

ISOLATION, ULTRASTRUCTURE, AND BIOCHEMICAL CHARACTERIZATION OF GLYCOGEN IN INSECT FLIGHT MUSCLE

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ABSTRACT

Glycogen from flight muscle of the blowfly, *Phormia regina*, has been characterized ultra-structurally and biochemically. *In situ*, glycogen is in the form of rosettes, which vary in size with diameters of up to 0.1 μ . Sedimentation analysis of pure glycogen, isolated by mild buffer extraction, reveals a polydisperse molecular weight spectrum, with larger particles having molecular weights of 100 million. Treatment of native glycogen with alkali, under conditions usual for the extraction of the polysaccharide from tissues, results in a 5- to 10-fold reduction in molecular weight, as well as a chemical alteration of the molecule. Flight muscle phosphorylase has a lower affinity for native than for alkali-treated glycogen. The maximum velocity of the enzyme is also lower with native substrate. The apparent K_m for inorganic phosphate is higher with native glycogen as cosubstrate. These kinetic differences with native and partially degraded glycogen demonstrate the importance of using the natural substrate in studies of biochemical control mechanisms.

When the blowfly initiates flight, an intense rate of glycogenolysis in the flight muscle is triggered (1). The mechanisms of the control of glycogen utilization in the muscle is being studied. As part of this study, glycogen from blowfly flight muscle has been examined. This is of significance because of the observations that the physical and biological properties of the polysaccharide depend on the tissue of origin, the metabolic state of the tissue, and the method of extraction (2, 3).

Heretofore, glycogen from insects has not been characterized. In this paper, we report the isolation by mild buffer extraction of glycogen from flight muscle of the blowfly, *Phormia regina*, and describe its biophysical and biochemical properties. The polysaccharide has been found to be

polydisperse, with a continuous spectrum of particle sizes up to 200 $m\mu$, with the largest particles having molecular weights of 100 million. Marked differences in the kinetics of flight muscle phosphorylases with native and partially degraded glycogens, as substrates, indicate the importance of using native glycogen in studies of regulatory mechanisms.

METHODS AND MATERIALS

Raising of Insects

Blowflies, *Phormia regina*, were maintained in laboratory culture at 23°C and 12 hr of light per day. The larvae were reared on horsemeat, and the adult flies were fed powdered milk and sucrose. 7 days fol-

lowing eclosion, flies, without regard to sex, were frozen in liquid nitrogen. The thoraces were isolated and maintained in the frozen state (-80°C) until used for the purification of glycogen.

Assay of Glycogen

Glycogen was determined by enzymatic methods (4-6) that enable degradation and estimation of products, glucose and glucose-1-phosphate, to be carried out simultaneously. To 0.085 ml of sample, containing from 0.005 to 0.010 mg of glycogen, was added 0.065 ml of a degradation mixture to give the following final concentrations: 0.4 M phosphate buffer, pH 7.2, 6.7 mM adenosine monophosphate (AMP), 7.0 mM ethylenediaminetetraacetic acid (EDTA), 0.2% bovine serum albumin, 0.35 units of 8-times recrystallized rabbit skeletal muscle phosphorylase b, and 40 units of amylo-1,6-glucosidase. After incubation for 2 hr at room temperature, glucose and/or glucose-1-phosphate were assayed spectrophotometrically by the addition of 0.47 ml of a reaction mixture to give the following final concentrations in a cuvette volume of 0.64 ml: 1.6 mM ATP, 13 mM MgCl_2 , 0.48 mM NADP, 8.6 μM glucose-1,6-diphosphate, 0.052 M imidazole, pH 7.2. Following the addition of glucose-6-phosphate dehydrogenase and phosphoglucomutase or hexokinase, as required, NADPH formation was measured at 340 $\text{m}\mu$.

