A Mild Procedure for the Isolation of Polydisperse Glycogen from Animal Tissues*

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More than 100 years ago, Claude Bernard (1) isolated glycogen from dog liver by a technique which involved prolonged heating of the tissue in hot alkali. Subsequently, this extraction procedure has been modified (2, 3) and has become a standard technique for the isolation of glycogen. A different method, which employs trichloroacetic acid, has been utilized by other investigators during the last few decades (4, 5). Extraction of glycogen also has been performed with hot water (6). Many variations of the methods mentioned above have been used for this purpose. Each of the products obtained with these three methods (alkali, acid, hot water) differs from the others with regard to its molecular weight. Other differences have been reported also (7).

Glycogen extracted with cold water has molecular weight values many times higher than that obtained with any of the above procedures (8–11). Cold water-extracted glycogen exhibits significant differences in its behavior toward enzymes when compared with glycogen extracted with strong alkali, acid, or heat (12). The present paper is concerned with a less drastic, quantitative method of extraction of glycogen, with the use of cold water.

EXPERIMENTAL PROCEDURE

Materials and Methods

Muscle of the parasitic nematode *Ascaris lumbricoides* was obtained as described previously (13). Livers were removed from well fed animals under phenobarbital anesthesia (100 mg of phenobarbital per kg, administered intravenously). Glycogen was determined by the use of the anthrone (14), phenolsulfuric acid (15), or a specific enzymatic method (16). Sedimentation coefficient distribution curves were determined, and molecular weights were calculated by procedures to be described in the accompanying paper (12). Protein concentration was determined by the method of Lowry et al. (17). The turbidity due to glycogen was corrected by means of a blank containing the sample and all reagents with the exception of the Folin reagent. Known amounts of added bovine serum albumin (equal to 0.02% of the glycogen present) were recovered quantitatively. If this method lacked specificity, the amounts of protein determined could only be greater than those actually present, and therefore represent maximal values.

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**Procedure for Extraction of Glycogen**

The tissue was homogenized at 2–4°C in a Waring Blender or in an all-glass homogenizer with 4 volumes of glycine buffer (0.2 M, pH 10.5) and 2 volumes of water washed chloroform. This was followed by centrifugation of the mixture at low speed (180 × *g*, 5 minutes, 2–4°C), decantation of the aqueous phase from the proteinaceous interphase and the lower chloroform phase, and storage at −20°C. The latter two phases were extracted in the Waring Blender with 2 volumes of the glycine buffer at 2–4°C; following low speed centrifugation (5 minutes, 180 × *g*, 0–2°C), the aqueous phase was combined with that from the first extraction and frozen. Re-extraction was repeated five times, and the combined, frozen aqueous phases were then thawed and centrifuged at 180 × *g* for 5 minutes in order to remove particulate material. The resultant supernatant solution was centrifuged either at 100,000 × *g* for 4 hours or at 70,000 × *g* for 16 hours, whichever was more convenient.1 In the preparative Spinco model L centrifuge. The gelatinous residue containing all the glycogen2 was homogenized and centrifuged at low speed (180 × *g* for 5 minutes), and enough water was added to this supernatant to adjust the concentration of glycogen to between 0.5 and 0.6%. The solution was shaken for 3 to 5 minutes with one-third of its volume of a mixture containing chloroform and 1-octanol in the ratio 3:1 (18). The supernatant aqueous phase was separated by aspiration from the interphase and the lower solvent phase after centrifugation at 180 × *g* for 5 minutes. Care was taken not to remove any of the interphase. A loss of as much as 10% of the aqueous glycogen solution can occur at each separation; however, the loss is not preferential for any molecular weight species present. This has been established by comparison of sedimentation coefficient diagrams of the material before the chloroform-octanol treatment with those of the purified material (12). The treatment with the organic solvent mixture was repeated four times for progressively longer periods (1, 2, 4, and 8 hours).

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1 These are not equivalent conditions; however, centrifugation at less than 70,000 × *g* does not result in a well defined pellet because of back-diffusion of the lighter components. Therefore, this force is used even for overnight centrifugation. Any increase in contaminating material brought down when this longer time is used does not appear to be significant.

2 The total amount of glycogen extracted is 90 to 100% of the amount present in the original tissue. This was determined by triplicate analysis (16) of weighed tissue samples, on one hand, and of an aliquot of the solution of the dissolved residue obtained after the high speed centrifugation (70,000 × *g* for 16 hours), on the other.
The treatment for 8 hours was repeated until no interphase containing denatured protein impurities was visible. After three to four treatments with the chloroform-octanol mixture, purification frequently was achieved more rapidly by adding to the aqueous phase 0.05 volume of M citrate buffer, pH 6.1. Glycogen from the aqueous phase was isolated after addition of LiBr (19) by precipitation with 1 volume of 100% ethanol, centrifugation at 1,600 × g for 15 minutes at 2–4°C, washing with 100% ethanol, centrifugation, and drying of the residue in a vacuum at room temperature over silica gel. This procedure could also be used for small tissue samples.

