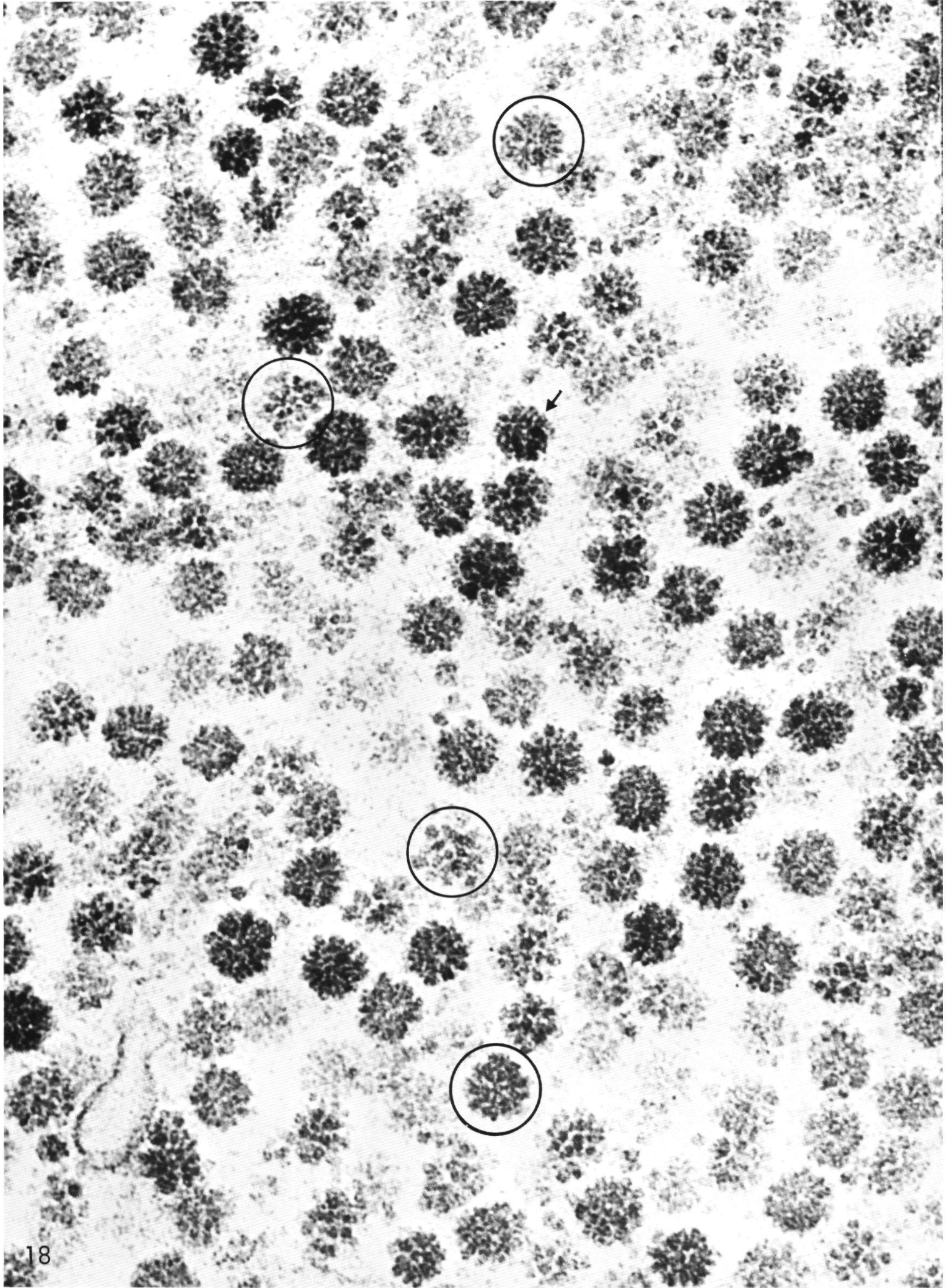
FIGURES 2 AND 3. The in situ fine structure of glycogen particles as depending on the type of the embedding medium employed. Tissue malpighian tubular epithelium of Periplaneta americana.
FIGURE 2. The glycogen particles in this methacrylate section have the appearance of vesicle-like structures with a fairly transparent centre. Such "glycogen-units" are hardly distinguishable from small Golgi- or endoplasmic reticulum vesicles. In the centre of the micrograph a cytosome is present, this cell-organelle is believed to have developed from multivesicular bodies.
Os, Meth, LH-Millonig. Magnification x 40.000
FIGURE 3. When Epon is used as the embedding medium, the glycogen particles stand out as star-like structures. No difficulties are encountered in distinguishing them from vesicle-like elements in the cytoplasm. Note the "septate desmosome" arrangement in the intercellular contact space. At (⟶) the section is cut normal to the cell membrane, here the arrangement has a ladder-like appearance. At (↦) the membranes are cut obliquely. Os, Ep, LH-Millonig. Magnification x 30.000