For the determination of glycogen in tissues, samples were digested at 100°C for 30 min in 1.0 N KOH. After cooling, the mixture was neutralized with 2.5 M phosphoric acid to a pH between 7.0 and 7.5 by using bromothymol blue as an external indicator. Glycogen was estimated enzymatically, as described above.

Electron Microscopy of Flight Muscle Glycogen In Situ

Flight muscle of 8-day-old flies was fixed in 2.5% glutaraldehyde in 0.1 M Sorensen's phosphate buffer (7), pH 7.4, for 2 hr at 4°C and then washed in two changes of buffer. The tissue was treated for 2 hr with 0.5% α -amylase in 10 mM phosphate buffer, pH 7.4, containing 20 mM NaCl (8). Control tissues were incubated in buffer without amylase. The tissues were rinsed in several changes of 0.1 M phosphate buffer, pH 7.4, stained with osmium tetroxide in Veronal-acetate buffer, dehydrated in graded methyl alcohols, and embedded in Epon 812. Silver sections were cut on an LKB ultratome III (LKB Instruments Inc., Rockville, Md.) with a diamond knife, and were placed on an uncoated 300 or 400 mesh copper grid. The sections were stained with 3% uranyl acetate in 50% ethanol for 5 min, followed by lead citrate for 5 min. The sections were examined in an RCA EMU-3H electron microscope.

1- μ sections were stained by the periodic acid-Schiff technique (PAS) to localize the glycogen for light microscopy.

Electron Microscopy of Isolated Flight Muscle Glycogen

Purified glycogen was examined by negative contrast after preparation of the samples, as follows. 1 volume of a 0.1% glycogen solution was mixed with 4 volumes of 2% potassium phosphotungstate, pH 6.9. The mixture was sprayed onto a 200-mesh copper grid coated with a pure carbon film peeled from mica. The films were made wettable by ion bombardment (9). Grids were examined with a Siemens Elmiskop I electron microscope.

Sedimentation Analysis of Glycogens

Sedimentation coefficient distributions of glycogens were determined with the Spinco Model E analytical ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). Runs were made at approximately 9300 rpm in the An D rotor. The measurement of plates, corrections for effects of concentration dependence, temperature, viscosity, and the normalizing routine have been described previously (3).

Assay of Protein

Protein was assayed by the method of Lowry et al. (10) following dialysis of the samples to remove interfering substances. When necessary, blanks containing sample without reagents were run to correct for turbidity. In the case of purified glycogen samples, the protein content was estimated from the analysis of amino nitrogen in total hydrolysates (11).

Materials

Phosphorylase was purified from rabbit muscle, obtained from Pel-Freez, by the procedure described by Fischer and Krebs (12). Amylo-1,6-glucosidase was prepared by the method of Bueding and Hawkins (4). Crystalline hexokinase, glucose-6-phosphate dehydrogenase, and phosphoglucomutase, as well as glucose-1,6-diphosphate and NADP were obtained from Boehringer Mannheim Corp., New York. AMP and ATP were purchased from P-L Biochemicals, Inc., Milwaukee, Wis. Imidazole, Grade III, and EDTA were bought from Sigma Chemical Co., St. Louis, Mo. Enzyme grade ammonium sulfate and "ultra pure" Tris were obtained from Mann Research Labs. Inc., New York. α -Amylase, 2-times crystallized, was purchased from Worthington Biochemical Corp., Freehold, N.J.

RESULTS

Glycogen in Flight Muscle, In Situ

Electron microscopic examination of flight muscle revealed that glycogen is located mostly in the intermyofibrillar sarcoplasm, with scattered deposits on the myofibrils. The glycogen is in the form of alpha particles or rosettes, which vary in size with diameters of up to 0.1μ (Fig. 1). The individual components comprising the rosettes, the beta particles, were not clearly delineated. Incubation of the flight muscle with α -amylase, following fixation, resulted in the disappearance of the glycogen rosettes, leaving only an amorphous network of electron-opaque material in this region.

