

# STUDIES OF NATIVE GLYCOGEN ISOLATED FROM SYNCHRONIZED *TETRAHYMENA PYRIFORMIS* (HSM)

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## ABSTRACT

Native glycogen was isolated from *Tetrahymena pyriformis* (HSM) by isopycnic centrifugation in cesium chloride density gradients. A density of 1.62 to 1.65 was isopycnic for glycogen. Most of the banded glycogen existed as 35 to 40  $m\mu$  particles which had a sedimentation coefficient of 214. These particles were composed of aggregates of 2 to 3  $m\mu$  spherical particles. Extraction of glycogen with hot alkali reduced the sedimentation coefficient of native glycogen from 214 to 64.7 and the particle diameter from approximately 40 to 20  $m\mu$  and smaller. Cell division was synchronized by a repetitive 12-hour temperature cycle, and glycogen was measured at several times during the cell cycle. The temperature cycle consisted of 9.5 hours at 12°C and 2.5 hours at 27°C. Approximately 90 per cent of the cells divided during the last 1.5 hours of the warm period. The carbohydrate/protein ratio of cells at the end of the cold period was 0.27 and was reduced slightly during the warm period. Glucose was incorporated into glycogen during both periods, although the rate of incorporation was greater during the warm period. No preferential incorporation on the basis of particle size was noted. Incorporation was measured in both native glycogen and KOH-extracted glycogen. *Tetrahymena* glycogen is compared with rat liver glycogen previously isolated by similar procedures, and the significance of using combined rate-zonal and isopycnic centrifugation for isolating native glycogen is discussed.

## INTRODUCTION

Most of the physical properties of glycogen, except molecular weight, are quite similar regardless of its source (1). For example, glycogen isolated from *Tetrahymena pyriformis* by hot KOH extraction resembles that isolated from mammalian liver and muscle, by similar extraction, in all properties examined except for its higher molecular weight (2). The hot KOH extraction procedure commonly used for preparing glycogen degrades the native material into units of much smaller size and correspondingly lower molecular weights (3). Mild isolation procedures are, therefore, necessary

for the studies of molecular size and sedimentation characteristics of native glycogen. Two such procedures have been recently reported (4, 5). Poly-disperse liver glycogen isolated by combined rate-zonal and isopycnic centrifugation have particle sizes up to 200  $m\mu$  with sedimentation coefficients up to approximately 4,000, indicating molecular weights much higher than the KOH-extracted material (5). Similar particle sizes and sedimentation coefficients for liver glycogen have been reported from several laboratories (6-8). It is not known whether the native glycogen of *Tetrahymena*

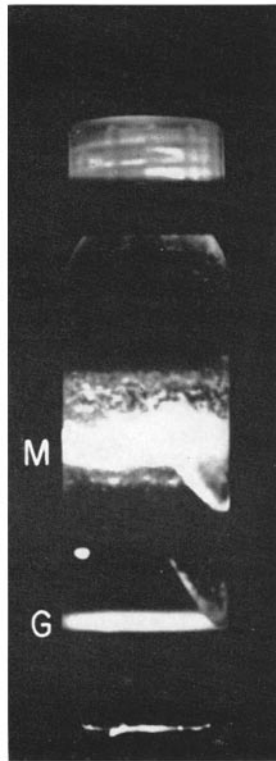


FIGURE 1 *Tetrahymena* homogenate banded in CsCl. Cells were obtained from the end of the cold period, stored in liquid nitrogen, thawed, homogenized, and diluted 1:10 (v/v) with buffer. Eleven ml of CsCl was layered underneath 16.0 ml of sample. The tube was centrifuged for 3 hours at 24,000 RPM and photographed with scattered light. A urea-formaldehyde density bead (density 1.46) was included as a reference marker. *M*, membrane fraction *G*, glycogen fraction.

occurs in these larger sizes, since its reported molecular weight was obtained from KOH-extracted material. Zonal centrifugation in cesium chloride gradients was carried out in order to compare the morphology and sedimentation properties of glycogen of *Tetrahymena* with those of glycogen isolated from rat liver by the same method. In addition, native glycogen and KOH-extracted glycogen were compared morphologically.

*Tetrahymena* contains a large amount of glycogen, which varies with culture conditions (9). Glycogen can represent up to 22 per cent of the cell's dry weight and is utilized during periods of anaerobiosis. The high content is also normally found in cells during the stationary phase of growth. Large amounts of glycogen also accumulate in exponen-

tially growing cultures exposed to repetitive supra-optimal temperature shocks which result in cell synchrony (9). In the present study, the content and morphology of native glycogen during cell growth and division were investigated in cells synchronized by a different repetitive temperature cycle that employs low-temperature shifts (10). Glucose incorporation into glycogen was also measured in different phases of the cell cycle.

#### MATERIALS AND METHODS

Axenic cultures (10 to 15 liters) of the ciliate *Tetrahymena pyriformis* (HSM) were grown on a 1 per cent (w/v) proteose peptone medium (Difco, Detroit) supplemented with 0.1 per cent (w/v) liver extract (Nutritional Biochemical Corporation, Cleveland). Division synchrony was induced by application of a repetitive temperature cycle (9.5 hours at 12° and 2.5 hours at 27°C) as previously described (10). Changes in cell number were continuously recorded, using the continuous cell monitor developed by James and Anderson (11).

At desired intervals, cells were collected from the 10 to 15 liter culture in 1.5-liter samples, concentrated to 40-ml volumes by centrifugation for 5 minutes using a hollow angle-head rotor at 1500 RPM in an International centrifuge, model PR-2. The cells were then pelleted by centrifuging for 3 minutes at 2000 RPM in the cold. The supernate was poured off and the cells were resuspended in cold buffer (0.01 M MgCl<sub>2</sub>, 0.1 M Tris-HCl, pH 7.0). This washing procedure was repeated two more times. The final pellet was resuspended to five times its volume in buffer, sampled for cell counting, and stored in liquid nitrogen.

