TECHNIQUES OF BIOCHEMICAL AND BIOPHYSICAL MORPHOLOGY

Edited by DAVID GLICK and ROBERT M. ROSENBAUM

VOLUME
1

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BIOCHEMICAL AND
BIOPHYSICAL MORPHOLOGY

Edited by **DAVID GLICK**
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VOLUME 1

WILEY-INTERSCIENCE, a division of
John Wiley & Sons, Inc., New York • London • Sydney • Toronto

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Library of Congress Catalog Card Number: 72–153

ISBN 0–471–30800–5

Printed in the United States of America.

10 9 8 7 6 5 4 3 2 1

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PREFACE

A pressing need has evolved for review volumes published at regular intervals, usually annually, to aid scientists in keeping up-to-date with developments in instrumentation, methodology, and techniques of biochemical and biophysical morphology. This need has been particularly accentuated by the remarkable expansion of the field in recent years and the growing importance of its contributions to many areas of biological and medical science. Therefore, we have initiated this series of review volumes to serve as an international authoritative source in the field. *Techniques of Biochemical and Biophysical Morphology* is designed to cover important new developments systematically and in a self-modernizing manner.

Each volume will be made up of chapters contributed by recognized authorities having intimate knowledge of, and experience with, the subjects they review. The general plan of the chapters will follow explanation of underlying principles and theory, a critical evaluation of past work, and a presentation of details of the instrumentation, methods, or techniques recommended by the author in a fashion to furnish the qualified scientist with practical laboratory information. To reach the greatest number of interested readers all chapters will appear in English.

The Editors and members of the Advisory Board wish to make this series as useful as possible, and therefore welcome any and all suggestions.

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January 1972

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TECHNIQUES OF BIOCHEMICAL
AND BIOPHYSICAL MORPHOLOGY VOLUME 1

Methods for Electron Microscopic Localization of Glycogen

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I. INTRODUCTION

In this chapter we attempt to discern the most practical and reproducible cytochemical techniques for the staining of glycogen in thin sections of tissues prepared for electron microscopy. Several reviews and reports concerning the cytochemical localization of glycogen have been published (13,43,44, 48,53), but a review of this literature is not undertaken here.

Glycogen, one of the most important energy reserves in animal cells, consists of chains of (1→4)-linked α-D-glucose residues interlinked by α-D-(1→6)-

glucosidic bonds forming a multibranched structure (11,24,49). However, most of the physical and chemical properties of glycogen are similar, regardless of its source (24). Protein is intimately associated with tissue glycogen and with isolated glycogen granules. In fact, two enzymes that synthesize and break down glycogen, synthetase and phosphorylase, are fixed to the glycogen molecule and are consistently found in glycogen fractions of tissue homogenates (19,21,52,57). The protein molecules which are thought to surround and intercalate between the polyglucose chains, may play an important role in keeping the macromolecular organization intact.

II. THE EFFECT OF PREPARATIVE TECHNIQUES ON THE PRESERVATION AND STAINING OF GLYCOGEN

The preservation, distribution, and appearance of glycogen varies considerably with the techniques of tissue preparation for electron microscopy (27, 29,34). Subsequent staining of glycogen is closely dependent on the chemical action of the fixative.

Osmium tetroxide has been widely used as a fixative for tissues prepared for electron microscopy. It is well known that hexoses, pentoses, and their polymers contain few chemical groups that bind or reduce osmium tetroxide (5,43). Under the conditions of osmium tetroxide fixation for electron microscopy, this lack of interaction does not aid the preservation or staining of glycogen. Glycogen that is preserved in tissue fixed with this compound may be stained by phosphotungstic or phosphomolybdic acid (50,58), or by lead stains at alkaline pH (44). However, neither of these heavy metal complexes can be considered a specific stain for glycogen, since each enhances contrast of several other cytoplasmic and extracellular components (e.g., cytomembranes, ribonucleoprotein particles, collagen).

Permanganate fixation (22) followed by osmium tetroxide treatment enhances preservation and staining of glycogen. However, this fixative significantly alters the morphology of the particles (12) and poorly preserves other cytoplasmic components.

