

Isolation of Native Glycogen by Combined Rate-Zonal and Isopycnic Centrifugation¹

ALBERT A. BARBER,² WARREN W. HARRIS, and
NORMAN G. ANDERSON,³ *Biology Division, Oak Ridge
National Laboratory,⁴ and the Technical Division, Oak
Ridge Gaseous Diffusion Plant,⁴ Oak Ridge, Tennessee*

SUMMARY

Rat liver glycogen was separated into fractions of uniform particle size by the rate-zonal centrifugation of homogenates through cesium chloride and sucrose density gradients. Glycogen was then isolated from other cell constituents having similar sedimentation coefficients by isopycnic-zonal centrifugation in CsCl density gradients to effect a density separation. A density of 1.62 to 1.65 was isopycnic for glycogen. A continuous spectrum of particle sizes up to 150 to 200 m μ was observed, and sedimentation coefficients up to 10,000 were measured. Sedimentation coefficients of particles of different sizes indicated a decrease in molecular packing with increasing cluster size. Microscopically, three levels of glycogen structure were resolved, including fundamental γ particles 2.5 to 3.5 m μ in diameter, subclusters of β particles 20 to 25 m μ in

diameter, and α clusters of increasing size up to maximum diameter of 200 m μ . The 20 to 25 m μ subclusters would accommodate the glucose residues required to explain the molecular weight of glycogen particles normally extracted in strong alkali. Membrane fragments were not observed microscopically and specific 260 to 280 m μ absorption was absent in isolated glycogen fractions. Zonal centrifugation combined with isopycnic banding, therefore, provides a method for isolating large amounts of native glycogen separated into fractions of uniform particle size with a high degree of purity. Such preparations are obviously needed for studies of enzyme binding to glycogen particles and studies of the free and fixed glycogen fractions in tissues.—*Nat Cancer Inst Monogr* 21: 285–302, 1966.

GLYCOGEN, the reserve polysaccharide of animal tissues, consists of chains of (1 \rightarrow 4)-linked α -D-glucose residues interlinked by α -D-(1 \rightarrow 6)-glucosidic bonds to form a multibranched structure (1–5). The length of the average chain appears to be 10 to 14 D-glucose residues. These may include or be attached to exterior chains averaging 6 to 9 residues in length, and interior chains 3 to 4 residues long (1, 2, 6). Between 8 and

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10 percent of all glucosidic linkages are of the (1→6) type. Glycogen has been reported to contain small amounts of protein (7) and RNA (8), while electron microscopic studies have suggested that glycogen particles may be bounded by membranes (9). The low partial specific volume of glycogen ($\bar{v} = 0.62$) suggests either a very open structure completely interpenetrated by water, or very close packing of the polyglucose chains.

The size distribution of isolated glycogen depends to a great extent on the isolation method, of which several have been used. These include extraction with concentrated potassium hydroxide at 100° C (10), trichloroacetic acid (TCA) (11, 12), hot water (13, 14), and cold water (7, 15, 16). Certain shortcomings have been noted with the KOH and TCA methods. For example, concentrated alkali destroys the larger molecular sizes noted when TCA or water is used (12, 17), whereas TCA does not remove all the glycogen present in tissues (12, 18–20). The failure of TCA to extract all the glycogen has led to the concept of free and fixed glycogen fractions within tissues. TCA presumably extracts only the free form. The existence of these two forms as artifacts of extraction, and therefore of no significance, has been reported (21). The complex literature on free and fixed glycogens has been summarized by Stetten and Stetten (3).

Using cold-water extraction, Lazarow (7, 22) reported the isolation of very large glycogen particles in moderately low centrifugal fields and showed that previous extraction methods degraded the large “native” particles. Recent studies support Lazarow’s conclusions and suggest that most of the physical studies previously reported have been done on partially degraded material (17).

Combined rate- and isopycnic-zonal centrifugation (s - ρ centrifugation) has been applied to problems of cell fractionation (23). The technique is particularly appropriate for isolation of polydisperse cell constituents, such as glycogen. It was therefore used to examine the polydispersity of glycogen separated from rapidly prepared homogenates at neutral pH, and to determine whether glycogen was uniform with respect to banding density. In the case of glycogen, the uniformity of banding density is of interest from the point of view of the uniformity of chain packing. Also, the presence or absence of substances such as protein, lipids, or nucleic acids might be detected by changes in the banding density.

The probability that attached or “fixed” glycogen would band at the same density as free glycogen is small.

Rate- and isopycnic-zonal centrifugation were combined in the present study to separate pure native glycogen into fractions of uniform particle size. The isolation was designed to avoid all procedures using strong alkali or acid, fat solvents, and high temperature. A preliminary report of this work has appeared (24).

MATERIALS AND METHODS

Rat livers were removed from adult Sprague-Dawley male rats after stunning, decapitation, and exsanguination. Livers were diluted 1:5 (w/v) and homogenized either in 0.25 M sucrose or 0.01 M phosphate buffer at pH 7.0 containing 0.15 M NaCl. All manipulations were carried out as rapidly as possible at 0 to 4° C.