1- μ sections stained with PAS showed a positive reaction in the intermyofibrillar region when examined by light microscopy. Sections examined following treatment with α -amylase were no longer PAS positive.

Isolation of Flight Muscle Glycogen

Frozen thoraces, usually 50 g, containing 10–15 mg glycogen per g wet weight (1.0–1.5%), were homogenized in a Waring blender with 10 volumes (w/v) of 0.25 M sucrose, 10 mM EDTA, 10 mM Tris, pH 7.4. The homogenization and all subsequent procedures were performed at 0–3°C. The homogenate was filtered through cheesecloth and centrifuged at 10,000 *g* for 10 min. The supernatant, containing 80% of the total glycogen, was filtered through glass wool into a chilled flask containing 0.25 volume of 0.5 M EDTA, 1.0 M glycine, pH 10.0. The glycogen was completely sedimented from this extract, pH 9.8, by centrifugation for 4 hr at 105,000 *g*. Following decantation of the supernatants, the gelatinous pellets were scraped from the tubes and redissolved in a volume of 0.1 M EDTA, 0.2 M glycine, pH 10.0, which was equal to the original weight of the tissue. The glycogen was then precipitated by the addition of 1 volume of ethanol, washed twice with cold 100% ethanol, and dried *in vacuo*.

The dry powder, 40% glycogen by weight, was further purified by a modified Sevag procedure (13) by redissolving in 0.1 M EDTA, 0.2 M glycine, pH 10.0, 200 ml per g powder, centrifuging at 5,000 *g* for 5 min to remove denatured protein, and shaking vigorously for 5 min with $\frac{1}{2}$ volume of a mixture of chloroform: 1-octanol,

3:1. The supernatant aqueous phase was removed by aspiration from the interphase and lower solvent phase after centrifugation at 180 *g* for 5 min and was filtered through a coarse sintered glass funnel. Care was taken not to remove any of the proteinaceous interphase. The treatment with the organic solvent mixture was repeated for progressively longer periods (1, 2, 4, 8, and 16 hr) by using a mechanical shaker. The treatment for 8- and 16-hr periods was repeated until the interphase was completely free of denatured protein impurities following low speed centrifugation to separate the phases. The purified glycogen was isolated from the aqueous phase, following addition of LiBr, by precipitation with 1 volume of 100% ethanol, and centrifugation at 1,600 *g* for 15 min. The product was redissolved in distilled water and reprecipitated with ethanol several times to remove traces of EDTA and glycine. Addition of a few crystals of LiBr was necessary to achieve complete precipitation of the glycogen. Finally, the product was washed with 80% ethanol, twice with 100% ethanol, and dried *in vacuo*.

Chemical and Physical Properties of the Isolated Glycogen

The product obtained by the above procedure (Table I) was a white amorphous powder, containing less than 0.1% protein. On incubation with phosphorylase b, amylo-1,6-glucosidase, AMP, and inorganic phosphate (P_i), this material was degraded completely to glucose-1-phosphate (90.5%) and glucose (9.5%) (Table II).

Sedimentation analysis (2) of the pure glycogen revealed that its molecular weight spectrum was quite polydisperse (Fig. 2), with larger particles having molecular weights of 50–100 million. This finding was confirmed by electron microscopic examination of preparations that had been negatively stained with phosphotungstic acid (Fig. 1 *d*). In these micrographs, a continuous distribution of particle size was seen, ranging up to 0.24μ in diameter. Treatment of native glycogen with 1.0 N KOH at 100°C for 30 min, conventional procedures for the extraction of glycogen from tissues, resulted in a 5- to 10-fold reduction in molecular weight, as well as a chemical alteration of the molecule (Fig. 2, Table II).