Formaldehyde does not adequately preserve soluble carbohydrates (29). Large amounts of protein and glycogen are extracted.

Dialdehydes (acrolein, glutaraldehyde, etc.) are being used extensively as fixatives for electron microscopy. The dialdehydes, especially glutaraldehyde, are considered excellent cross-linking agents that react with the polyhydroxy groups of polysaccharides and with amino- and imino-groups of associated proteins (47). The molecular bridges formed between the polysaccharides and proteins are thought not only to stabilize (insolubilize) the protein–polysaccharide complexes, but also to enhance their stainability. Glutaraldehyde fixation coupled with postfixation in osmium tetroxide appears to have

several effects: it preserves the macromolecular structure of glycogen, enhances staining, and restricts glycogen displacement in cells. Further information on the effect of fixatives on glycogen preservation and staining may be obtained from the work of Minio et al. (27), Millonig and Marinozzi (29), Paluello and Rosati (34), and Revel (43).

The buffers used in fixation greatly influence the degree of extraction of proteins and glycogen from tissues. During osmium tetroxide fixation, for example, approximately three times more protein is lost from tissues fixed in *s*-collidine buffer than from tissues fixed in bicarbonate, phosphate, or other buffers (23). Very little critical analysis is available on the effect of various buffers during dialdehyde fixation. From our experience, tissues fixed in formaldehyde–glutaraldehyde mixtures in cacodylate or phosphate buffers (16) and postfixed in osmium tetroxide show excellent preservation and staining of glycogen and of other polysaccharides (3). Following dehydration in acetone or alcohol, embedment in epoxy resins (Epon, Araldite, Epon–Araldite mixtures) permits good cytochemical staining of glycogen in thin sections.

Although routine glutaraldehyde–osmium-tetroxide–epoxy resin methods allow good preservation and staining of glycogen, it still seems desirable to develop preparative techniques that require minimal chemical fixation, dehydration in organic solvents, and embedding in plastics. Freeze-substitution, frozen section, and frozen-dried section techniques (18,19,41,55) may become extremely useful in determining the *in situ* form and structure of glycogen. Indeed, in the isolation of glycogen, Drochmans has emphasized the need for very mild techniques that neither degrade nor disturb the macromolecular arrangement of the polymer nor denature the proteins bound to glycogen (57). Such techniques are similarly required for precise cytochemical demonstration of glycogen.

III. RECENT ULTRACYTOCHEMICAL TECHNIQUES FOR THE STAINING OF GLYCOGEN

Although several investigators have made significant contributions to the development of techniques for the cytochemical demonstration of glycogen in thin sections (14,25,43,48,54), the periodic acid–Schiff (PAS) procedure developed for the light microscopic demonstration of polysaccharides remains the prototype for most successful electron microscopic procedures (Figure 1). The periodate treatment of glutaraldehyde–osmium-tetroxide-fixed plastic embedded sections is common to all cytochemical procedures, but individual techniques reveal the periodate oxidation product through a variety of dissimilar reactions, and all these reactions produce an electron-dense final product. Although the reactions demonstrate periodate-engendered aldehydes in glycogen, other more complex polysaccharides, primary and second-