A stock of cell culture to be labeled with glucose-C<sup>14</sup> was removed from the synchronized parent culture and subcultured on the temperature program of the parent culture. The cells were then exposed to 0.04 μc/ml of uniformly labeled D-glucose-C<sup>14</sup> (New England Nuclear Corp., Boston, Specific Activity: 3.3 mc/millimole) to which sufficient carrier D-glucose had been added to give a final concentration of 1 × 10<sup>-3</sup> M. 500-ml samples were then removed after exposure for varying lengths of time. The cells were then harvested and washed as outlined above, except that "cold" glucose (10<sup>-3</sup> M) was added to the buffer.

Cell counts were performed on suitably diluted samples with a Coulter counter, model B (Coulter Electronics, Hialeah, Florida), previously calibrated for this cell. Radioactivity was measured using a Tracerlab Omni/Guard low-background counting system with a C<sup>14</sup> efficiency of 13 per cent.

Zonal centrifugation was carried out in the B-IV rotor system using cesium chloride as the gradient material (12). Sucrose, generally used as the gradient

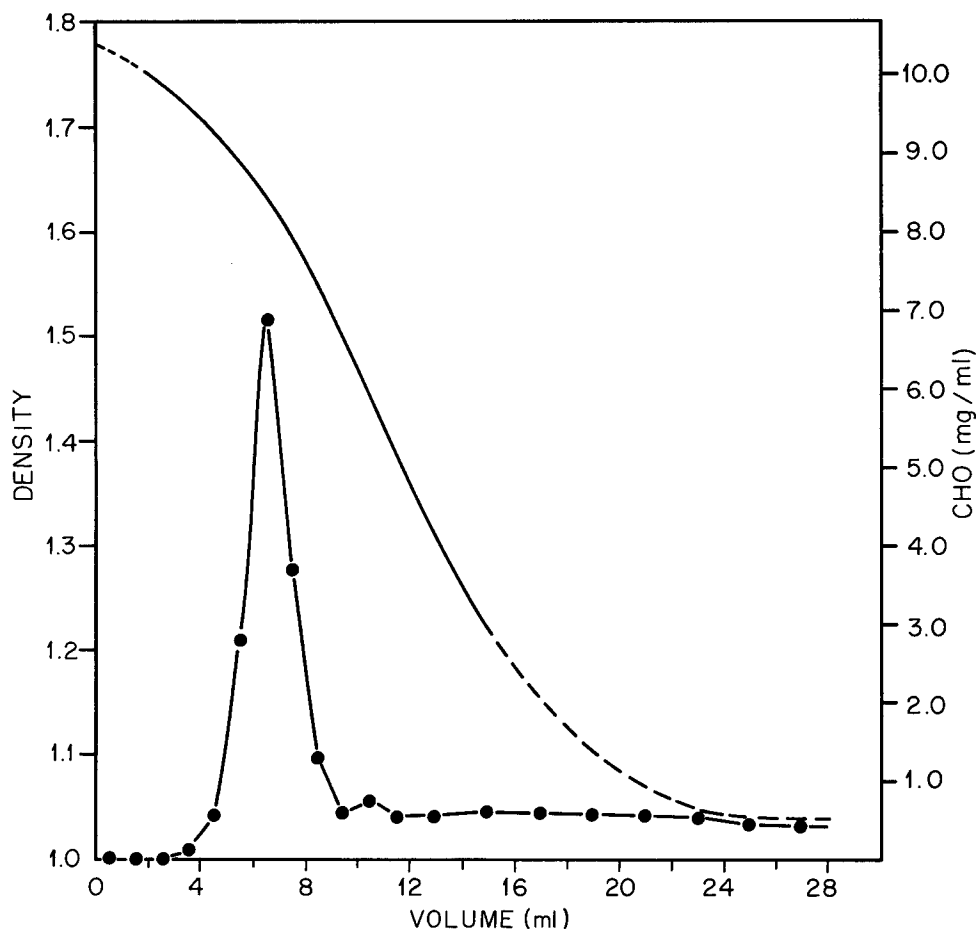


FIGURE 2 Cesium chloride density and carbohydrate content in fractions collected following banding of *Tetrahymena* in CsCl density gradient. Samples were collected from the tube photographed in Fig. 1. Density was determined by refractive index measurements of each collected aliquot. The extrapolated portion (-----) of the density curve between 1.0 and 1.2 represents CsCl densities too low to be measured by refractive index. ●—● carbohydrate distribution.

material, was not used because it interfered with the chemical determination of glycogen. Twenty ml of cell homogenate (1:5, v/v) were placed in a rotor containing a gradient volume of 1200 ml and a cushion of about 300 ml. Inboard of the sample layer, 200 ml of buffer was used. Separations were carried out at 20,000 RPM for 30 minutes (approximately 9500  $w^2t$ , including acceleration and deceleration). The rotor contents were emptied by displacement with a high-density solution from the outboard side, and forty-two fractions of 40 ml each were collected in tubes maintained in ice. The effluent was continuously monitored at 260  $m\mu$  during emptying. The density of solution in each collected fraction was determined from measurements of refractive index, using an American Optical Company refractometer.

Isopycnic banding was carried out by density gradient centrifugation in Spinco No. 30 angle head rotors. Acceleration of density gradients from rest to high speeds and deceleration back to rest causes relatively little shearing of the gradients formed. This has been studied both in mathematical models and in experimental studies using virus particles and DNA (13). Tubes containing 11 ml of CsCl (saturated at 0°C and containing 0.01 M potassium citrate, pH 7.0) layered beneath 16 ml of sample volume were centrifuged at 24,000 RPM for 3 hours. Centrifuged samples were photographed using scattered light. Banded glycogen was collected from centrifuged samples using a band recovery apparatus (Model BRA I) developed in this laboratory (14). The apparatus utilizes a flat end spinal needle connected to a 2-ml syringe whose plunger is driven by a low-speed motor.

