I. INTRODUCTION

The principal use of periodate oxidation in histochemistry has been the demonstration in tissue sections of materials, chiefly carbohydrates, containing 1,2-glycol groups. The investigation of the effects of periodate on tissue sections has required the re-study of fundamental chemistry, and lead to histochemical information and to other techniques. These studies have led to the accumulation of a considerable amount of data which has presented a problem to the reviewer, whether to include all the relevant material or to restrict the discussion to the periodic acid Schiff
(PAS) reaction. The reviewer has decided upon the latter, using it as the central theme, and referring only in passing to other applications of periodate oxidation.

II. THEORY OF METHOD

A. Periodic Acid Oxidation in Organic Chemistry

Malaprade (1928a, b) introduced periodic acid (HIO₄) as a reagent for alcohols. The reaction takes the form of splitting vicinyl carbon atoms when both bear a hydroxyl group with the formation of aldehyde:

\[ R\text{CHOH-CHOHR} + \text{HIO}_4 \rightarrow 2\text{RCHO} + \text{H}_2\text{O} + \text{HIO}_3 \]

Specificity of the reaction for 1,2-glycols was discovered by Fleury and his associates (Fleury and Courtois, 1950; Fleury and Lange, 1932; Fleury and Paris, 1933).

From the viewpoint of the present discussion, the further reactions of importance are those in which aldehyde is produced, e.g. from α-ketols:

\[
\begin{align*}
\text{CH}_2\text{OHCOCH}_2\text{OH} + \text{HIO}_4 & \rightarrow \text{HCHO} + \text{CH}_2\text{OHCOOH} + \text{HIO}_3 \\
\text{CH}_3\text{COCHOHCH}_3 + \text{HIO}_4 & \rightarrow \text{CH}_3\text{CHO} + \text{CH}_3\text{COOH} + \text{HIO}_3
\end{align*}
\]

as described by Clutterbuck and Reuter (1935) and Fleury and Lange (1932, 1933).

Similarly, compounds possessing an amino group replacing an hydroxyl group, RCHOH.CHNH₂R, as in serine (Nicolet and Shinn, 1939), or threonine (Shinn and Nicolet, 1941) or hydroxylysine (Van Slyke et al. 1940) can produce aldehyde on periodate oxidation. Formaldehyde is formed from the periodate oxidation of the structure RCHOH.CHNR. R (Nicolet and Shinn, 1941).

In summary as Novikoff (1955) points out, aldehyde is formed from periodate oxidation of 1,2-glycol groups if:

\[ R \]

\[
\begin{align*}
R & \quad H - C - OH \\
& \quad H - C - OH \\
& \quad R
\end{align*}
\]

(1) Unsubstituted

\[ R \]

\[
\begin{align*}
R & \quad H - C - OH \\
\quad H - C - OH \\
\quad R \quad H - C - NH₂ \\
\quad R
\end{align*}
\]

(2) Amino alcohol substitution of one hydroxyl group
(3) Alkyl-amino alcohol substitution of one hydroxyl group

\[
\begin{align*}
\text{R} & \quad \text{H—C—OH} \\
\text{R} & \quad \text{H—C—N—H} \\
\text{R} & \quad \text{R}
\end{align*}
\]

(4) Carbonyl substitution of one hydroxyl group

\[
\begin{align*}
\text{R} & \quad \text{H—C—OH} \\
\text{R} & \quad \text{H—C=O} \\
\text{R} & \quad \text{R}
\end{align*}
\]

Jackson (1944) reviews evidence suggesting that the mechanism of \(\alpha\)-glycol oxidation by periodic acid is the splitting of the carbon chain, as a third step in the esterification of a hydroxyl group by para periodic acid (\(\text{H}_5\text{IO}_6\) or \(\text{HIO}_4 + 2\text{H}_2\text{O}\)).

\[
\begin{align*}
\text{R} & \quad \text{H—C—OH} \\
\text{R} & \quad \text{H—C—OH} \\
\text{R} & \quad \text{H—C—OH}
\end{align*}
\]

\[
\begin{align*}
\text{R} & \quad \text{H—C—OH} \\
\text{R} & \quad \text{H—C—OH} \\
\text{R} & \quad \text{H—C—OH}
\end{align*}
\]

\[
\begin{align*}
\text{R} & \quad \text{H—C—OH} \\
\text{R} & \quad \text{H—C—OH} \\
\text{R} & \quad \text{H—C—OH}
\end{align*}
\]