Enzymatic degradation revealed that the degree of branching is not greatly different from that of glycogens obtained from a variety of

species (14). However, the outer branches are quite short; only 24.8% of the total glucosyl residues are released by phosphorylase alone (Table II). The apparent increase in outer branch length after hot alkali treatment, which has now been observed consistently with native glycogen from other tissues (M. Berman, E. Bueding, and M. Reissig, unpublished observations), probably reflects the availability of additional terminal residues that previously were resistant to enzymatic attack perhaps because of steric hindrance before the large glycogen particles were degraded by this drastic treatment.

The purified glycogen was completely free of amylase-like activity. When a 1% aqueous solution of the final product was incubated for 24 hr at room temperature, in the presence of penicillin and streptomycin to prevent microbial degradation, neither an increase in reducing sugar nor an alteration of the sedimentation coefficient distribution resulted. Amylase-like activity was bound to the partially purified glycogen (Table I), but the enzyme activity was removed by the repeated chloroform-octanol treatments. The use of high EDTA and high pH following the initial homogenization and throughout the purification procedure prevented any measurable enzymatic alteration of the glycogen structure.

Other Fractions of Glycogen

As noted in Table I, during the first centrifugation, at 10,000 *g*, 20% of the total tissue glycogen was sedimented with the large cellular particulate fraction. This sedimentation of glycogen appears to be due to mechanical occlusion, since the polysaccharide could be quantitatively extracted from the particulate fraction by repeated washing with homogenization medium followed by recentrifugation at 10,000 *g*. In routine purification experiments, this fraction of the total glycogen was discarded. This did not result in a preferential loss of any molecular weight species, as this fraction of glycogen, when purified, had the same sedimentation coefficient distribution as found for the bulk of the glycogen.

Approximately 20% of the flight muscle glycogen was insoluble in 10% trichloroacetic acid (TCA) solution and was firmly bound to the tissue proteins. This fraction of the total glycogen was removed during extractions with chloroform-octanol and, thus, was not present in the final product. Repeated dissolving in 0.1 N NaOH and

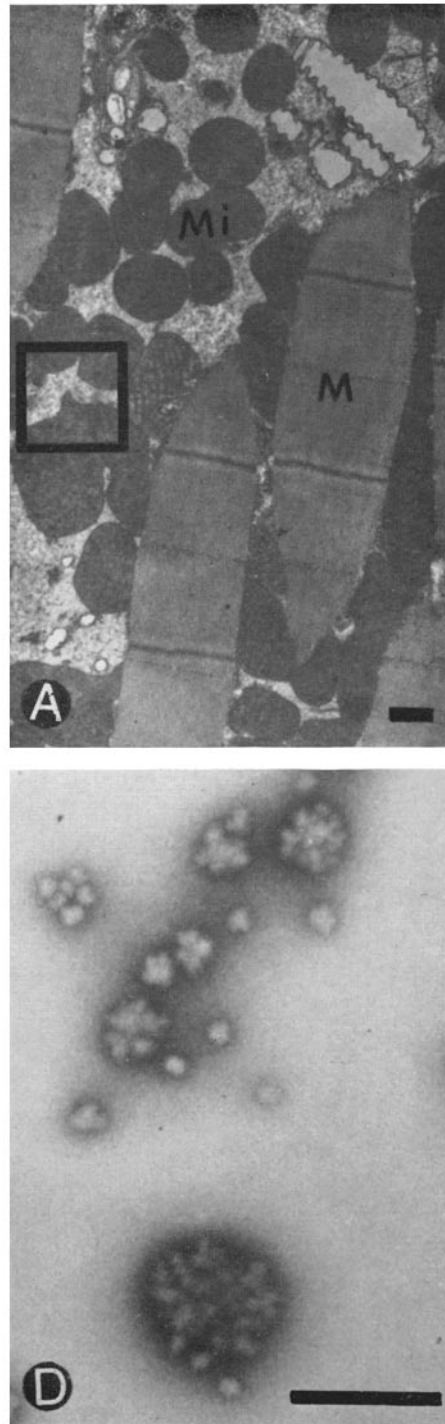


FIGURE 1 *A* and *D* See legend on opposite page.