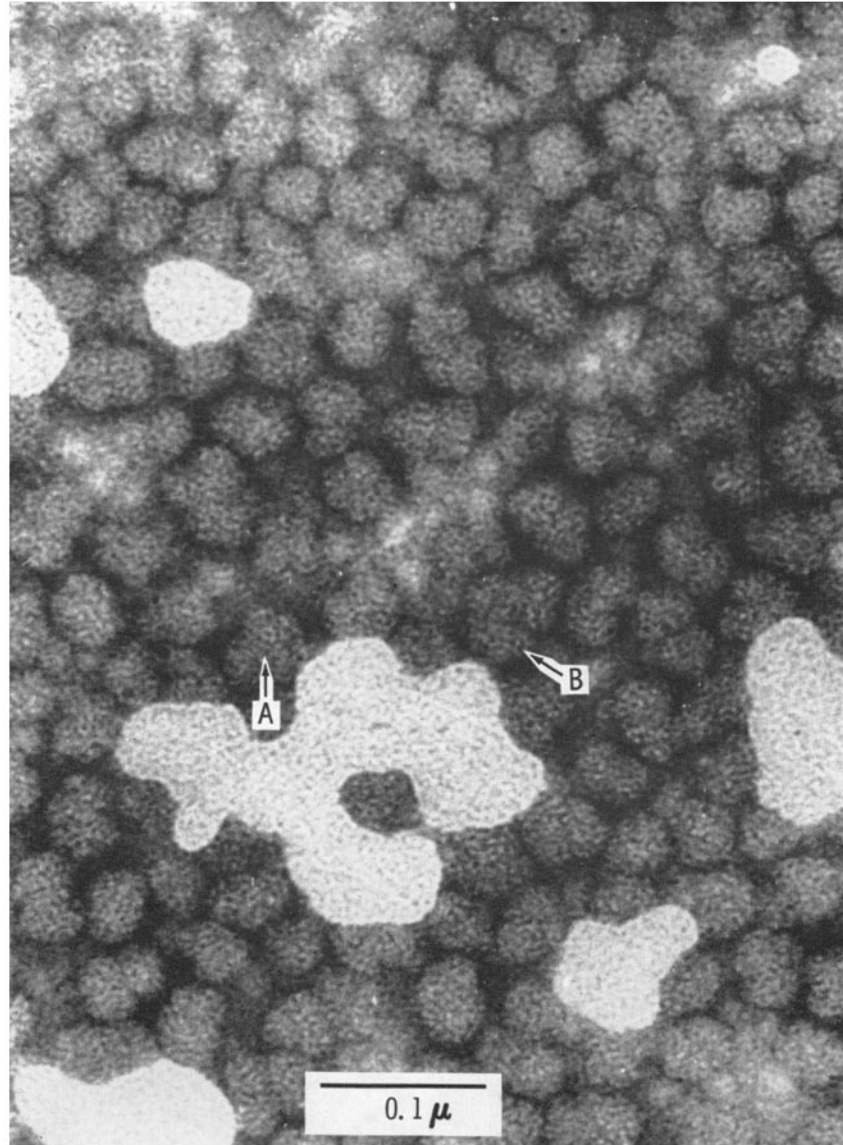


FIGURE 3 Electron micrograph of negatively stained glycogen collected at the level of 1.65 density from a *Tetrahymena* homogenate banded in CsCl. A, 35- to 40- $\mu$  particle; B, 2- to 3- $\mu$  particles. Original magnification, 300,000.

The apparatus also consists of a tube mount and a light source. The total volume of certain samples was collected in 1-ml aliquots, starting from the bottom of the tube, with a New Brunswick PA-56 pump.

Carbohydrate was measured by the phenol-sulfuric acid method (15). To 1 ml of sample containing 5 to 50  $\mu$ g of carbohydrate were added 1 ml of 5 per cent phenol and 5 ml of concentrated sulfuric acid. The optical density at 490  $m\mu$  was measured, after 1 hour at room temperature, using a Bausch and Lomb

Spectronic 20 spectrophotometer. A standard curve was prepared using reagent grade glucose. Glycogen was identified as the carbohydrate band at density 1.62 by comparing cell homogenates extracted with potassium hydroxide and precipitated with alcohol with untreated samples. Glycogen was also identified microscopically from treated and untreated samples.

Sedimentation coefficients were determined using the Spinco model E analytical ultracentrifuge.