FIGURES 4, 5 AND 6. Comparison of thick lightmicroscopic and adjacent thin electronmicroscopic sections as a method for localizing the glycogen deposits at the subcellular level.
FIGURE 4. Lightmicrograph of a PAS-stained 1 μ section of fetal rat liver (20 day's old embryo). The cell areas that contain glycogen are indicated PAS. In order to facilitate orientation, the section areas, which are depicted in the figs. 5 and 6, are outlined in black lines. Note the presence of a megakaryocyte. Os, Ep, PAS. Magnification x 925
FIGURE 5. At this low electronmicroscopic magnification, the fine granularity in the PAS-positive area of the hepatocyte on the right is hardly distinguishable from the basophilic granularity of the hepatocyte in the centre of the micrograph. Os, Ep, LH-Millonig. Magnification x 5.200
FIGURE 6. At higher magnification one discerns star-like glycogen particles in the PAS-positive areas. Note that the space, occupied by the large mitochondrion above the nucleus (N) is shown as being negative in the lightmicroscope section. Part of an early erythroblast with numerous polyribosomes is present bottom left. Os, Ep, LH-Millonig. Magnification x 15.700

FIGURES 7 AND 8. Direct methods for the subcellular identification of glycogen.
FIGURE 7. Malpighian tubular epithelium of Periplaneta americana, treated with Best-carmine in accordance with the prescriptions of Themann (1963). Top right a number of glycogen particles demonstrate high electrondensity. The basal cell-membrane invaginations stand out sharply, due to unspecific carmine precipitation. Os, Ep. Magnification x 29.500
FIGURE 8. Methacrylate section of malpighian tubular epithelium, treated with 5% K-permanganate in accordance with the prescriptions of Drochmans (1960). The glycogen particles are strongly stained in this methacrylate section, but a generalized destructive effect on tissue fine structure is also apparent. Os, Meth. Magnification x 47.750

FIGURES 9a/b AND 10. Possible complications of lead-staining procedures with respect to the identification of glycogen at the subcellular level.
FIGURES 9a/b. Two successive thin sections of frog liver, treated in such a way as to show the effectivity of the glycogen-staining with lead, as dependent on the pH of the staining solution. Section 9a was treated with a half-saturated lead-acetate solution, pH 7. Section 9b was stained with the same staining solution, but after the pH was raised to 10. Note the transparent, hole-like appearance of the unstained glycogen particles. Os, Ep. Magnification x 13.000
FIGURE 10. The glycogen particles in this section of a fetal rat liver (21st day of gestation) show a finely stippled aspect. Such a punctated appearance of the glycogen granules crops out when the lead-stained sections are exposed to a strong radiation with electrons. This fine stippling cannot be taken to represent a particular arrangement of glycogen-particle subunits.
Os, Ep, LH-Reynolds. Magnification x 83.000