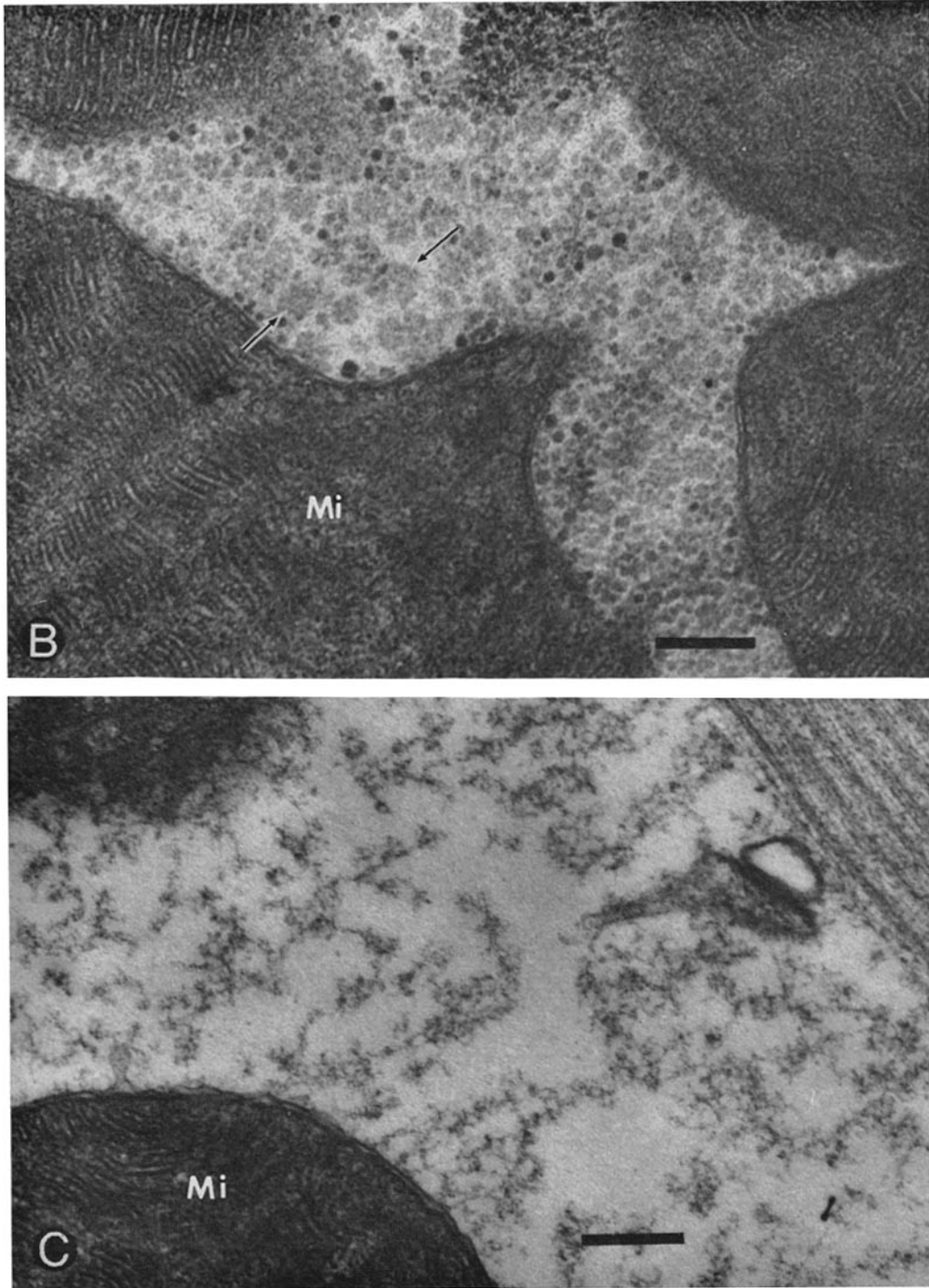


FIGURE 1 *A-D* Ultrastructure of flight muscle glycogen. (*A*) *Phormia* flight muscle incubated in 0.01 M phosphate buffer, pH 7.4, containing 0.02 M NaCl, at 37°C for 2 hr, as control for effect of α -amylase. Mitochondria (*Mi*) and glycogen rosettes are located between the myofibrils (*M*). $\times 5,820$; bar represents 1.0 μ . The area within the square is shown in higher magnification in *B*. (*B*) Glycogen rosettes (arrows) are prominent adjacent to the mitochondria (*Mi*). $\times 75,000$; bar represents 0.2 μ . (*C*) Insect flight muscle incubated with 0.5% α -amylase in 0.01 M phosphate buffer, pH 7.4, containing 0.02 M NaCl, for 2 hr at 37°C. Only amorphous electron-opaque material can be seen adjacent to the mitochondria (*Mi*). $\times 75,000$; bar represents 0.2 μ . (*D*) Rosettes of glycogen isolated by mild buffer extraction. Negatively stained with phosphotungstate. $\times 100,000$; bar represents 0.2 μ .

TABLE I
The Isolation of Native Glycogen

Fraction	Protein	Glycogen	Glycogen: Protein	% recovery
	<i>Total mg</i>			
Homogenate	—	600	—	100
10,000 g precipitate	—	120	—	20
10,000 g supernatant	1355	480	0.35	80
100,000 g precipitate or first crude powder	725	482	0.66	80
Aqueous phase following first CHCl ₃ -octanol shake	85	300	3.5	50
Final product	<0.2	180	>900	30
Proteinaceous CHCl ₃ -octanol phases (combined)	720	240	0.33	40

TABLE II
The Enzymatic Degradation of Flight Muscle Glycogen

Sample	Glucose-1-phosphate released by phosphorylase alone	Glucose-1-phosphate released by phosphorylase + amylo-1,6-glucosidase	Glucose released by phosphorylase + amylo-1,6-glucosidase
Native	24.8 ± 0.5	90.5 ± 0.2	9.5 ± 0.1
KOH-treated	31.0 ± 1.1	90.5 ± 0.2	9.5 ± 0.1

The values shown represent the means and their standard errors of the per cent of the total glucosyl residues contained in the glycogen sample. Preparation of the alkali-treated sample is described in the text.