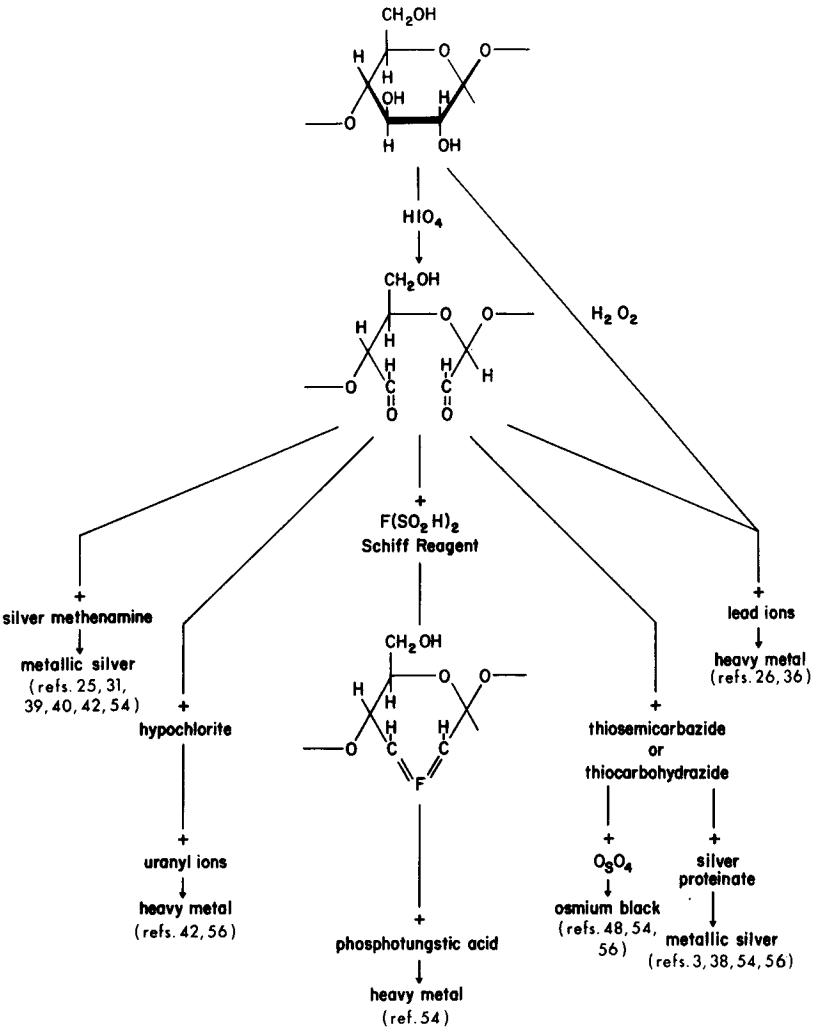

Figure 1. Formulation for the periodic acid–Schiff reaction and related modifications developed for the staining of glycogen in thin sections for electron microscopy.

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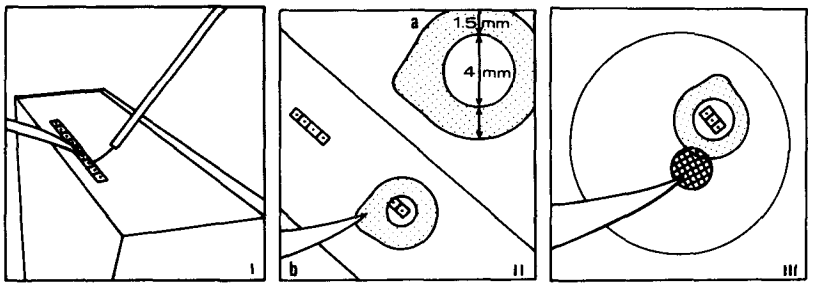

Figure 2. The sections floating on the surface of the trough fluid (I) are collected within the lumen of a thin (less than 0.5 mm thick) but rigid plastic ring (II*a*, *b*). The ring carrying the sections is refloated on the surface of the reagents within a depression chamber or small Petri dish. After treatment for the desired interval, the solutions are aspirated and replenished with a Pasteur pipet. Sections are mounted by touching the grid surface to the floating sections (III).

ary hydroxyamine groups, hydroxyaldehydes, and ketone groups may also be revealed (10,20). Therefore, Thiery (54) underlines the necessity for control tissues not treated with periodic acid. Controls are particularly required after glutaraldehyde fixation, where nonspecifically bound aldehydes persist despite thorough washing.

1. Blockage of Free Aldehydes

Free aldehydes may be neutralized by chemical blockage (51). This may be accomplished in the following manner.

Procedure. Sections mounted on inert gold or platinum grids* or carried in plastic rings (26, see Figure 2), are floated on the surface of blocking and rinsing solutions. Sections are floated on either: (*a*) 2% sodium bisulfite solution for 15 min at room temperature or (*b*) 2% dimedone solution in 1% acetic acid for 60 min at 60°C.

The sections are washed thoroughly in distilled water and then subjected to periodic acid oxidation and glycogen staining procedures.