Periodic acid oxidation of a variety of other structures and at a variety of temperature solvents, pH and concentration conditions are referred to in Jackson (1944). The reactions described as producing aldehyde from \(\alpha\)-glycols are those at room temperature, aqueous solution, and \(0.022 \text{ M to } 0.044 \text{ M}\) concentration. However, it should be mentioned that \(\alpha\)-glycols can be oxidised by periodic acid in non-aqueous solvents such as ethanol, acetic acid, butyl alcohol and dioxane (p. 186).
Pigman (Pigman and Goepp, 1948) points out two instances in which α-glycols were resistant to periodic acid oxidation, namely 1,6-anhydroglucofuranose (Dimler et al., 1946) and tetraacetylinositol (Dangschat and Fischer, 1942). It is more than possible that in the latter compound the α-glycol grouping might be masked by acetyl groups.

B. Periodate Oxidation in Histochemistry

It is a general principle in histochemistry that where aldehyde is produced by some means, or is normally present, in a tissue, it can be coloured by Schiff’s (1865) reagent as in Feulgen’s (Feulgen and Rossenbeck, 1924) reaction where aldehyde is produced from nucleic acid hydrolysis by HCl, or in Bauer’s (1933) reaction for glycogen where aldehyde is produced by chromic acid oxidation. The ability of periodic acid to produce aldehyde from 1,2-glycols leads naturally to its use in histochemistry.

The first publication (McManus, 1946) described the colouring of mucin by Schiff’s reagent after periodic acid as well as a variety of other structures: “certain pituitary cells, the colloid of the pituitary stalk and thyroid, granules in some nerve cells in the medulla of the rat and in the human intestine, and the basement membranes of the tubular epithelium and of the glomerulus of the kidney”. Marchese (1947) applied the method to the demonstration of glycogen. McManus (1948) summarized many applications and introduced the term periodic acid-Schiff (PAS) reaction.

Quite independently, Lillie (1947) discovered acidified periodate for the demonstration of reticulin and of glycogen. The unpublished periodic acid method of Hotchkiss (1948) had been used in a number of studies on the pituitary (Catchpole, 1947) and thyroid (Gersh, 1947), antedating the other methods of McManus and Lillie by a year or more.

While each of the techniques using periodic acid as an oxidant in histochemistry depended upon the demonstration of the aldehyde by Schiff’s reagent, the techniques differed in several important features as outlined in Table I.

Hotchkiss (1948) noted that aqueous solutions of periodic acid were “usually somewhat more rapid and vigorous in action” than the alcoholic solution. Reasons were advanced why colouring with Schiff’s reagent after periodic acid oxidation could be used for the “staining of polysaccharide structures in fixed tissue sections”. There was an analysis of the chemistry involved, and the suggestion of the use of a “spot-test” for confirmation in the test-tube of results in sections.
PERIODATE OXIDATION TECHNIQUES

TABLE I
ORIGINAL PERIODATE METHODS

<table>
<thead>
<tr>
<th>Technique</th>
<th>Oxidant</th>
<th>Solvent</th>
<th>Reducing rinse</th>
<th>Schiff’s reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>McManus, 1946</td>
<td>$\frac{1}{2}%$ HIO$_4$</td>
<td>Water</td>
<td>None</td>
<td>Lison (1936)</td>
</tr>
<tr>
<td>Lillie, 1947</td>
<td>No Metaperiodate Nitric acid</td>
<td>Water</td>
<td>None</td>
<td>Lillie (1947a)</td>
</tr>
<tr>
<td>Hotchkiss, 1948</td>
<td>0.8% H$_2$IO$_4$</td>
<td>70% Ethanol (water)</td>
<td>Thiosulphate</td>
<td>Hotchkiss (1948)</td>
</tr>
</tbody>
</table>

The chemistry of the reaction was given by Hotchkiss in the case of an anhydroglucose residue of starch as follows:

\[
\begin{align*}
\text{H} & \text{C} \\
\text{HCOOH} & \text{O} \\
\text{HCOH} & \text{O} \\
\text{HC} & \\
\text{HC} & \\
\text{HCH} & \text{O} \\
\text{H} & \\
\end{align*}
\]

\[
\begin{align*}
\text{H} & \text{C} \\
\text{HCO} & \text{O} \\
\text{HCO} & \text{O} \\
\text{HC} & \\
\text{HC} & \\
\text{HCH} & \text{O} \\
\text{H} & \\
\end{align*}
\]

\[
\begin{align*}
\text{H} & \text{C} \\
\text{OH} & \text{O} \\
\text{HC-SO}_2^- & \\
\text{HC-SO}_2^- & \\
\text{HC} & \\
\text{HC} & \\
\text{HCH} & \text{O} \\
\text{H} & \\
\end{align*}
\]

It was pointed out that the periodate-fuchsin staining procedure gives a positive result with any substance which satisfies all the following requirements:
(1) Proper linkages.
(2) Not diffused away in fixation.
(3) Non-diffusable oxidation product.
(4) Present in sufficient concentration for colour.