FIGURE 11 UP TO AND INCLUDING FIGURE 14. Examples of tissue- or celltypes in which glycogen is present in beta particle form.
FIGURE 11. Neutrophilic leucocyte from human bone marrow. Glycogen particles and ribosomes are hardly distinguishable from each other. Os, Ep, LH-Millonig. Magnification x 23.500
FIGURE 12. Electronmicrograph of part of an epithelial cell from a kidney tumor of Balb/C mice, maintained by subcutaneous transplantation. There is only a small difference in size between the glycogen beta-particles (250 A) and the ribosomes, which are present in the cell at the bottom of the micrograph. (Courtesy of U.v.Haelst.) Os, Ep, LH-Millonig. Magnification x 23.500
FIGURE 13. Electronmicrograph of rat skeletal muscle, showing longitudinally sectioned myofibrils. The length of the repeating unit of striations (sarcomere) is from Z-line to Z-line. The glycogen beta-particles are present predominantly in the intermyofibrillar space, where one also finds the vesicular structures of the sarcoplasmic reticulum and the sarcosomes.
Os, Ep, LH-Millonig. Magnification x 27.800
FIGURE 14. Electronmicrograph of the stratum-spinosum layer of fetal human skin (20 weeks old embryo). Parts of three cells are pictured. At this developmental stage large amounts of glycogen are present in the stratum-spinosum cells. Note the presence of numerous desmosomes. From these desmosomes the tonofilaments extend into the cytoplasm. (Courtesy of J.M.Janssen).
Glut, Ep, LH-Reynolds. Magnification x 22.750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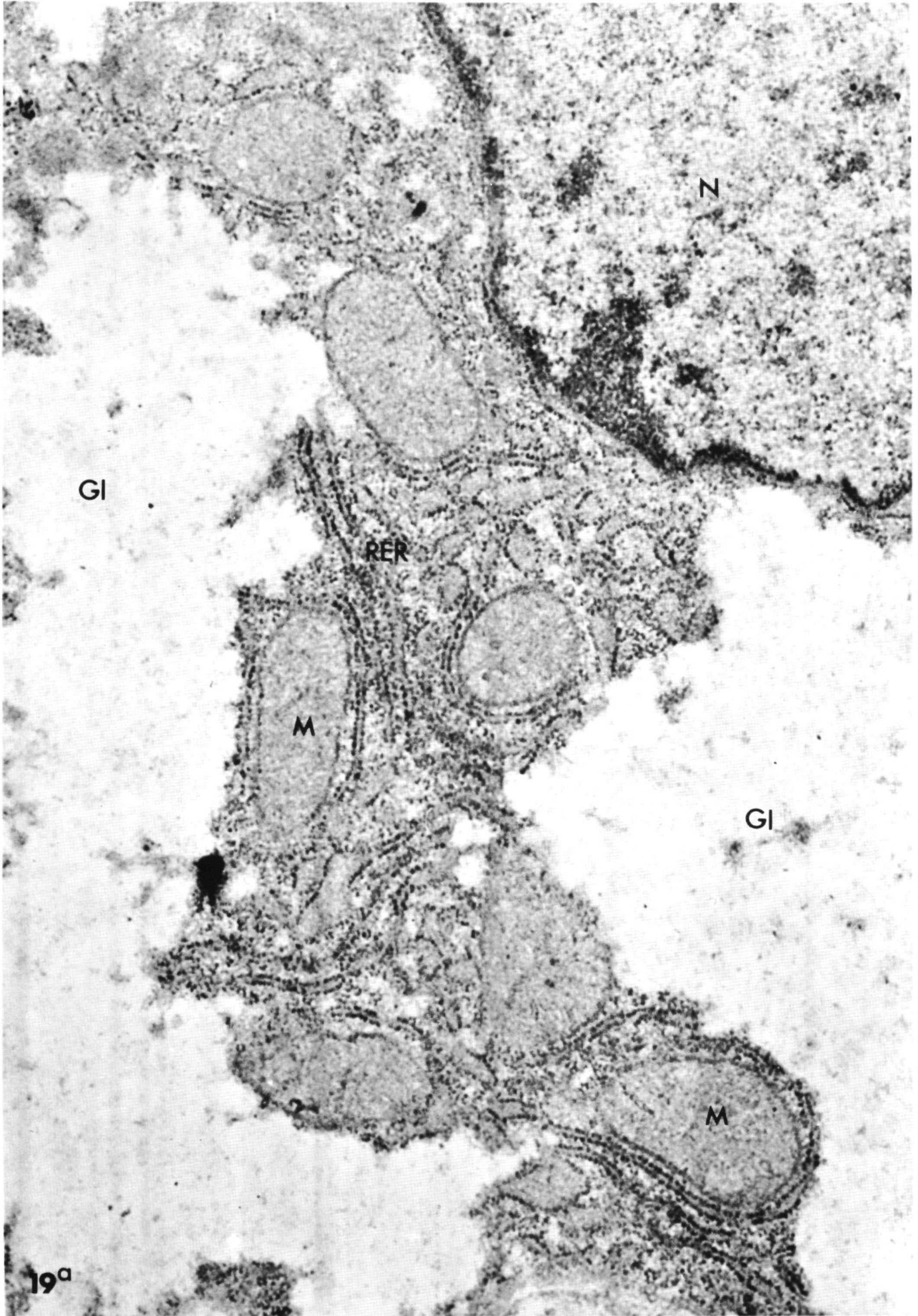
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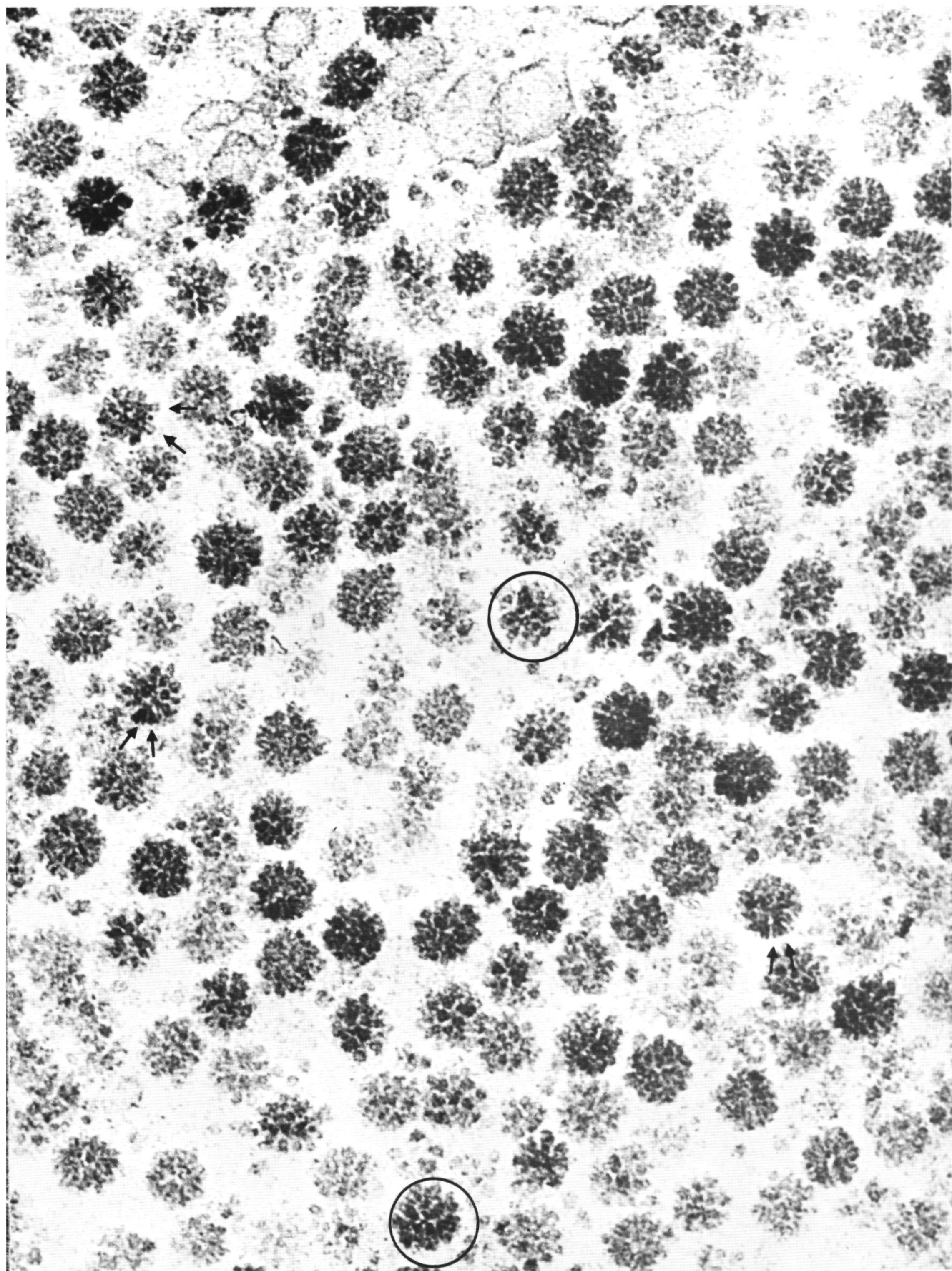