Samples were prepared for microscopy by the

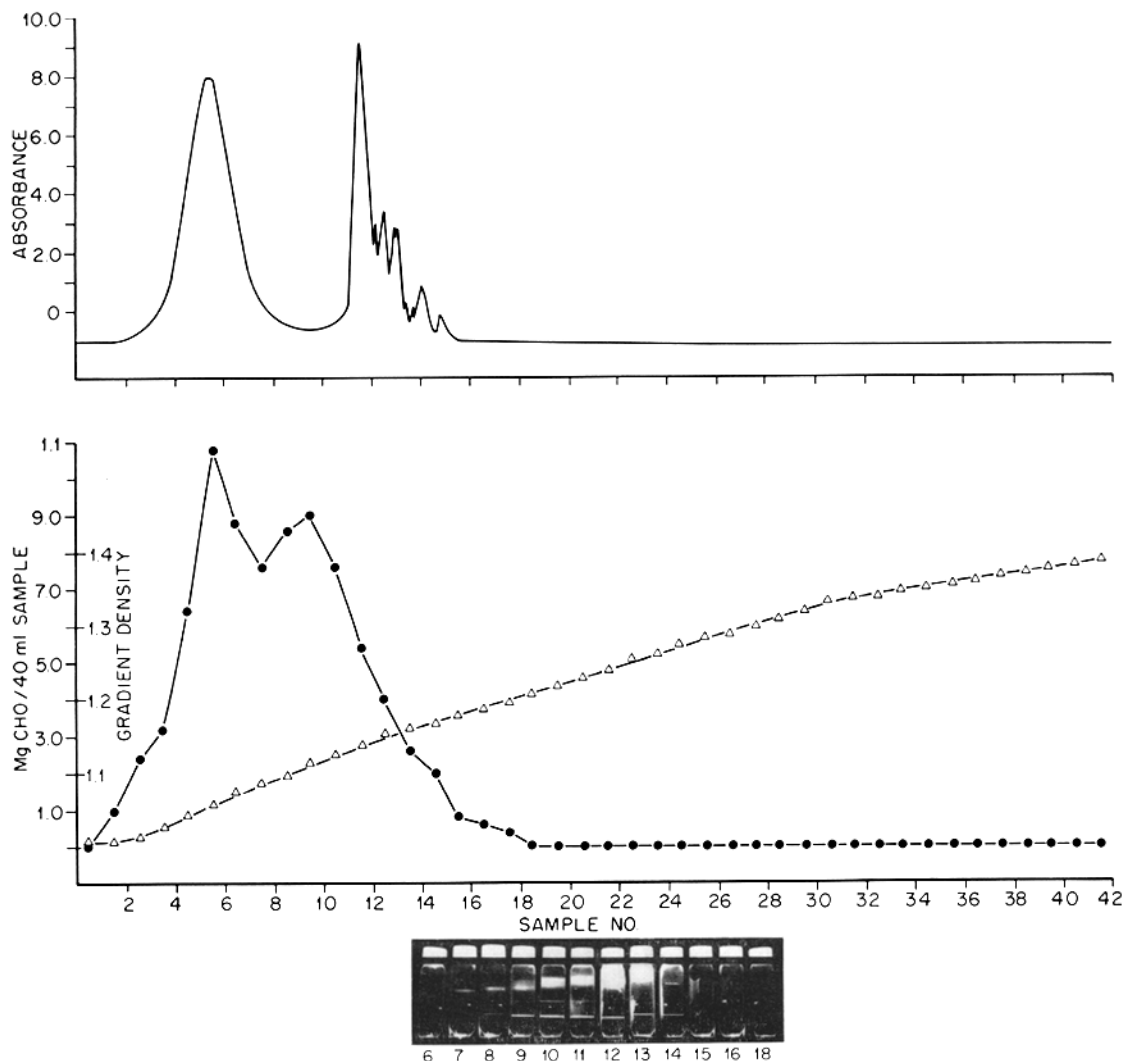


FIGURE 4 Distribution of *Tetrahymena* glycogen following centrifugation at 20,000 RPM for 30 minutes using a cesium chloride gradient in the B-IV rotor. Twenty ml of a 1:5 (v/v) homogenate in buffer was used as starting sample. Absorbance at  $260\text{ m}\mu$  was used to monitor the eluant. Soluble proteins (peak in sample 6) and membranes (peak in sample 12) were the major  $260\text{-m}\mu$  absorbing fractions. Glycogen turbidity absorbs only slightly under these conditions. Centrifuge tubes containing banded samples were photographed using scattered light. Banded glycogen is observed as the lower band in tubes 8 through 15. The absence of visible bands in tubes 6 and 7 indicates that either the particles are too small to be sedimented or are too small to scatter light even if present as a band of material. ●—● carbohydrate distribution,  $\triangle$ — $\triangle$  CsCl gradient density.

negative staining method of Brenner and Horne (16). A single drop of sample was placed on each of two carbon-coated Formvar specimen screens and allowed to stay on the screen for 20 to 30 seconds in a large Petri dish in the presence of osmium tetroxide vapor. The drop of sample was removed by touching

the edge of the screen with filter paper, and a drop of 2 per cent phosphotungstic acid (PTA) (adjusted to pH 7.0 with potassium hydroxide) was added immediately. The PTA was left on the screen for approximately 30 seconds, removed, and the screen was dried under ultraviolet light. Glycogen is constructed

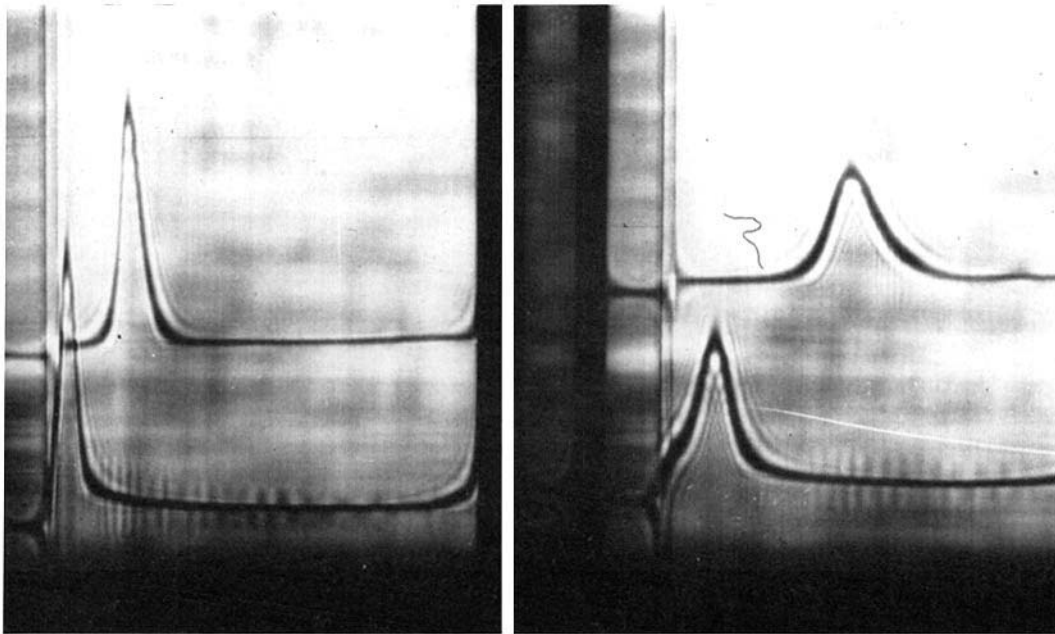


FIGURE 5 Sedimentation analysis of native glycogen and KOH-extracted glycogen. Native glycogen (top) was collected from zonal sample 10 after banding in CsCl. KOH-extracted glycogen (bottom) was obtained by boiling sample 10 in 20 per cent KOH for 40 minutes. Centrifugation was done in a Spinco model E ultracentrifuge at 20,410 RPM. Photos taken at 4 (left) and 12 (right) minutes.

from low-density elements and is observed microscopically with more difficulty than other materials of higher electron density.