Zonal centrifugation was carried out in the B-IV rotor systems with either cesium chloride or sucrose as the gradient material (25, 26). Twenty ml of homogenate was placed in the rotor containing a gradient volume of 1200 ml. About 300 ml of high-density solution was used as the underlay and 200 ml of buffer as the overlay. Separations were normally carried out at 20,000 rpm for 15 minutes ($G_c = 4700 \times 10^6$, including acceleration and deceleration⁵). The rotor contents were emptied by use of high-density solutions, and the eluant was monitored at 260 m μ . Forty-two fractions of 40 ml each were collected in tubes maintained in ice. The density of each tube was determined from measurements of refractive index with an American Optical refractometer.

Isopycnic banding was carried out by density gradient centrifugation in Spinco No. 30 angle-head rotors (27). Tubes containing CsCl (saturated at 0° C and containing 0.01 M potassium citrate at pH 7.0) layered underneath 18 ml of sample volume were centrifuged at 24,000 rpm for 3 hours in a Spinco Model L ultracentrifuge. Banded samples were photographed with scattered light (23). The glycogen band was removed from some samples with a Band-Recovery Apparatus⁶ (BRA I) equipped with a square-tipped spinal needle connected to a 2 ml syringe (23). The total contents of other banded samples were collected in 1 ml portions, starting from the bottom of the centrifuge tube, with a New Brunswick PA-56 pump. The density of each sample was determined from refractive index measurements.

Glycogen was measured by a phenol-sulfuric acid colorimetric method (28), which has been successfully used as a rapid and simple procedure for measuring liver glycogen (29). One ml of 5 percent (w/v) phenol and 5 ml of concentrated reagent-grade (Dupont) sulfuric acid were added to 1 ml samples containing 5 to 40 μ g of carbohydrate. Absorbancy was measured at 490 m μ after 1 hour at room temperature and compared with standards prepared from glucose. All spectrophotometric measurements were made with either a Bausch and Lomb Spectronic or a Beckman DB spectrophotometer.

Sedimentation coefficients were read directly from computer plots programmed for sucrose density gradients in the B-IV rotor system (23).

Samples were prepared for microscopy by the negative staining technique of Brenner and Horne (30). Phosphotungstic acid (PTA) has been used successfully for morphological studies of glycogen by negative staining (31, 32). A single drop of sample was placed on each of two

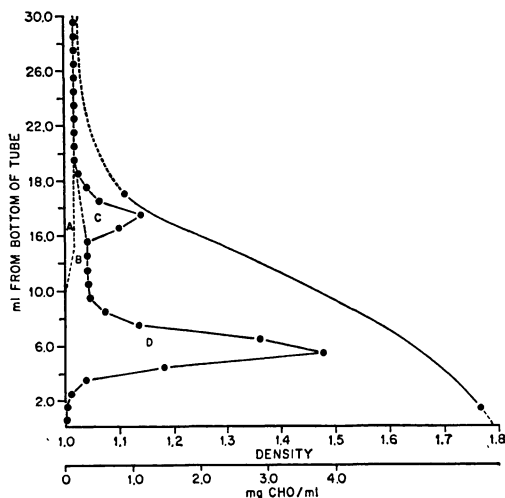
⁵ Where $G_c = \int \omega^2 dt$.

⁶ Information on drawings available from N. G. Anderson.

carbon-coated Formvar specimen screens and allowed to remain on the screen for 2 to 3 minutes in a large petri dish in the presence of osmium vapor. The drop of sample was removed by touching the edge of the screen with filter paper, and a drop of 2 percent (w/v) phosphotungstic acid (adjusted to pH 7.0 with KOH) was added immediately. The PTA was left on the screen approximately 30 seconds, removed, and the screen dried under ultraviolet light. Glycogen was somewhat difficult to examine with the electron microscope because of its low density to electrons. Screens were examined with one or more of the following RCA model electron microscopes: EML, EMU3-F, or EMU3-G.

RESULTS

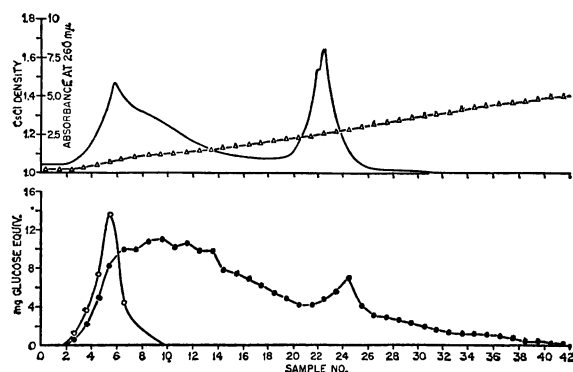
Initially, it was of interest to determine whether all glycogen banded in a narrow zone in CsCl or whether some glycogen was associated with cell structures that banded at a different level. Centrifugation of a rat liver homogenate in CsCl separated the membrane and glycogen fractions into two distinct bands visible with scattered light (fig. 1). The CsCl density and the carbohydrate distributions within the centrifuge tube are plotted in text-figure 1. The banding density of glycogen, under the conditions used, was approximately 1.65. Membrane-associated carbohydrates banded at a density of 1.25. In addition to the banded glycogen (area D, text-fig. 1), representing approximately 65 percent of all carbohydrate, and the membrane-associated carbohydrate (area C, text-fig. 1), representing approximately 12 percent of the carbo-



TEXT-FIGURE 1.—Cesium chloride density and carbohydrate distribution in rat liver homogenate banded in CsCl (same tube as photographed in fig. 1). A total of 16.0 mg of the 16.2 mg placed into the tube was recovered. ●—●—●—●—● Carbohydrate distribution; ----- extrapolated values; ●—●—●—●—● CsCl density.