precipitation with 10% TCA failed to release any glycogen. Detergents or 8 M urea did not prevent this fraction of glycogen from coprecipitating with the insoluble protein. However, treatment with 0.03% trypsin effected a rapid release of the glycogen into the TCA-soluble fraction; essentially all the bound glycogen was solubilized within 4 hr of incubation with the proteinase. Following trypsin digestion, the glycogen still appeared to be associated with peptide, since it was detained on Dowex 50-H⁺ (The Dow Chemical Co., Midland, Mich.). Several fractions were obtained by elution with increasing concentrations of HCl, each of which yielded several amino acids upon total hydrolysis in 6 N HCl. The nature of the association between this glycogen fraction and tissue protein was not characterized further. It was noted, however, that the amount of glycogen present in an aliquot, as assayed enzymatically, seemingly increased during trypsin digestion. This probably indicates that a portion of the protein-bound glycogen is not available to the degradative

enzymes used in the assay until after it is released by the trypsin treatment.

Glycogen in the blowfly abdomen, principally in the fat body, is utilized as a major energy reserve after depletion of the polysaccharide in the flight muscle (1). Analyses of mild buffer-extracted, purified, abdominal glycogen indicate that the degrees of branching and chain lengths of the glycogens from fat body and flight muscle were identical. Electron micrographs of the isolated fat body glycogen showed rosettes and subunits with size distributions similar to those of the muscle. Sedimentation analysis of one preparation revealed a polydispersed molecular weight spectrum at least equal to or, perhaps, slightly heavier than those from flight muscle.