Screens were examined with one or more of the following RCA electron microscopes: EML, EMU3-F, or EMU3-G.

## RESULTS

Native glycogen was isolated from other cell components of *Tetrahymena pyriformis* by banding the cell homogenate in a cesium chloride density gradient. The glycogen banded in a narrow zone near the base of the centrifuge tube and was well separated from other cell constituents (Fig. 1). Approximately 70 per cent of the total carbohydrate of the homogenate was banded at a density of approximately 1.65 (Fig. 2). The remaining carbohydrate was presumed to be glycogen, but of smaller size, which did not sediment under the centrifugation conditions employed (24,000 RPM for 3 hours). The minimum size of glycogen particles that sedimented completely under these conditions has not been determined. Little or no carbohydrate was associated with the membranous zone of the banded homogenate.

Glycogen recovered from the band at density 1.65 existed as particles with maximum diameters between 35 and 40  $\mu$  (Fig. 3). Certain of these were visibly subdivided into 20 to 25  $\mu$  particles. The smallest particles observed were spherical  $\gamma$  particles with diameters of 2 to 3  $\mu$ . The larger particles were formed from the aggregation of many of these  $\gamma$  units. The 2 to 3  $\mu$  and 35 to 40  $\mu$  particles were quite uniform in size. Zonal centrifugation in a gradient density, which separates mixtures of different glycogen particle sizes into zones of uniform particle size, demonstrated the uniformity of the 35 to 40  $\mu$  particles, which sedimented as a single zone away from the starting boundary (Fig. 4). A second carbohydrate zone, which did not migrate from the starting boundary, was also noted. The extent of glycogen sedimentation was also determined by banding each of the collected fractions in CsCl and photographing the banded samples with scattered light. Under the conditions of the experiment, no glycogen was observed, chemically, beyond sample 18. The particulate glycogen collected from banded samples 8 and 14 was similar in size and structure to that banded directly from whole homogenates. These

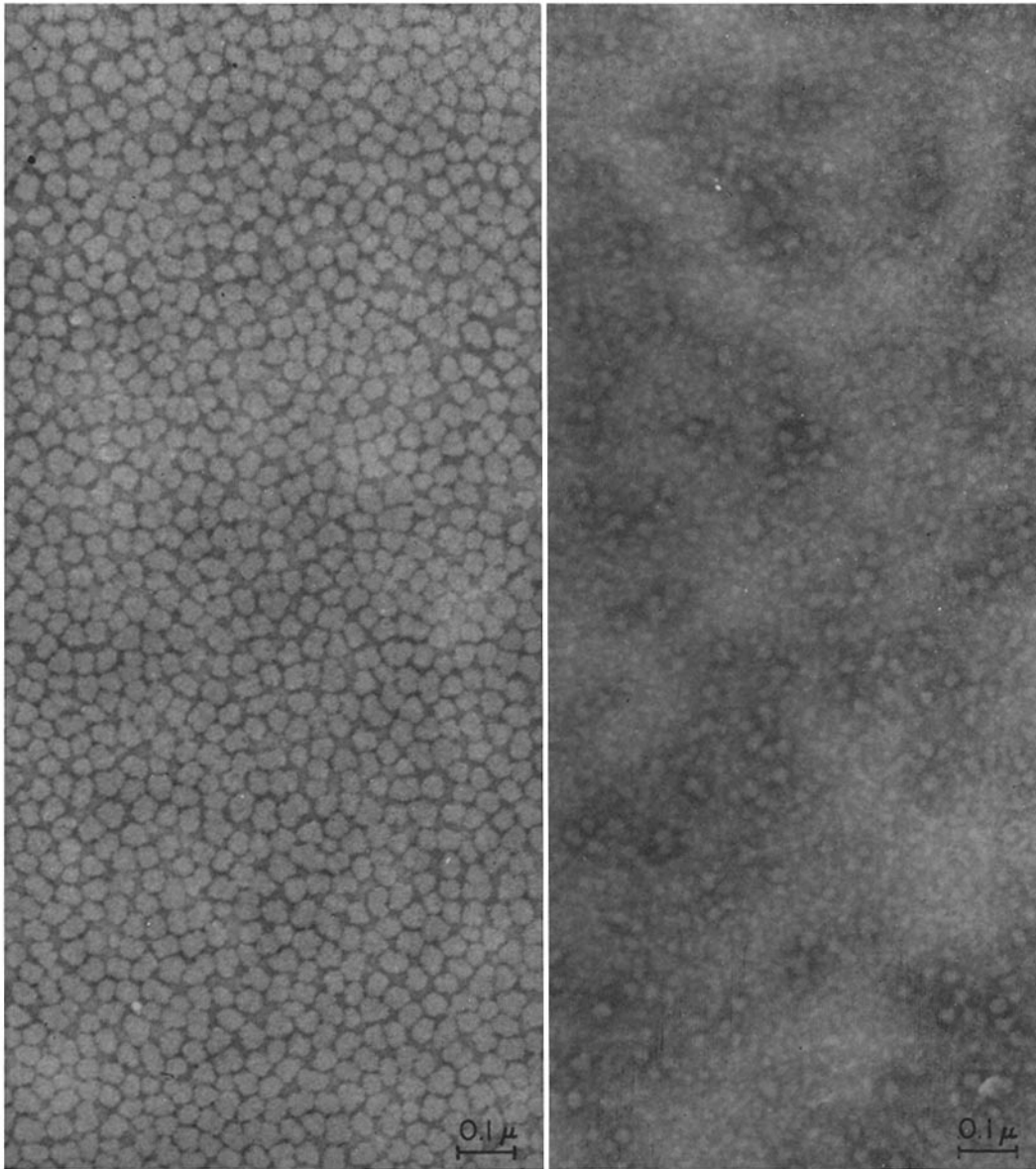


FIGURE 6 Electron micrographs of native (left) and KOH-extracted glycogen (right). Same preparations as used for the sedimentation analysis shown in Fig. 5. Samples were dialyzed before negative staining. Original magnification, 80,000.