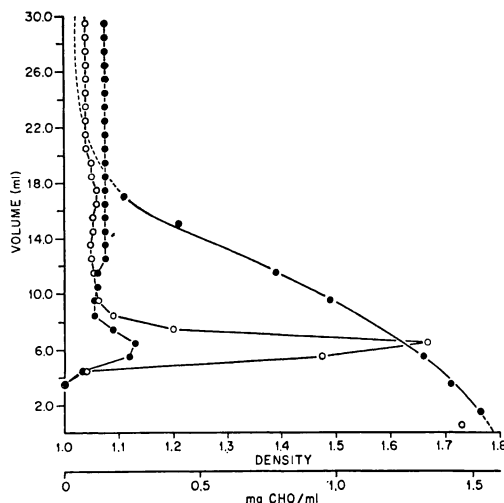
hydrate, two other carbohydrate areas were identified. These included partially sedimented glycogen (area B, text-fig. 1) and unsedimented glycogen. The distribution of nonsedimentable glycogen was determined by measurement of the distribution of glucose in a separate tube centrifuged under similar conditions. As expected, the amount of glycogen banded was less when the centrifugal force was reduced. The ratio between completely and partially banded material, therefore, depended on the centrifugal force and suggested that glycogen exists as a continuous spectrum of particle sizes.

Rate-zonal centrifugation separates mixtures into zones of particles having similar sedimentation coefficients. The polydispersity of native glycogen was confirmed by a rat liver homogenate sedimented in the zonal centrifuge at 17,500 rpm for 15 minutes with CsCl. Cesium chloride was used in place of sucrose because the latter interferes with the analysis for glycogen. Glycogen was sedimented throughout the B-IV rotor (text-fig. 2). The curve of glycogen concentration passed through a maximum a short distance from the starting zone, and a gradual decrease in the concentration was noted in successive samples. An abrupt increase in concentration was noted, however, in the samples containing the larger membrane fractions (tubes 22–26, text-fig. 2). Therefore, as in the banded homogenate, a membrane-associated glycogen fraction was observed. The samples collected from the region of the starting zone contained banded, partially sedimented, and nonsedimented carbohydrate as determined by isopycnic zonal centrifugation in CsCl. Tubes 5 through 12 were banded, since they represented the extremes of glucose distribution under similar conditions of zonal centrifugation (text-fig. 2). The carbohydrate distribution in banded tubes 6 and 10 is plotted in text-figure 3. Sample No. 6 contained only small amounts of banded



TEXT-FIGURE 2.—Distribution of rat liver carbohydrates following density gradient centrifugation in the B-IV rotor at 17,500 rpm for 15 minutes. A total of 20 ml of a 1:5 (w/v) homogenate made in buffer and containing 185 mg of carbohydrate (equivalent to 46 mg/g tissue) was used as sample; 188 mg was measured in the collected samples. ●—● Carbohydrate distribution; Δ — Δ CsCl gradient density; ○—○ glucose distribution determined in a separate run; — absorbance.

ZONAL CENTRIFUGE



TEXT-FIGURE 3.—Cesium chloride density and carbohydrate distribution in zonal samples 6 and 10 banded in CsCl. Samples obtained from the experiment described in text-figure 2 were banded as described for text-figure 1. ●—●—● CsCl density. Sample 6; ○—○ sample 10; ●—●—● CsCl density.

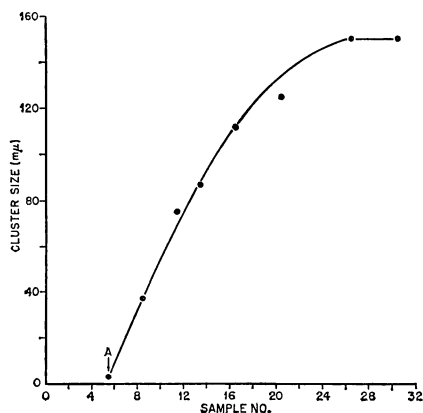
carbohydrate, whereas more than 70 percent of the carbohydrate of sample 10 was banded. The analytical data from the isopycnic banding of tubes 5 through 12 showed that the total amount of nonsedimentable carbohydrate present was similar to that found in homogenates banded directly in CsCl.

