CARBOHYDRATE HISTOCHEMISTRY STUDIED BY ACETYLATION TECHNIQUES

I. Periodic Acid Methods

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Plate 42

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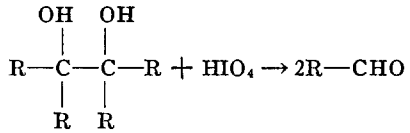
Periodic acid forms aldehydes from 1,2-glycols (1). This reaction, much used in organic chemistry, was introduced into histochemistry for the demonstration of carbohydrates in tissue sections (2–4). Schiff's reagent is used to color the aldehyde. Materials which have been colored with Schiff's reagent after periodic acid are taken to consist of, or contain, carbohydrate. However, amino or alkylamino substituents of 1,2-glycols can produce aldehyde when oxidized by periodic acid (2). Further confirmation of the carbohydrate nature of the material which colors with Schiff's reagent after oxidation by periodic acid is desirable.

Earlier attempts in this direction have produced questionable results. Extracts of malt diastase are used to remove glycogen from tissue sections (5). Crude commercial enzyme preparations, fungal in origin, containing pectinesterase, pectinase, and polygalacturonidase, as well as purified polygalacturonidase, have been used to extract the non-glycogen carbohydrates from tissue sections (6). The results of the use of diastase and of the pectic group of enzymes are limited by inadequate knowledge of the necessary substrate linkage as well as inhomogeneity of both enzyme and substrate. Further chemical confirmation of the presence of the 1,2-glycol linkage in the materials which color with Schiff's reagent after periodic acid oxidation is reported here. Since such glycol groups are rarely present in materials other than carbohydrates, it can be assumed safely that test indicates the presence of carbohydrates.

It was decided to attempt the blockage of the hydroxyl groups of the 1,2-glycols. This is ordinarily done in organic chemistry by acetylation, using acetic anhydride or ketene. The reaction has the advantage of being easily reversed. The chemistry of the various procedures will be outlined as a preliminary to the description of the experiments which were set up.

The reaction of a carbohydrate with periodic acid is considered to be the following:—



When a section is acted upon by Schiff's reagent after periodic acid, a colored complex is formed between Schiff's reagent and the aldehyde. The procedure of periodic acid oxidation of a tissue section and coloring with Schiff's reagent is referred to by us as the PAS technique. As Hotchkiss (2) has pointed out, the coloration of materials in tissue sections by the PAS technique takes place when a material with the appropriate linkage is present in sufficient quantity and when the original material and its oxidation product are non-diffusible.

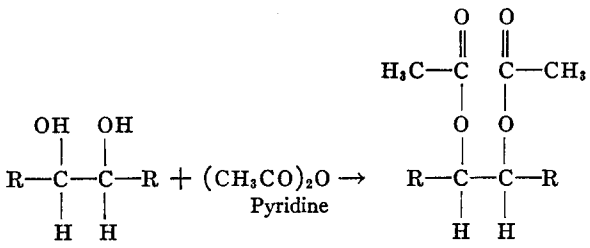
The PAS technique for tissues (3, 7) is the following:—

1. Paraffin sections, or frozen sections, to water.
2. 0.5 per cent (or 1 per cent) aqueous periodic acid, 5 minutes.
3. Wash, distilled water.
4. Schiff's reagent, 15 minutes.
5. Sulfurous acid rinses, 3 × 2 minutes each.
6. Wash tap water to red-purple color.
7. Counterstain as desired, mount with or without dehydration.

Result: Mucin, glycogen, basement membranes, reticulin, etc., red to purple. The sections are best examined *without* a blue filter, using frosted glass in the microscope lamp (Figs. 1 and 4).

The acetylation of tissue sections is a new procedure. We first tried soaking the sections overnight in pure acetic anhydride in a Coplin jar. On the advice of Dr. W. W. Pigman a mixture of acetic anhydride with pyridine was used. With this mixture the blockage of hydroxyl groups was accomplished in 45 minutes. Pure acetic anhydride required overnight action.