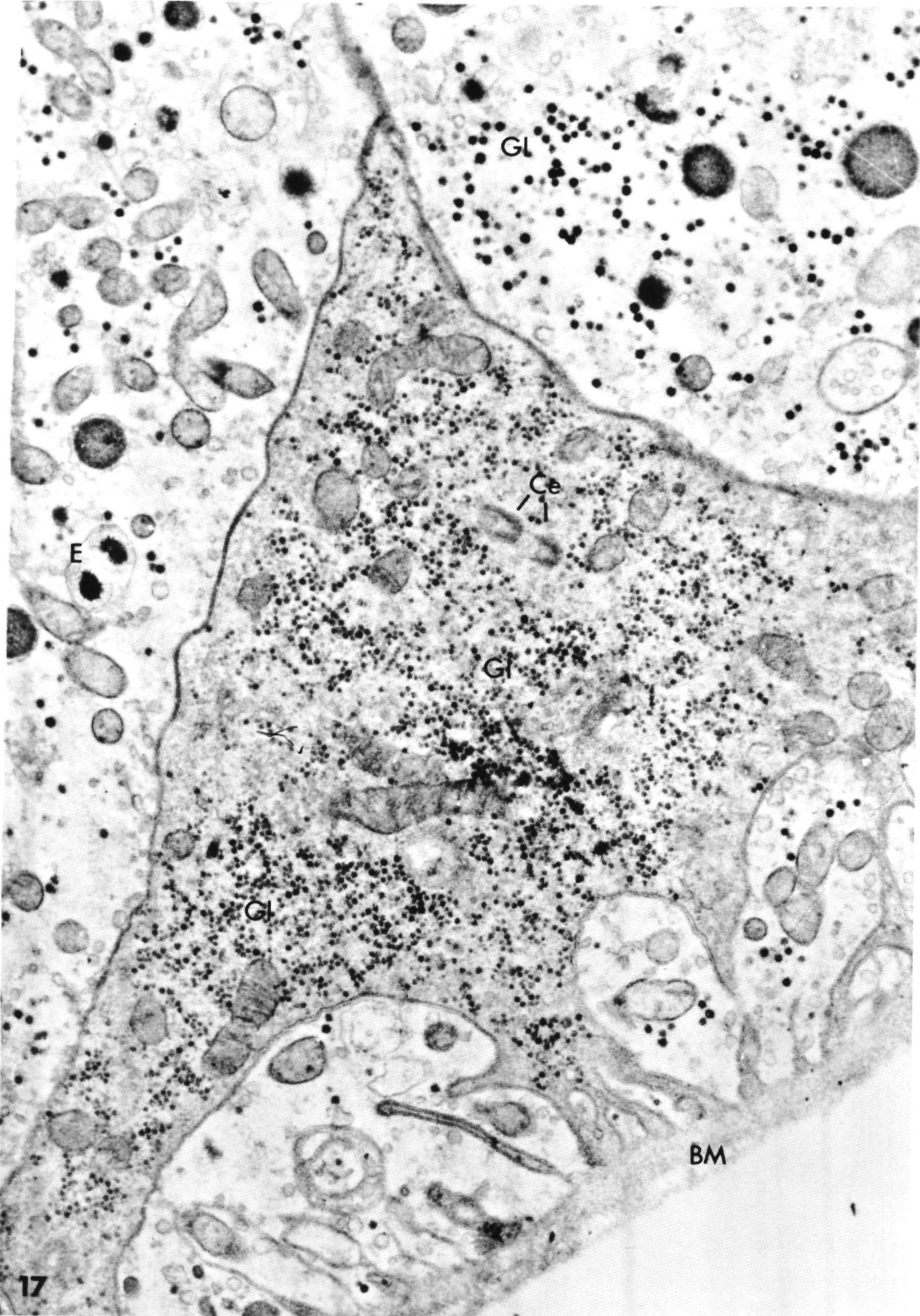
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STELLINGEN