Zonal centrifugation of a rat liver homogenate was also carried out with a sucrose density gradient. Each collected sample was then centrifuged in CsCl gradients. The presence of glycogen, banded at its characteristic density level, was determined by photography with scattered light (fig. 2). The presence of glycogen bands throughout the rotor again indicated a continuous spectrum of particle sizes. The equivalent sedimentation coefficient of glycogen particles at various positions within the rotor was read directly from the computer plot, with 1.62 as the density of glycogen. Computer plots of several experiments, with different centrifugal forces and sucrose gradients, indicated an equivalent sedimentation coefficient for the largest glycogen particles of approximately 10,000 S^* . Generally the maximum was somewhat less. Minimum equivalent sedimentation coefficients were not determined in these rate-zonal separations, since the low centrifugal forces used did not sediment the small particles out of the starting zone. A continuous spectrum of particle sedimentation coefficients was observed. Assuming the same shape for the glycogen distribution curves and the same positions of maximum glycogen concentration in sucrose and the CsCl gradients, the peak of glycogen concentration occurred in the particle size range of 500 to 1000 S^* .

Glycogen particles collected in samples from several positions in the rotor demonstrated a progressive increase in particle size as one moves

centrifugally from the starting zone (fig. 3). The particles in any given sample were similar in size. Large α particles, or clusters (No. 3 in fig. 3), were constructed from subclusters or β particles which measured 20 to 25 $m\mu$ in diameter (No. 2 in figs. 3 and 4). The 20 $m\mu$ subclusters were aggregates of many fundamental γ units 2.5 to 3.5 $m\mu$ in diameter (No. 1 in fig. 4). The latter have been reported to be rodlike and approximately 20 $m\mu$ long (31). However, it is difficult to distinguish rods from beaded aggregates at this level.

The large α clusters attain sizes of 150 to 200 $m\mu$ in diameter. The progressive increase in cluster size as related to their position in the gradient is plotted in text-figure 4. The largest commonly observed α particle was 150 $m\mu$ in diameter, its equivalent sedimentation coefficient was approximately 3000. The most rapidly sedimenting particles appeared in the electron microscope to be aggregates of α clusters. The shape of the curve in text-figure 4 strongly suggests that the largest α particles existed in the form of α cluster aggregates in solution.



TEXT-FIGURE 4.—Relationship between glycogen cluster size and sample number after centrifugation in sucrose density gradients. Average cluster size for each sample plotted was determined from figure 3. A, position of sample placed into rotor.

It should be emphasized that the equivalent sedimentation coefficients calculated apply strictly only to the conditions employed in a given experiment. This is shown by simple calculations relating particle size to sedimentation rate using the formula:

$$(2a)^2 = \frac{18\eta s}{\rho' - \rho} \quad (1)$$

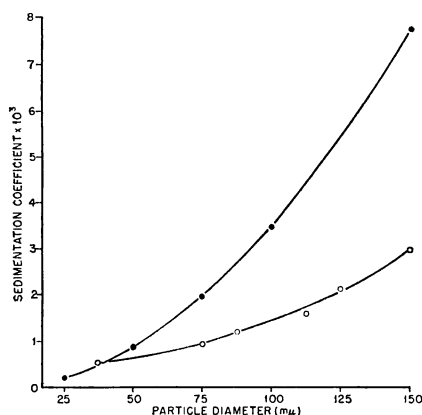
where $2a$ is the diameter of the particle in cm; η is the viscosity of the medium; ρ' is the particle density; ρ is the medium density; and s is the sedimentation coefficient ($s \times 10^{13}$ is the sedimentation coefficient in Svedberg units). In water at 20° C the above expression can be reduced to

$$(2a)^2 = \frac{0.18s}{\rho' - 1.0} \quad (2)$$

and for glycogen of density 1.62 the expression further reduces to

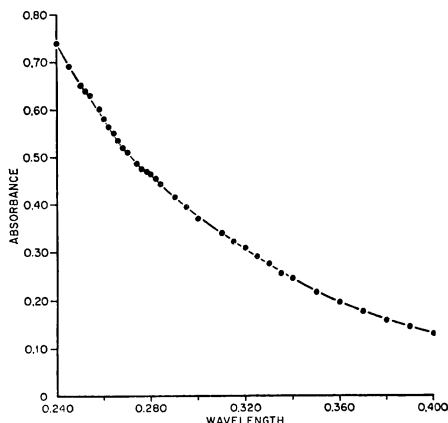
$$(2a)^2 = 0.29s \quad (3)$$

Calculated theoretical sedimentation coefficients for different-sized particles of 1.62 density were compared with experimentally determined values of measured glycogen particles (text-fig. 5). The two curves intersect at a particle dimension of approximately $37\text{ m}\mu$ but become increasingly divergent with increasing particle sizes. Close packing of glycogen subclusters ($20\text{--}25\text{ m}\mu$ particles) was therefore indicated, and an increasingly open structure, as observed, must be postulated for the larger clusters.



TEXT-FIGURE 5.—Relationship of size and sedimentation coefficient for theoretical particles having a density of 1.62 compared with the measured size and sedimentation coefficient of glycogen particles of increasing size. Theoretical curve is plotted from the formulation $(2a)^2 = 0.29 s$ and the experimental curve is plotted from figures 2 and 3. ●—● Calculated; ○—○ observed sedimentation coefficients in conventional Svedberg units.