During the earlier stages of this study, the initial step consisted in the homogenization of the tissue with cold distilled water. This procedure proved satisfactory for rabbit liver, Ascaris muscle, and Fasciola hepatica glycogens. When a crude aqueous extract of rabbit liver was kept at pH 5.5 for 30 minutes, changes in the sedimentation coefficient spectrum were observed. No such changes were found when the initial extraction was carried out at any pH between 7.0 and 11 at 2–4°C. Since it is well known that tissue extracts often are acidic, the precaution was taken of ensuring a slightly alkaline pH during the initial extraction by using glycine buffer, pH 10.5, instead of water. This procedure is referred to as a cold water extraction since the glycine buffer is merely used to prevent an acid pH. While the original method could be used for certain tissues, only extraction with the chloroform-glycine mixture has been found to yield quantitative results with all of the following tissues: liver of man, chimpanzee, rabbit, and guinea pig, Ascaris muscle, Fasciola hepatica, and Hymenolepis diminuta.

Glycogen from rabbit muscle (frozen in situ (20)) was isolated in the same manner as from other tissues until the residue, after high speed centrifugation, was obtained. Thereafter, this residue was homogenized with 0.2 M glycine (pH 10.5) and chloroform, with the use of volumes equal to one-half of those used for the original extraction of the tissue. After centrifugation at low speed (180 × g, 5 minutes, 0–2°C) and separation of the supernatant, the interphase and lower chloroform phase were homogenized with 0.2 M glycine, pH 10.5 (one-half of the volume used in the preceding step); the mixture was centrifuged at 180 × g for 5 minutes, and the upper aqueous phase was combined with the first extract and frozen. Re-extraction was repeated three times, and the combined, frozen extracts were thawed and centrifuged at low speed. To the supernatant, 1 volume of ethanol and a few crystals of LiBr were added, and the resulting precipitate was separated by centrifugation at 1600 × g for 20 minutes at 0–2°C and dried in a vacuum at room temperature. The powder was shaken with water (10 ml per g of powder) and the chloroform-octanol (3:1) mixture (5 ml g of powder) for 2 to 3 minutes in a glass-stoppered tube. After centrifugation at low speed, the supernatant was removed, and the interphase and lower phase were re-extracted with the same mixture (5 ml per g of powder) for 2 to 3 minutes in a glass-stoppered tube. After centrifugation at low speed, the supernatant was removed, and the interphase and lower phase were re-extracted. Glycogen precipitated from the combined supernatants can be purified further by treatment of its aqueous solution with the chloroform-octanol mixture in the manner described for glycogen from other tissues (see above).

The product obtained by either of the above procedures is a white amorphous powder, soluble in water (up to 40%); its solutions are highly turbid. On incubation with phosphorylase b, amylo-1,6-glucosidase, 5'-AMP, and Pi, this material is degraded completely to glucose-1-P (approximately 92%) and glucose (8%) (16).

DISCUSSION

It has been known for some time that glycogen can be obtained by extraction with cold water and subsequent centrifugation. Using such a technique for rat liver glycogen, Lazarow (8) obtained a product that had a molecular weight many times higher than the molecular weight of alkali-extracted glycogen. He attributed this property of cold water-extracted glycogen to some form of aggregation caused by protein extracted with the glycogen. The residual protein content of this material amounted to approximately 1%. The observations described in the present and the subsequent (12) papers require a different interpretation because a progressive reduction in the concentration of contaminating protein from 13%, in the case of rabbit liver glycogen, to 0.01% has no influence whatsoever on either the molecular weight average or distribution (12). Also, it is of interest that when glycogen is extracted with hot alkali (3), the residual protein concentration is 5 to 10 times higher than that of cold water-extracted glycogen, which has 50 times the molecular weight.

Prolonged centrifugation at high speed is necessary to ensure a quantitative yield of the lower molecular weight components of glycogen. Insufficient centrifugation accounted for the lack of quantitative recovery of glycogen obtained by Lazarow (8) because of the loss of the lighter components of glycogen in the supernatant. Similarly, if the low speed centrifugation is performed at an excessive speed or for too long a time, a preferential loss of the higher molecular weight components can occur. Any such selective losses must be avoided carefully throughout the isolation procedure because they would result in a distortion of the molecular weight spectrum of the highly polydisperse product.

Treatment of glycogen with strong alkali produces a 10- to 100-fold reduction in molecular weight and chemical alteration of the molecule (7, 9). Extraction of glycogen with cold trichloroacetic acid or hot water also decreases the molecular weight by a factor of 10 to 50. On the basis of the observations reported in this and the subsequent paper (12), it is suggested that henceforth none of the drastic procedures (alkali, acid, or heat) be used for studies concerned with the characterization of glycogen, and that care be taken to ensure that no selective losses result from differential sedimentation during the isolation.

SUMMARY

In an attempt to obtain a product more closely approaching the state of glycogen in intact animal tissues, a procedure for the extraction of this polysaccharide is described. This procedure is designed to avoid physical and chemical alteration by strong alkali, acid, or high temperature and distortion of the molecular weight spectrum resulting from selective losses during sedimentation.

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