The acetylation of the 1,2-glycols can be expressed as follows:—



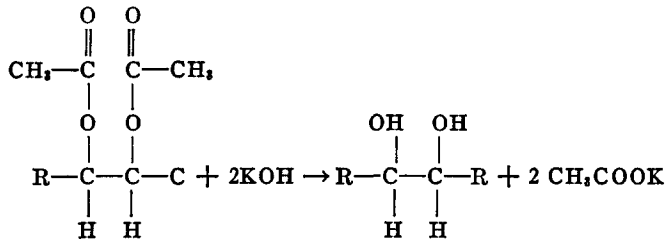
The reaction of the acetylated 1,2-glycol with periodic acid does not produce an aldehyde detectable with Schiff's reagent.

To demonstrate this the following test was carried out:—

1. Paraffin sections to water.
   (*a*) Place sections in: Acetic anhydride 13 cc.
   Pyridine 20 cc.
   Leave for 45 minutes at room temperature.
   (*b*) Wash tap and distilled water.
2. Periodic acid 0.5 per cent aqueous for 5 minutes.
3. Following steps as above.

Result: The structures which do not color after acetylation are listed after the next section (Figs 2 and 5).

The acetyl groups can be removed readily from acetylated 1,2-glycols by weak alkali. The alkali action on acetylated 1,2-glycols is shown below.



The 1,2-glycol linkage is returned. It should be susceptible to oxidation by periodic acid with aldehyde formation.

The test was performed as follows:—

1. Sections to water.
   (*a*) Place sections in: Acetic anhydride 13 cc.
   Pyridine 20 cc.
   Leave for 45 minutes at room temperature.
   (*b*) Washed distilled water.
   (*c*) Place in 0.1 N KOH for 45 minutes at room temperature.
   (*d*) Wash in tap and distilled water.
2. 0.5 per cent periodic acid, 5 minutes.
3. Following steps as above.

The materials coloring with Schiff's reagent after periodic acid previous to acetylation, color after acetylation if the tissue has been acted upon by weak alkali (Figs. 3 and 6). The materials we have colored with PAS on untreated sections and on acetylated sections followed by alkali, which do not color in acetylated sections, include the following:—

Renal basement membrane, splenic reticulin, epithelial mucin, cartilage ground substance, glomerular hyaline of intercapillary glomerulosclerosis, arterial and arteriolar hyaline, intercellular substance or vessels, glycogen, amyloid, thyroid and pituitary colloid, cytoplasm of Gaucher's cells in splenic sinuses (8), cytoplasm of certain anterior pituitary cells, renal tubular casts, and the free surface of the epithelium of the first convoluted renal tubules. All these appear to consist of, or contain, carbohydrate.

DISCUSSION

Acetylation of tissue sections prevents the coloration by the PAS technique. Removal of the acetyl groups with alkali returns the ability to color. This is strong evidence that the materials which color with Schiff's reagent after periodic acid oxidation are carbohydrate. It is unlikely that the original PAS coloration was the result of the presence of amino groups such as might be present in glucosamine since the *N*-acetyl of the acetylated sections would probably be stable to alkali under the conditions used for the saponification.

It is suggested that the study of tissue carbohydrates histochemically is most

exact if three reactions are used: (1) color section with Schiff's reagent after periodic acid; (2) acetylate section, attempt coloring with Schiff's reagent after periodic acid; (3) acetylate section, leave 45 minutes in 0.1 N KOH, color with Schiff's reagent after periodic acid. It can be assumed that any material coloring in 1 and 3 and not in 2 possesses numerous 1,2-glycol linkages and very likely is carbohydrate.

There are further corollaries of the procedure of acetylation of tissue sections which may prove important. In the first place, it is shown that available hydroxyl groups are present in tissue carbohydrates fixed in formalin, Bouin's, Zenker's, and Helly's fixatives. Secondly, acetylation may find use in studying other reactions of tissue, such as metachromatic staining, fat coloring, and silver impregnations.

CONCLUSIONS

An acetic anhydride pyridine mixture acetylates hydroxyl groups in tissue sections. The acetyl groups can be removed from tissue sections by weak alkali.