No membrane elements were observed microscopically in banded glycogen fractions. The absence of membranes was also suggested by the absence of specific 260 or $280\text{ m}\mu$ -absorbing materials in the collected fractions (text-fig. 6). It therefore appears that glycogen clusters are not held together by membranous elements.



TEXT-FIGURE 6.—Absorption spectrum of glycogen isolated by banding in cesium chloride. Wavelength is in μ .

DISCUSSION

Polydisperse rat liver glycogen was separated into zones of uniform particle size by combined rate- and isopycnic-zonal centrifugation. Forty-two samples were collected following rate-zonal separation and were

banded in CsCl to effect a density separation of the glycogen from other cell constituents having similar sedimentation coefficients. Liver glycogen particles exist as a continuous spectrum of sizes.

Glycogen was identified as the 1.62 to 1.65 density material in banded samples by both chemical analysis and amylase digestion. Phenol-sulfuric acid was used as the chemical method for measuring glycogen on the assumption that all the carbohydrate of liver was present as glycogen. The validity of this assumption was indicated both by comparison of the carbohydrate values of 45 to 50 mg per g tissue with the published values for glycogen (18, 19) and by the results of the carbohydrate distribution in liver homogenates banded in CsCl. Centrifugation in Spinco No. 30 rotors for 3 hours at 24,000 rpm, in the absence of density gradients, totally sedimented particles with sedimentation coefficients (in water) of larger than 110. The amount of smaller material sedimented decreases with molecular size. Calculations of the banding profile of a heterogeneous mixture in a density gradient is very complex. However, if one uses particles of $S^* 100$ as the minimum for banding in the procedures used, the carbohydrates distributed in the partially banded and nonbanded fractions of the banded sample represent the size spectrum of glycogen particles up to this size. This entire spectrum (excluding material which banded with the membrane fraction) would constitute only 23 percent of the total carbohydrates present. Assuming a size distribution curve for particles below $S^* 100$ similar to that for larger particles, the contribution of non-glycogen carbohydrates to total carbohydrate would be negligible. This would suggest that nearly all carbohydrates of liver are present in the form of glycogen, and glycogen exists in a continuous size spectrum from small molecules to clustered particles containing several million glucose residues. A gradual decrease in concentration occurs with increasing particle size. It is evident that further studies with high-speed zonal centrifuges will be required to determine whether such a size spectrum of small molecules does, in fact, occur, or whether there is a minimum-sized molecule larger than glucose from which larger units are formed.

The three levels of structural organization observed in glycogen were referred to as α , β , and γ particles (31). The γ units were spherical or oblong particles 2.5 to 3.5 $m\mu$ in diameter. These were combined into 20 to 25 $m\mu$ β particles. Increasing numbers of β particles per cluster resulted in a spectrum of α particle sizes ranging from 30 to 200 $m\mu$. The α particle is the large, sedimentable unit of glycogen described by Lazarow (7, 22). Similar measurements and levels of organization for glycogen were found in this study. The major size differences in these studies concern the γ particle. Drochmans (31) considers them rodlike structures 3 by 20 $m\mu$, whereas the 3 $m\mu$ particles observed in the present study were primarily spherical and occasionally occurred in short chains.

The large clusters, characteristic of native glycogen from liver, are preserved only by relatively mild isolation procedures. The dissociation of large clusters by KOH and acid treatment has been observed by both centrifugal and microscopic methods (17, 22, 31). TCA extraction meth-

ods cause less breakdown than do methods involving KOH digestion (12). The particles isolated from liver following KOH digestion have molecular weights in the range of 1 to 8×10^6 (1), which indicates the dissociation of clusters into smaller units. Drochmans considered the product of KOH digestion normally used for physical studies to be the $3 \text{ m}\mu$ particles (31). Particle dimensions and molecular weight calculations, however, indicate the $20 \text{ m}\mu$ particle as the product. Assuming a spherical, $3 \text{ m}\mu$ particle, the volume occupied by an anhydrous glucose molecule of molecular weight 162 and density of 1.62, the $3 \text{ m}\mu$ particle would contain approximately 85 glucose residues. Assuming a $3 \text{ m}\mu$ by $20 \text{ m}\mu$ cylinder, the particle would contain approximately 850 residues. Neither of these values can be easily correlated with specific units in the branching model proposed for glycogen (1). However, both are too small to represent the glycogen particle studied after KOH extraction. On the other hand, 20 to $25 \text{ m}\mu$ spherical particles would contain the appropriate number of glucose residues indicated by the molecular weight of KOH-extracted material. Morphological transition in glycogen structure at the level of the 20 to $25 \text{ m}\mu$ particle was indicated by the divergence in the curves of the theoretical and experimental sedimentation coefficients at that point. This divergence could be interpreted as the transition point from a relatively closed to a relatively open structure. The chemical bonds leading to the open structure could be the point of attack of KOH during extraction. The amount of opalescence, used by Drochmans (31) as indicative of the $3 \text{ m}\mu$ particle as the digestion product, would likewise be reduced in solutions of $20 \text{ m}\mu$ particles.