samples were representative of the zone whose peak occurred in sample 10. The carbohydrate peak which remained at the starting zone presumably contained the smaller units of glycogen, and possibly other carbohydrates, which did not move under the centrifugation conditions employed. The  $S_{20,w}$  value of glycogen collected

from sample 10 was found to be 214 when measured in the Spinco model E analytical ultracentrifuge (Fig. 5). Uniformity of particle size from this sample was indicated in the sedimentation pattern.

The sedimentation coefficient of 35- to 40-m $\mu$  glycogen particles collected in sample 10 following zonal centrifugation was reduced from 214 to 64.7

TABLE I

*Carbohydrate and Protein of Tetrahymena*

Cells were grown on the repetitive cycle described in Methods. At the times indicated, 1.5-liter samples were removed from the stock culture. The warm period of the cycle started at 0930 hours and the cold period at 1200 hours. The cell population in the stock culture went from about 70,000 to 130,000 per ml during the warm period. Each sample was banded in CsCl for 3 hours at 24,000 RPM as described under Methods. The percentage banded was measured from plots of each sample, similar to the plot shown in Fig. 2.

Time	Total cells	Total	Total	Carbohy-	Total
	harvestd	protein	carbohydrate	drate	glycogen
	× 10 <sup>6</sup>	mg	mg	Proteins	banded
					per cent
0930	10.7	76	20.6	.27	69
1030	11.2	78	20.0	.26	68
1130	16.2	76	17.0	.23	65
1230	20.0	79	18.1	.23	66
1330	20.0	79	17.0	.22	67

by boiling in KOH for 40 minutes (Fig. 5). The relationship between particle diameter ( $d$ ) and sedimentation coefficient ( $S$ ) is  $S = d^2$ . Therefore, the reduction of 214 $S$  particles with 35- to 40- $\mu$ m diameters to 64.7 $S$  particles by KOH digestion should result in particles having a diameter of 20  $\mu$ m. This was the maximum size observed microscopically (Fig. 6). The reduction in particle size was accompanied by an increase in polydispersion. The decrease in sedimentation homogeneity in the digested sample suggests the presence of particles smaller than 20  $\mu$ m. These smaller units were also observed microscopically (Fig. 6).

It was of interest to know what changes in glycogen occurred during cell division. The carbohydrate and protein contents of synchronized *Tetrahymena* populations, sampled before, during, and shortly after the warm period of the temperature cycle treatment, are summarized in Table I. The cell number per sample nearly doubled during the 2½-hour warm period, but the protein content remained unchanged. The slight decrease in total carbohydrate resulted in a 15 per cent reduction in the carbohydrate/protein ratio. There was no change in the ratio of banded carbohydrate and nonbanded carbohydrate in CsCl gradients, indicating that all glycogen particle sizes are used.

The results at 1330 hours indicated that carbohydrate and protein syntheses were negligible during the first 1.5 hours of the ensuing cold period. No morphological differences were observed in the glycogen of any of these samples following banding in CsCl.

Glucose incorporated into *Tetrahymena* during the entire 10-hour cold period of the repetitive temperature cycle was distributed uniformly throughout all carbohydrate fractions (Fig. 7). Cells harvested from 500 ml of culture fluid contained 18 mg of carbohydrate, of which 1.35 mg was incorporated from the glucose added to the medium. Approximately 75 per cent of the C<sup>14</sup> activity and carbohydrate was localized in the band at density 1.65. Similar carbohydrate distribution was noted when cells were exposed to radioactive glucose during the warm period of the cycle, but the rate of uptake in the warm period was higher (Fig. 8). The distributions of radioactivities and carbohydrate within tubes containing banded samples were similar regardless of the length of exposure to C<sup>14</sup>-glucose. The rapid appearance of the glucose label uniformly distributed in all carbohydrates indicated direct addition to both the banded glycogen and the non-banded carbohydrates. Glucose incorporation was also measured following the isolation of glycogen by the classical KOH extraction—alcohol precipitation method. The radioactivities and carbohydrate distributions following banding in CsCl were similar to those noted in non-extracted glycogen (Fig. 9). Therefore glucose, incorporated into glycogen, was stable in boiling KOH. The presence of small amounts of carbohydrate throughout the centrifuge tube, containing the glycogen extracted by KOH, indicated that small glycogen particles were present and did not band under the relatively short times used. The similarity in the carbohydrate distribution in banded samples of extracted glycogen and non-extracted glycogen (compare Figs. 7 and 9) indicates that the carbohydrate fraction of non-extracted homogenates which did not band under the conditions of these experiments contained smaller glycogen particles.