2. The Periodic Acid Oxidation Procedure

Sections mounted on gold or platinum grids or carried in plastic rings are floated on the surface of 1% aqueous periodic acid at room temperature for

*Although it is easier to handle grids than to float sections that are unmounted, both methods yield good results. With grids, however, thorough rinsing is necessary to remove precipitated reagents from the bars. In all the following procedures, both methods of carrying sections are applicable.

20 to 30 min. It is advisable to use a freshly prepared periodic acid solution. Treatment with periodic acid for 30 min is sufficient to oxidize 1:2-glycol groups and to remove osmium from subcellular components. The sections are then rinsed for 30 min in three successive changes of distilled water. Control tissues are floated on distilled water instead of periodic acid solution.

IV. GLYCOGEN STAINING PROCEDURES

1. The Periodic Acid–Schiff–Phosphotungstic Acid Procedure (PAS–PTA)

Rationale. Periodic acid (HIO_4) oxidizes the 1:2-glycol groups of glucosidic molecules exposing two aldehydes. Under the conditions employed, periodic acid does not further oxidize dialdehydes as do other oxidants (e.g., chromic acid, potassium permanganate, and hydrogen peroxide). The exposed dialdehydes combine with the fuchsin–sulfurous acid Schiff reagent to form a colored product. Since the product does not contain heavy atoms, very little electron opacity is produced by the Schiff reaction.

Thiery (54) has successfully modified the classical PAS procedure for use at the electron microscopic level. Following the periodic acid-rinse treatment (111–2), the following procedure is employed:

Procedure. Thin sections are floated on Schiff reagent for 1 to 2 hr at room temperature. This procedure is performed in subdued light.

Sections are rinsed for 2 min each in five successive changes of freshly prepared sulfurous acid solution containing either:

1. 10 ml of 10% sodium metabisulfite.
   10 ml of *N* hydochloric acid.
   200 ml of distilled water, or
2. Equal parts of 1% sodium or potassium metabisulfite and 0.1*N* hydrochloric acid.

Sections are then rinsed in three 2-min changes of distilled water and stained for 30 to 60 min in 2% aqueous phosphotungstic acid. The PTA solution may be adjusted to pH 4.5 or 7.0.

Finally the sections are rinsed three times in distilled water and mounted on grids.

The nature of binding of the Schiff complex and PTA is unknown, but the reaction probably involves ionic interaction between the two complexes. In any event, under the conditions employed by Thiery, the electron opacity of the dialdehyde Schiff complex is significantly enhanced by PTA. The dialdehyde Schiff complex may also be stained with other heavy metal salts including silver nitrate and lead citrate (54).

Although the PAS–PTA procedure stains glycogen well, it cannot be considered specific, since nonglycogen tissue components also stain intensely (e.g., collagen). An additional limitation is the lack of fine detail of the glycogen particles. One distinct advantage gained by use of this technique is excellent correlation of PAS positive regions in thick sections with PAS–PTA stained glycogen in adjacent thin sections. Formulation for the electron microscopic demonstration of glycogen by the PAS–PTA procedure is illustrated in Figure 1.

2. The Periodic Acid–Silver Methenamine Procedure

A technique involving periodic acid and silver methenamine has been used extensively for the cytochemical demonstration of glycogen and other polysaccharides (25,31,39,40,42,54). In this technique, aldehydic groups engendered by periodate oxidation reduce the silver tetramine contained in the methenamine silver reagent, with consequent release of metallic silver.

Procedure. Periodic acid oxidized-rinsed sections are floated on the surface of a freshly prepared silver methenamine incubation medium* prepared in either of the following ways:

1. A quantity of 5 ml of 5% silver nitrate is added to 45 ml of a 3% methenamine (hexamethylenetetramine) solution. A white precipitate forms which disappears after vigorous agitation. Then 5 ml of 2% sodium borate in distilled water is added to this solution. The final solution is filtered through two sheets of Whatman #42 filter paper. [Formulation according to Rambourg (39).]