The particulate glycogen isolated in the present study was collected in fractions of uniform particle sizes throughout the entire spectrum of sizes without appreciable membrane contamination. Such preparations are necessary for studies of enzyme attachment to glycogen particulates. Attachments of α -glucan phosphorylase and uridine diphosphate glucose-glycogen transferase to glycogen particles have been clearly established (33, 34). The major technical difficulties in these studies involved separation of glycogen particulates from membranous elements. Such difficulties are normally overcome by studies of specific activities during glycogen enrichment. Assuming that the presence of CsCl does not dissociate the bound constituents, isopycnic banding would provide a rapid method for collecting large amounts of uncontaminated glycogen. Rate-zonal separation can also be used to isolate large quantities of the various particle sizes for study of the specificity between particle sizes and amount of enzyme bound. The significance of such studies has been discussed (33).

Free and fixed glycogen fractions in tissues have been isolated by TCA extraction (3). In liver, 10 to 15 percent of the glycogen is considered fixed (18), although the existence of two fractions has been denied (21). In homogenates banded in CsCl, 12 percent of the carbohydrate was associated with the membrane fraction. Rate-zonal separations demonstrated membrane-associated glycogen in the region of mitochondria and

large fragments. No microsomal-bound glycogen was noted in samples banded from the microsomal region. Further studies using rate-zonal and isopycnic centrifugation are needed to determine if this membrane-associated glycogen represents the fixed glycogen fractions identified by TCA extraction.

One problem arising from these studies is the relationship of the three levels of structure observed with the electron microscope to the statistical models of branched glycogen deduced from chemical studies. One published consideration was concerned with the problem of the maximum size reached by a continuously branching open structure (35). Assuming that each chain branches into two at regular intervals, a maximum size of 26 to 40 $m\mu$ was calculated. At this size the entire surface is occupied by the ends of chains and further growth is not possible. The assumption that all chains divide in two is unlikely, since uniform growth probably would not occur. Although the 26 to 40 $m\mu$ size compares favorably with the observed β particle, and α particles could form from irregularly branched β particles, the chemical nature of the classical branched structure of glycogen cannot be directly correlated with the morphology of the particle.

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PLATES



FIGURE 1.—Rat liver homogenate banded in CsCl. A total of 11.0 ml of saturated CsCl was layered beneath 18.0 ml of sample containing a formaldehyde bead of density 1.46. The tube was centrifuged at 24,000 rpm for 3 hours and photographed with scattered light.

PLATE 55

FIGURE 2.—Distribtuion of rat liver glycogen following density gradient centrifugation in the B-IV rotor at 20,000 rpm for 15 minutes. A total of 20 ml of a 1:5 (w/v) homogenate made up in 0.25 M sucrose solution was used as the sample. The absorbance of the collected stream was monitored at 260 m μ . Glycogen distribution was determined by banding collected samples in CsCl as described for figure 1. The banded samples were photographed with scattered light. Relationship between sample number and banded tube is indicated by the numbers below the photographed tubes. The sedimentation coefficients of particles of known density and the sucrose density gradient were computer-plotted. Sedimentation coefficients are in conventional Svedberg units. \triangle —— \triangle Sucrose density.