Sections acetylated by acetic anhydride pyridine no longer produce aldehydes after oxidation by periodic acid. Acetylated sections subjected to weak alkali regain the ability to produce aldehydes after oxidation by periodic acid.

Acetylation of tissue sections and the removal of acetyl groups by weak alkali can be used as histochemical confirmation of the carbohydrate nature of materials coloring with Schiff's reagent after periodic acid.

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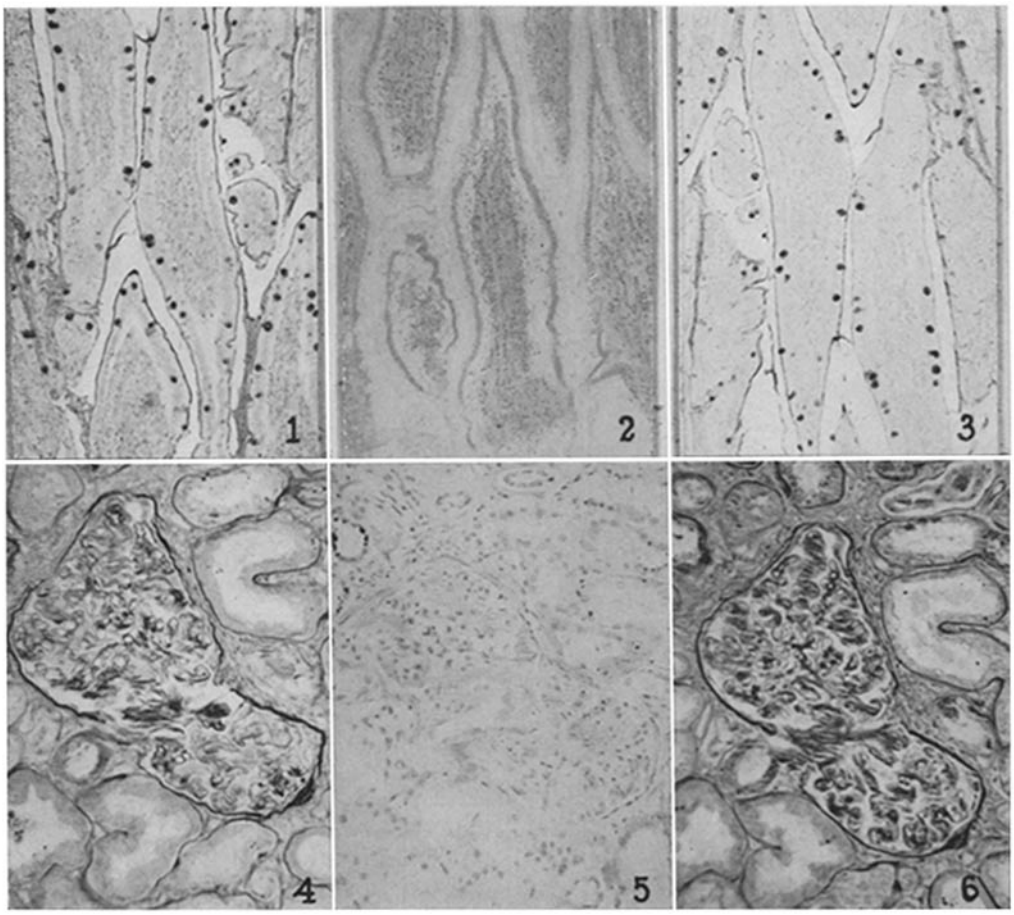
EXPLANATION OF PLATE 42

Figs. 1, 2, and 3. Jejunum. Figs. 4, 5, and 6. Amyloidosis of kidney. × 125.

FIGS. 1 and 4. PAS staining. × 125.

FIGS. 2 and 5. PAS staining on acetylated sections. Hematoxylin stain of nuclei. × 125.

FIGS. 3 and 6. Acetylated sections treated with weak alkali. PAS staining. × 125.



(McManus and Cason: Carbohydrate histochemistry. I)