Or:

2. To 100 ml of methenamine (3% in distilled water) is added 5 ml silver nitrate solution (5% in distilled water). The white precipitate is dissolved by agitation. To 12 ml of sodium borate (5% in distilled water) is added 100 ml of distilled water. [Formulation according to Thiery (54).]

Sections are incubated in the silver methenamine solution within a covered Petri dish in a 60°C oven. It is essential to perform this step in subdued light or in a darkroom. After 45 to 60 min, sections that show a yellow-brown tinge are rinsed several times in distilled water, with a subsequent rinse for 5 min in sodium thiosulfate (5% in distilled water). After they have been rinsed in distilled water, sections are mounted on copper grids.

For controls, sections are not treated with periodic acid prior to staining with silver methenamine.

*Consult Marionozzi (25), Movat (31), or Swift and Saxton (51) for other formulations of the silver methenamine incubation medium.

This technique suffers from the following disadvantages:

1. The duration time of the reaction is often difficult to estimate.
2. Some tissue components show argentaffinity even without periodate oxidation (e.g., collagen, mast cell granules, chromosomes).
3. After the periodic acid–silver methenamine treatment, some background precipitation occurs throughout the tissue.
4. The reduced silver particles tend to obscure the fine structure of the glycogen.

In summation, this technique stains glycogen moderately well (42,54), but its many pitfalls make it impractical for routine use.

3. The Periodic Acid–Lead or the Hydrogen Peroxide–Lead Procedure

When employed at alkaline pH, lead ions enhance the contrast of many cytoplasmic components, including glycogen (59). According to Marinozzi (26), it is possible to stain glycogen and ribosomes selectively with lead stains if bound osmium is first removed from tissue sections by oxidation with either periodic acid or hydrogen peroxide.

Procedure. Sections are treated with periodic acid or hydrogen peroxide solutions— for 30 min in 0.8 to 3.2% periodic acid (36) or for 10 to 15 min in 1.5% hydrogen peroxide. Afterward, sections are rinsed in distilled water and stained in lead citrate (44) for 10 to 15 min. After rinsing, floating sections are mounted on grids.

This procedure is expeditious and stains glycogen moderately well (1,36). Other cytoplasmic components are also stained. The chemical basis of the reaction is not known, but it is considered unspecific for glycogen *per se*. Overoxidation of thin sections causes extraction of glycogen, whereas staining with uranyl acetate obscures the detail of the particles.

4. The Periodic Acid–Thiosemicarbazide or Thiocarbohydrazide–Osmium Tetroxide Procedures (PATO, PATCO).

Formulation for the electron microscopic demonstration of glycogen by the PATO procedure is illustrated in Figure 3. Aldehydic groups formed by periodic acid oxidation condense with hydrazino groups of thiocarbamyl compounds. Subsequent reaction of osmium tetroxide with the thiocarbamyl moiety of the aldehyde thiosemicarbazone yields "osmium black." The "osmium black" produced at the site of the reaction is intensely electron

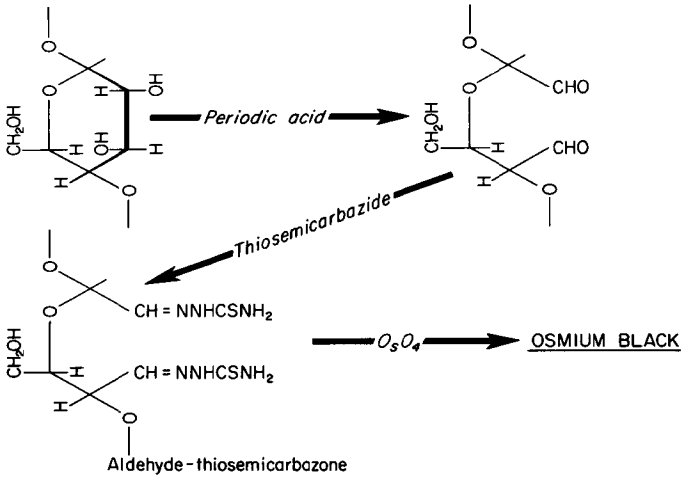

Figure 3. Formulation for the ultracytochemical demonstration of glycogen by the periodic acid–thiosemicarbazide–osmium textroxide (PATO) procedure of Seligman and coworkers.

opaque but does not obscure the fine structure of the glycogen particles. This technique was developed by Hanker and Seligman and coworkers (14,48).