The Kinetics of Flight Muscle Phosphorylase with Native and Alkali-Treated Glycogens

Phosphorylase a, purified from flight muscle of the blowfly (C. C. Childress and B. Sacktor,

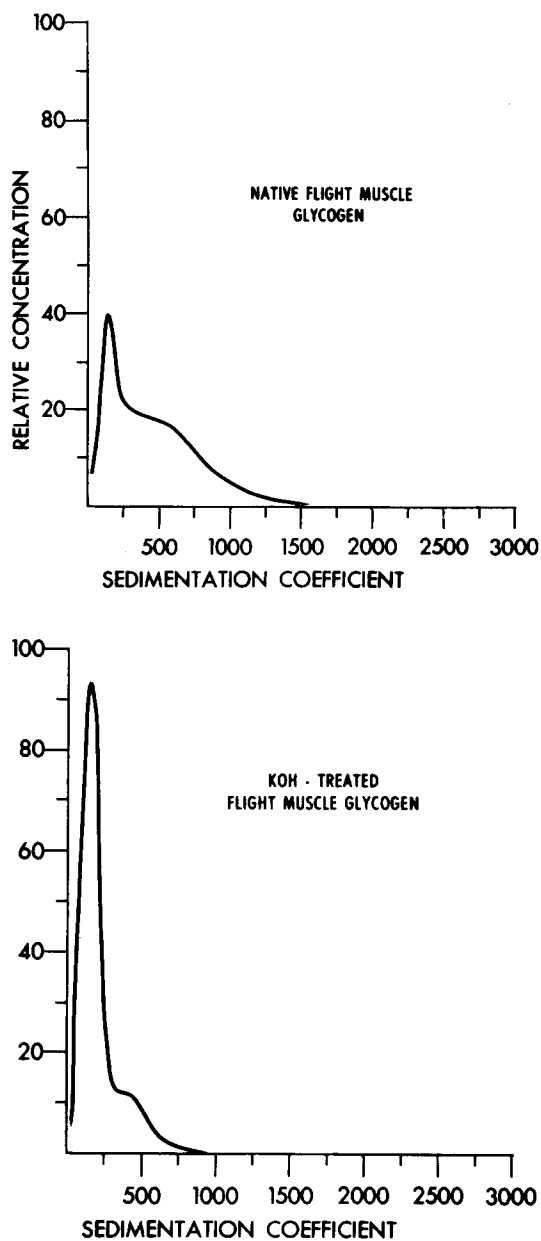


FIGURE 2 The sedimentation coefficient distributions of native and alkali-treated glycogens.

submitted for publication), exhibited a lower affinity for native polydisperse glycogen than for the same substrate following its exposure to 1.0 N KOH for 30 min at 100°C (Fig. 3). The apparent K_m values are 0.09 and 0.29 mM for the alkali-treated and native glycogens, respectively, in the presence of saturating levels of inorganic phos-

phate (P_i) and AMP. A much greater difference between K_m values for the two substrates was observed at low AMP levels, 0.27 as compared to 2.7 mM. The value for maximum velocity with the native substrate is approximately 50% of the value obtained with alkali-treated glycogen or