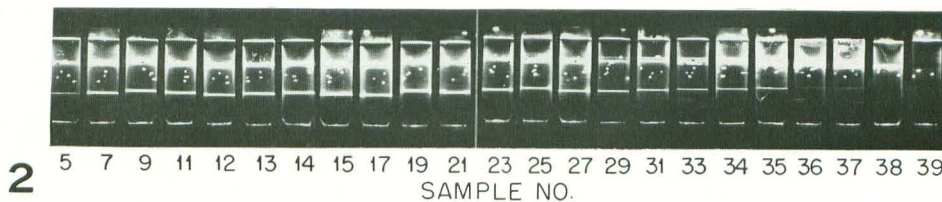
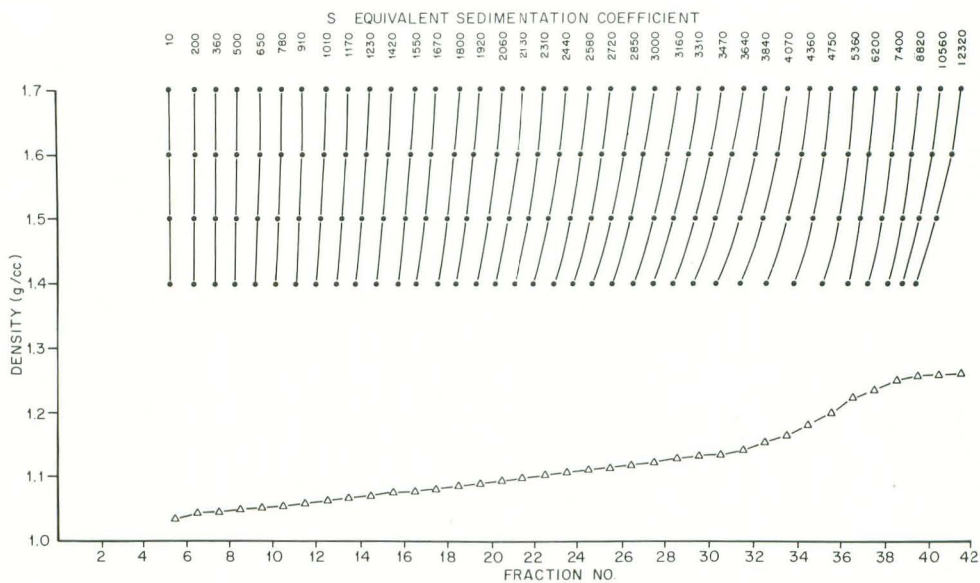
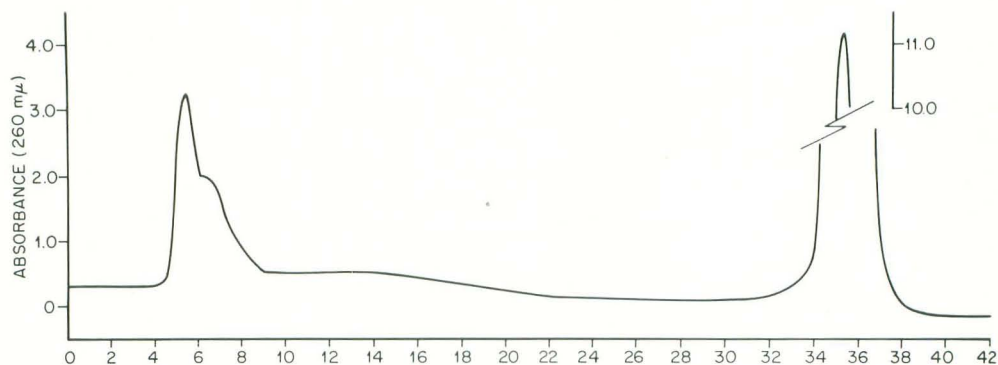
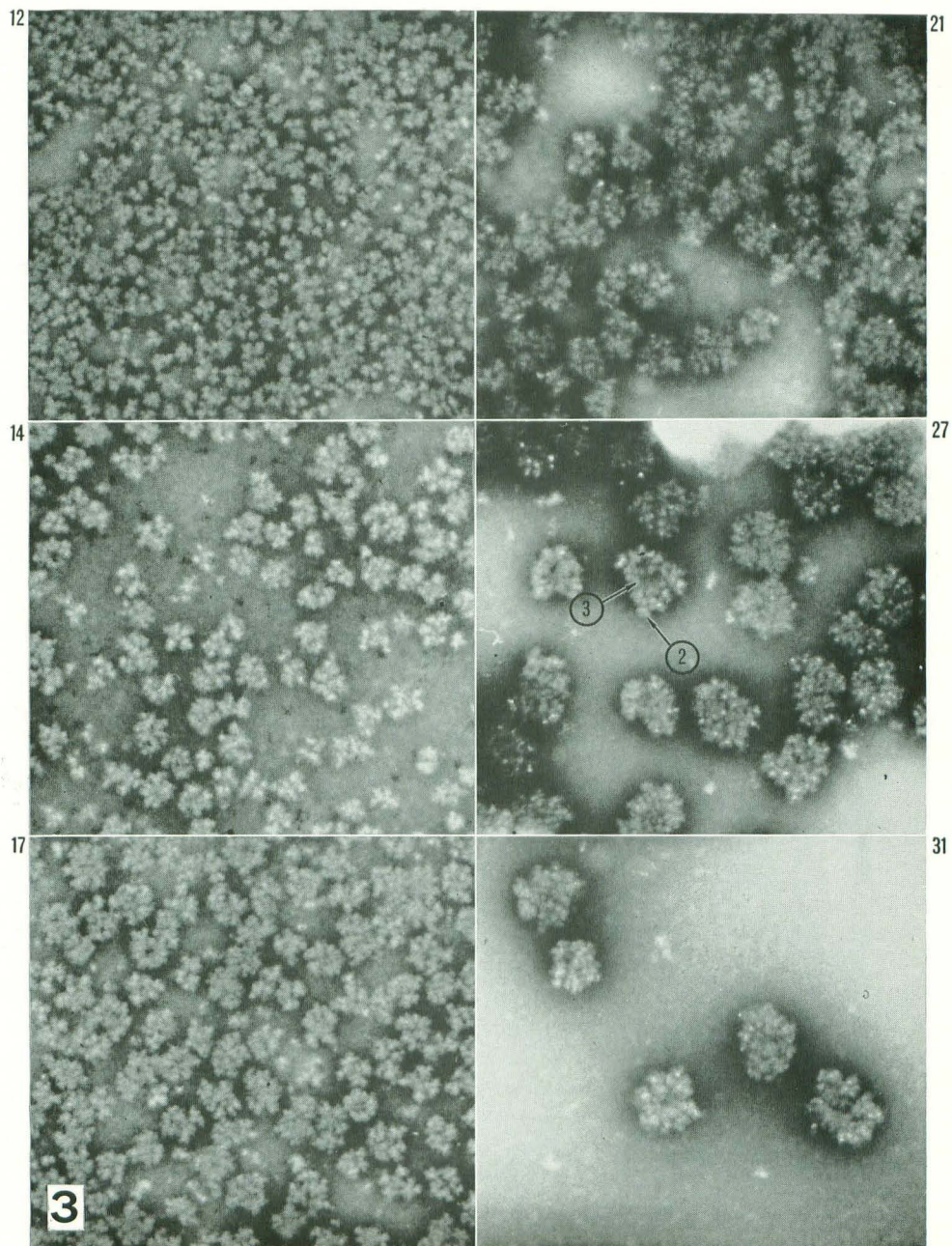