I

Het gladwandig endoplasmatisch reticulum der levercellen is niet betrokken bij de glycogenese en glycogenolyse.

Dit proefschrift

II

De opvatting van Dale, dat de enzymen lactaat-dehydrogenase en aldolase een verhoudingsconstante groep vormen, is onjuist.

R.D.Dale, Biochem. J., 96 (1965), 347

III

De door Drochmans gegeven interpretatie van de opbouw der glycogeen partikels uit de lever is onhoudbaar.

P.Drochmans, J.Ultrastruct.Res. 6 (1962) 141
Dit proefschrift

IV

De vorming van autophage vacuolen geschiedt onafhankelijk van de reeds aanwezige lysosomen.

V

De mening van Olszewska en Gabara, dat bij de cytokinese een scheiding van de dochter-protoplasten voorafgaat aan de vorming van de celwand, moet als onjuist worden verworpen.

M.J.Olszewska; B.Gabara, Protoplasma, 59 (1963) 163

VI

Er bestaan aanwijzingen dat de intergenische recombinaties na de chromosomen-verdubbeling plaatsvinden.

W.O.Abel, Z.Vererbungsl. 96 (1965) 228

VII

Geen der argumenten, waarmee Weiling pleit voor het vervangen van de term pinocytose door de term potocytose, is steekhoudend.

F.Weiling, 56 (1963) 523

VIII

Het proces van de mitotische kerndeling van Neurospora crassa is tot op heden niet opgehelderd.

C.E.Somers et al. 45 Genetics (1960) 801

J.Weijer et al. Canad.J.Genet.Cytol. 7 (1965) 140

IX

Het gevoel van onbehagen over de huidige toestand van het onderwijs in de biologie, in het bijzonder bij het V.H.M.O., stoelt op een gebrek aan integratie van recente gegevens der biologische wetenschappen.

X

De keuze van de volgens de vigerende regelingen in openbare gebouwen aan te brengen voorwerpen van beeldende kunst dient niet uitsluitend te worden bepaald door de architect.

THOBEN
OFFSET
NIJMEGEN