## DISCUSSION

Recently a two-step centrifugation procedure has been developed for the isolation of native glycogen (5). The first step is a rate separation which sediments mixtures into zones of uniform particle size, and the second is an isopycnic one which



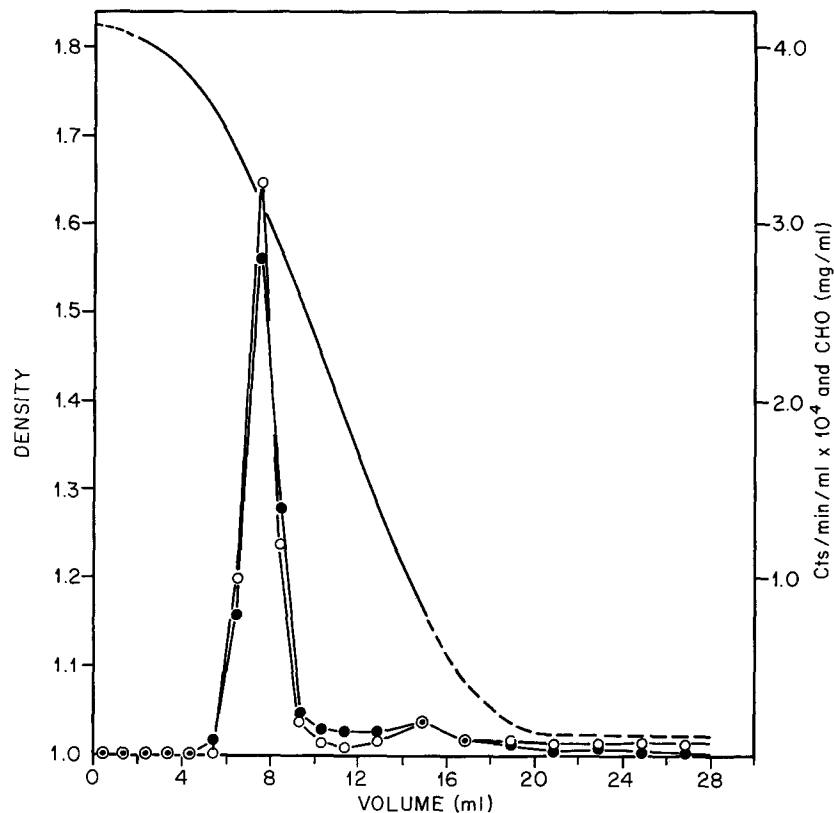


FIGURE 7 Carbohydrate and radioactivity distributions in *Tetrahymena* homogenates banded in CsCl. A 500-ml culture was removed from the stock at the beginning of the cold period and labeled with  $20 \mu\text{C}$  of glucose. The cells were harvested at the end of the cold period.  $8 \times 10^7$  cells, containing 18 mg carbohydrate, were frozen, thawed, homogenized, and diluted to 40 ml. Sixteen ml were used as sample for banding. Centrifugation was done at 24,000 RPM for 3 hours. ●—● carbohydrate, ○—○ radioactivity.

separates particles into zones of uniform density. Rat liver glycogen isolated by this method was uncontaminated by other cellular particles when examined both microscopically and spectrophotometrically. Liver glycogen existed as a continuous spectrum of particle sizes up to  $200 \text{ m}\mu$  with sedimentation coefficients up to several thousand. Native *Tetrahymena* glycogen, isolated by the same method, occurred with a maximum size of  $40 \text{ m}\mu$  and a sedimentation coefficient of 214.

Several methods have been used to extract glycogen from cells and tissues. These include boiling in KOH (17), extraction with trichloroacetic acid (18), hot water (19), and cold water (4). The sedimentation coefficients and molecular weights of liver glycogen are much reduced by treatment with either KOH or trichloroacetic acid (20). Native particulate glycogen was first

isolated centrifugally from liver by Lazarow (21), but his procedure did not indicate the continuous spectrum of sizes or permit collection of samples containing only particles of uniform size.

Degradation of native *Tetrahymena* glycogen by boiling in KOH was observed by both microscopy and centrifugation procedures. Forty-millimicron glycogen particles with a sedimentation coefficient of 214 were broken down to particles of  $20 \text{ m}\mu$  and less with a maximum sedimentation coefficient of 64.7. Liver glycogen extracted with hot KOH has been reported to have a lower molecular weight than *Tetrahymena* glycogen extracted by the same method, in spite of its larger native size (2). It is not known why a larger product is obtained by KOH digestion of glycogen from *Tetrahymena*. One possibility indicated from the present studies is that many of the  $40\text{-m}\mu$  particles

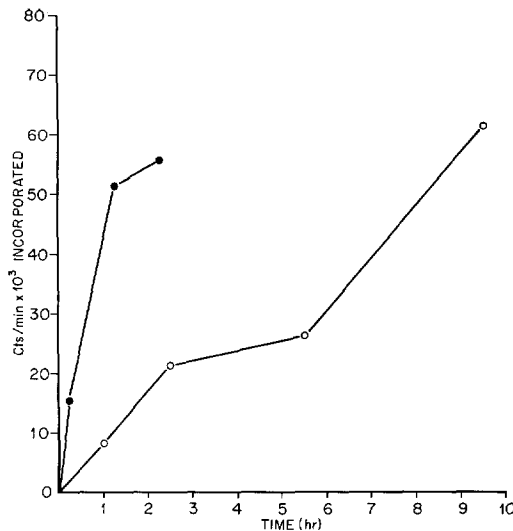


FIGURE 8 Rate of incorporation of radioactive glucose into glycogen. Label was added to subcultures of the stock culture as described in Methods. ●—● warm period, ○—○ cold period.

in *Tetrahymena* appear to be undivided, whereas in liver the structure is characterized by aggregates of uniform 20 to 25  $m\mu$  particles. Consequently, KOH digestion of liver could yield somewhat smaller products.

In both rat liver and *Tetrahymena*, the larger glycogen particles were generally formed from aggregates of 20- $m\mu$  particles, although many 40- $m\mu$  particles from *Tetrahymena* did not appear to be so subdivided. The 20- to 40- $m\mu$  particle has been considered the maximum size allowable in the branching structure proposed for glycogen (22). This is in the size range of one of the unit particles found in both liver and *Tetrahymena*. However, growth of particle size in liver presumably occurs by the continued aggregation of these 20- to 40- $m\mu$  particles, whereas in *Tetrahymena* it does not. Therefore, whatever process is responsible for continued growth of particle size in liver, such as irregular branching (8), does not occur in *Tetrahymena*. However, except for the larger particle sizes in liver, the physical and morphological properties of glycogen from both are similar. The two glycogens are also chemically similar in that both are branched  $\alpha$ -1,4-glycogen, containing 8-10 per cent  $\alpha$ -1,6- interchain linkages (23).