Procedure. Sections mounted on inert gold or platinum grids or carried in plastic rings (25) are treated with periodic acid, rinsed, and floated for 30 to 45 min on the surface of either 1% thiosemicarbazide in 10% acetic acid or 0.2% thiocarbohydrazide in 20% acetic acid in distilled water.

Thin sections are then rinsed thoroughly for 30 min (10 min in 10% acetic acid solution, then 5 min in 5% and 1% acetic acid solutions, and finally in several changes of distilled water).

Sections are mounted on copper grids and exposed to osmium tetroxide vapor at 60°C (48,54).

The simplest method for exposing sections to osmium tetroxide vapor is described by Vye and Fischman (56). Grids bearing sections are mounted on cover slips that are placed in small Coplin jars containing crystals of osmium tetroxide. The jars are sealed with stopcock grease and incubated for 60 min to 3 hr in a 60°C water bath.

Glycogen particles are stained intensely with either the PATO or the PATCO procedure (Figure 4). Very little nonspecific staining or background precipitation is apparent. The α– and β– particles are readily recognizable, but fine details of these particles cannot be distinguished (Figure 4*b*).

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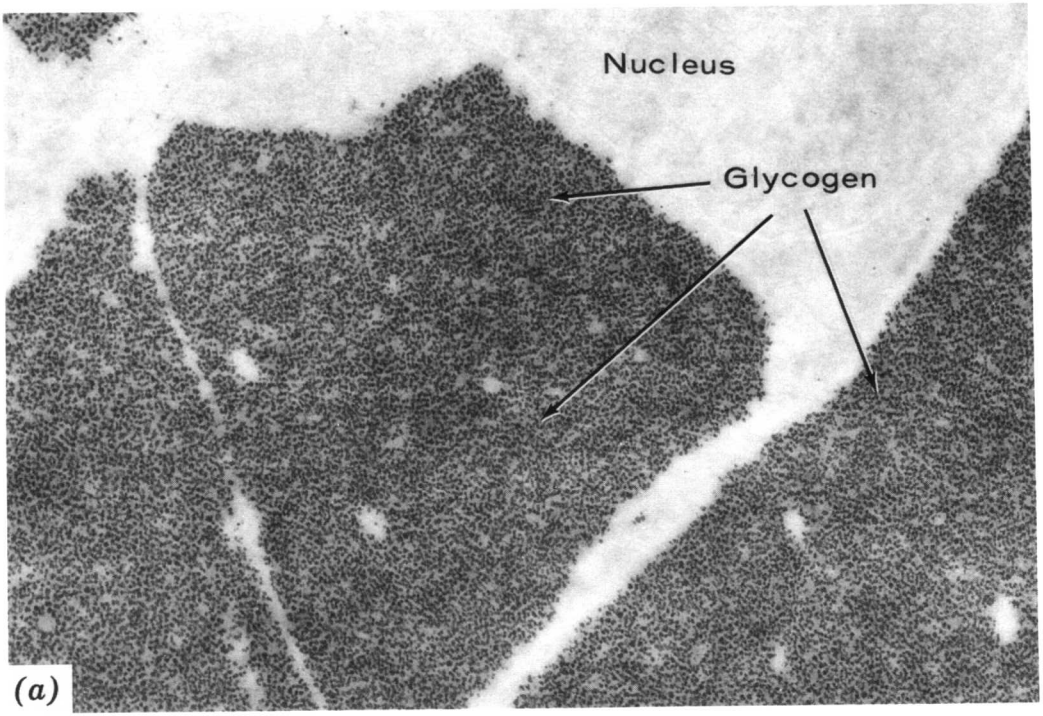

Figure 4. The PATO procedure is used to stain glycogen in the glycogen body from a 20 day chick embryo: (*a*) At low magnification (X15,000) intensely stained pools of glycogen fill the cells; (*b*) at higher magnification (X250,000) the fine structure of the interconnected β-particles are not discernible.