PLATE 56

FIGURE 3.—Electron photomicrograph of negatively stained glycogen. The number in the corner of each photograph indicates the zonal sample number from which glycogen was banded. The lower band of each sample (*see* photograph in fig. 2) was collected, rapidly dialyzed, and viewed following negative staining. Original magnification 80,000. Numbers 2 and 3 refer to the subclusters and clusters of glycogen, respectively (*see* text).



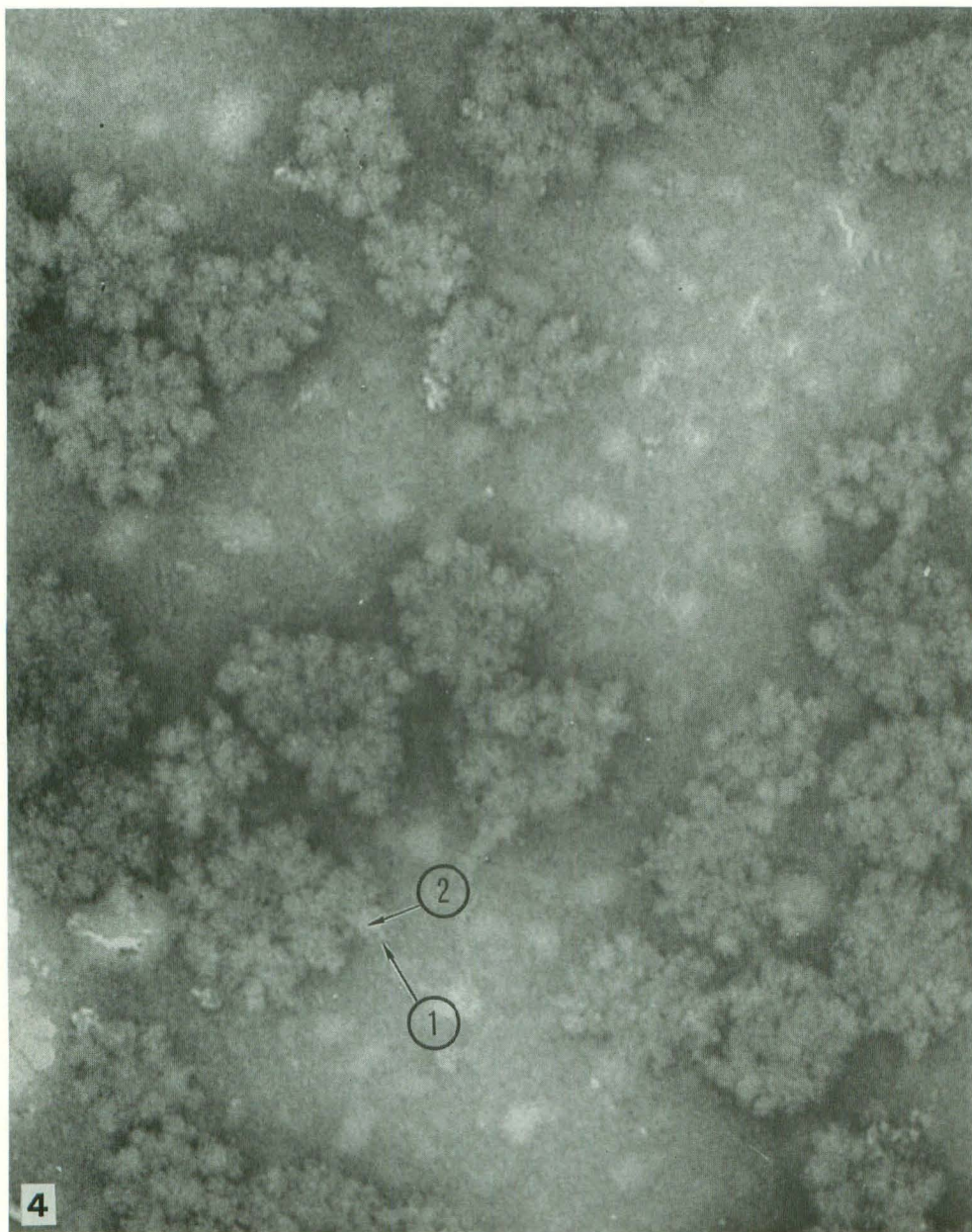


FIGURE 4.—Electron photomicrograph of negatively stained glycogen. Numbers 1 and 2 refer to the fundamental unit and the subclusters of glycogen structure, respectively (*see text*). Original magnification, 320,000