Three levels of structural organization in liver

glycogen, called  $\alpha$ ,  $\beta$ , and  $\gamma$  particles (6), have been described morphologically. In *Tetrahymena*, similar dimensions of 2 to 3  $m\mu$  for the  $\gamma$  and 20  $m\mu$  for the  $\beta$  particles have been seen. It was only the size range for  $\alpha$  particles which differed. Although in certain cases the larger particles in *Tetrahymena* did not appear to be formed from 20  $m\mu$  particles, their existence was indicated by KOH extraction. Boiling in KOH reduced the size of the 40- $m\mu$  clusters to a maximum size of approximately 20  $m\mu$ , with no intermediate sizes noted. The 20- $m\mu$  particle undoubtedly represents the size normally used for the physical studies on glycogen when KOH is used in the extraction procedure (2). The sedimentation coefficient reported here for KOH-extracted glycogen was similar to that reported for other glycogens treated the same way (24). The morphology of KOH-extracted glycogen of *Tetrahymena* was also similar to that of KOH-extracted glycogen of rat liver (8, 25).

Correlation of the observed structural units with the models of glycogen structure proposed from chemical data is difficult (1). Using the molecular weight of anhydrous glucose and a density of 1.62, the volume occupied by a single glucose residue is approximately  $1.66 \times 10^{-22}$  cc. A spherical 3- $m\mu$  particle could contain approximately 85 glucose residues. The 3- $m\mu$  particle could be tentatively assumed to represent the exterior side chains of the branching model for glycogen with a certain amount of associated structure. The 2- $m\mu$  particles would contain 25 residues and would more nearly represent the number of glucose residues in single chains. Similar calculations have been applied to rat liver glycogen (5). No chemical counterpart of the 20- $m\mu$  particles can be postulated except for the maximum size cluster proposed by Pollard (22).

The amount of carbohydrate per cell at the end of the cold period of the repetitive temperature cycle and at a population density of 70,000 cells per ml was  $1.92 \times 10^{-4}$   $\mu$ g, and the carbohydrate/protein ratio was 0.27. The value for carbohydrate per cell was similar to that reported by Scherbaum and Levy (9). Also, the carbohydrate/protein ratio was similar to that measured at the end of a different temperature cycle treatment used by the above authors to induce synchrony. This is a higher ratio than is found in exponentially growing cells (9).

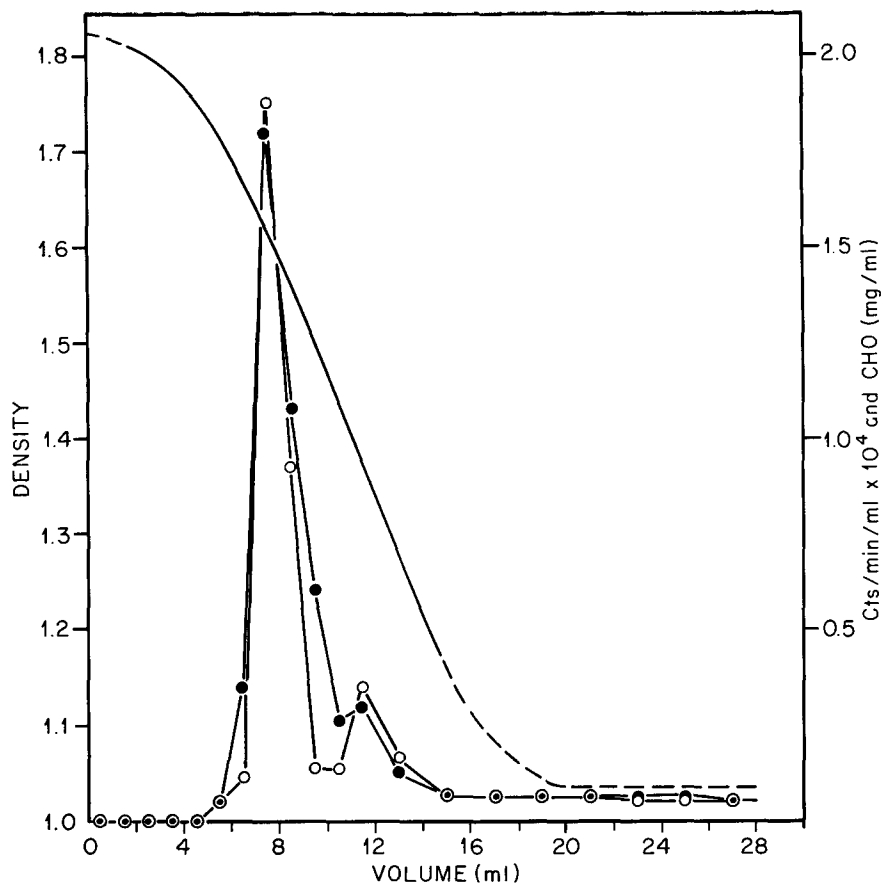


FIGURE 9 Carbohydrate and radioactivity distributions in KOH-extracted glycogen banded in CsCl. Glycogen was extracted from cells collected at the end of the cold period. Cells had been exposed to radioactive glucose for the entire 12-hour temperature cycle. ●—● carbohydrate, ○—○ radioactivity.

Although glucose is not needed for the growth of *Tetrahymena*, it was rapidly incorporated into glycogen when added to the culture medium. Glucose was incorporated during both the cold and warm periods of the repetitive temperature cycle. However, a net reduction of carbohydrate occurred during the warm period. Greater utilization would be expected during the warm period, since the oxygen tension is much reduced during this period. The cells presumably use part of their glycogen stores during these periods of anaerobiosis (2). Glucose was incorporated uniformly into glycogen of all molecular sizes, even during the shortest time intervals measured. The addition of glucose to all particle sizes has also been reported for rat liver glycogen (26). The similarities in

structure and chemical composition of liver and *Tetrahymena* glycogens would indicate that glucose addition in *Tetrahymena* occurs also at the non-reducing end of all particles (26).

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