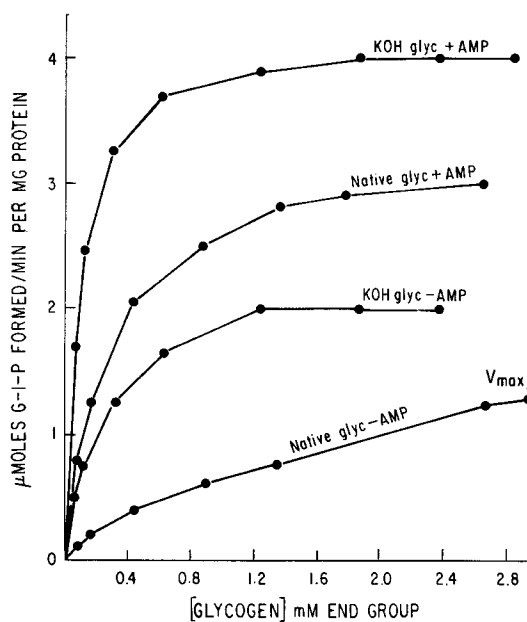


FIGURE 3 Initial velocity of flight muscle phosphorylase a as a function of the concentration of native and alkali-extracted glycogens. The concentration of P_i was 80 mM and of AMP, when present, was 1 mM.

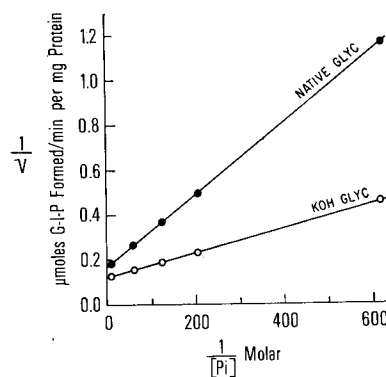


FIGURE 4 Initial velocity of phosphorylase a as a function of P_i concentration at saturating levels of native and KOH-extracted glycogens. The concentration of both glycogens was 2.5 mM expressed as end groups at the nonreducing ends of the chains. The AMP concentration was 0.16 mM.

commercially available shellfish glycogen. The lower V_{\max} is not due to the presence of an inhibitor in the native glycogen preparation, since a mixture of native and alkali-treated glycogens resulted in the velocity attainable with KOH-glycogen alone.

It was also found that the affinity of flight muscle phosphorylase a for P_i was dependent upon the nature of the glycogen present as cosubstrate (Fig. 4). In the presence of saturating levels of AMP and glycogen, the apparent K_m values for P_i are 4.5 and 9.5 mM, respectively, for KOH-treated and native glycogens.

DISCUSSION

Flight muscle glycogen exists in the form of particles ranging in size from 0.03 μ to large rosettes, approximately 0.2 μ in diameter. Glycogen of this type, having a polydisperse distribution of molecular size, has been found in the muscle of the invertebrate, *Ascaris lumbricoides* (15). On the other hand, glycogen from mammalian skeletal and cardiac muscle appears to be present only in single particle form (16, 17).

Sedimentation and chemical analyses demonstrate that native glycogen, prepared by mild buffer extraction, differs significantly from glycogen partially degraded by treatment with alkali. 56% of the native glycogen has sedimentation coefficients greater than 300. On the other hand, only 18% of the KOH-treated polysaccharide have S-values over 300. Alkali treatment also

results in an apparent increase in outer branch length; approximately 31% of the glucose-1-phosphate is released by phosphorylase alone, in contrast to 25% with native glycogen.

Phosphorylase a from blowfly flight muscle exhibits unique kinetic behavior with native glycogen as compared with alkali-treated glycogen. These findings, as well as those with phosphorylase and native glycogen from mammalian liver (3), suggest that native glycogen is bound to phosphorylase with less affinity than the smaller alkali-degraded substrate, perhaps as a result of steric hindrance. Furthermore, the enzyme-glycogen complex apparently has a lower affinity for P_i in the presence of native glycogen than in the presence of glycogen prepared in KOH. This result is consistent with other observations that lowering the level of glycogen increases the K_m value for P_i (C. C. Childress and B. Sacktor, submitted for publication). Thus, it appears that the use of native glycogen has the same effect upon the K_m for P_i as lowering the glycogen concentration. Differences of this kind, between native substrate and substrate artificially modified by its isolation procedure, demonstrate the importance of using the natural substrate in kinetic measurements that attempt to predict parameters involved in the control of enzyme activity in vivo.

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