C. Demonstration of the Aldehyde by Schiff’s Reagent

Pararosaniline or parafuchsin is turned into a colourless compound by addition of sulphite groups. The process is considered by Wieland and Scheuing (1921) to be as follows:

\[
\begin{align*}
&\text{Or} \\
&\text{Or}
\end{align*}
\]

Either (I) or (II) above, parafuchsin leucosulphoric acid, is the active material in Schiff's reagent. The compound is colourless but has the property of combining with aldehyde to form a red, magenta or purple quinonoid dye. The process of formation of the coloured dye goes through stages (III) and (IV) on p.177, which are colourless, to form compound (V) which is coloured.

It is well known that ketones as well as aldehydes can colour Schiff's reagent (Lison, 1936). From time to time other materials are described as able to colour Schiff's reagent, but usually there is no proof that the materials themselves are aldehyde-free. It is true, however, that simple oxidation, ageing, exposure to air and to sunlight can colour Schiff’s reagent. For these circumstances, controls should be used where possible.
PERIODATE OXIDATION TECHNIQUES

Periodate, a reagent well known for the production of aldehydes from 1,2-glycols, is used in histochemistry for the detection of carbohydrate-containing materials. The aldehyde produced in the sections can be coloured by Schiff's reagent, and by a number of other aldehyde reagents and ammoniacal silver.

III. PROCEDURES OF ORIGIN

A. McManus (1946) (Nature 158, 202)

Zenker-formol sections were passed to water, after iodine and hypo, and placed for 2 min in a 0·5% solution of periodic acid in distilled water. The sections were then washed in tap and distilled water and placed in
Schiff's reagent for 15 min at room temperature. The customary rinsings in sulphurous acid, as for Feulgen's test, followed, and the sections were dehydrated in alcohols and mounted in balsam after xylene.

B. McManus (1948) (Stain Technology 23, 99)

1. Routine for Paraffin Sections

1. Paraffin sections to water.
2. Wash in running tap water 5 min if iodine and hypo were used.
3. 0.5% periodic acid in water 5 min.
4. Rinse in distilled water.
5. Schiff's reagent, 15 min.
6. Rinse in three changes of sulphurous acid, each 2 min.
7. Wash in running water 3 to 5 min.
8. Stain in Harris haematoxylin 20 to 30 sec.
9. Wash in running water 5 min.
10. Dehydrate in two changes of 95% alcohol.
11. Two changes of absolute alcohol.
12. Clear in xylene and mount in balsam.

(Note: Steps 8 and 9 are optional.)

2. Preparation of Schiff's Reagent

1. Weigh out basic fuchsin, 1 g.
2. Weigh out anhydrous sodium bisulphite, 1 g.
3. Boil 200 ml distilled water.
4. Add fuchsin and stir.
5. Cool to 50°C.
6. Filter.
7. Add 20 ml $\text{N HCl}$ (98.3 ml of HCl, S.G. 1.16, made to 1000 ml with distilled water).
8. Cool to 25°C.
9. Add sodium bisulphite.

(Keep in dark. The fluid takes about two days to become orange or straw coloured; then it is ready for use.)

3. Sulphurous Acid Rinse

1. 10% sodium metabisulphite, 6 ml.
2. Normal HCl, 5 ml.
3. Distilled water, 100 ml.

Results: Known carbohydrates in tissue, mucin and glycogen, for example, are red to purple. Many other sites are similarly coloured,
renal basement membrane, B cells of the human hypophysis, many types of colloid and various pigments.

C. Lillie (1947)

1. The Periodic Acid, Schiff Sulphite Leucofuchsin Reaction, 
Short Variant

1. Deparaffinize and hydrate through xylene, alcohols, and distilled water as usual.
2. Oxidise 10 min in 0·69% (0·03 M) KIO₄ in 0·3% HNO₃.
3. Wash 5 min in running water.
4. Immerse for 10 min in Schiff’s reagent.
5. Transfer quickly and directly to three successive baths: 1,2 and 2 min in 0·5% sodium metabisulphite (Na₂S₂O₅). Replace sulphite rinses daily or more often.
6. Wash 5 min in running water.
7. Counterstain nuclei and cytoplasm as desired: e.g.,
   (a) Stain 2 min in a 2% acetic haemalum solution of about 0·1% haematoxylin content (Mayer’s or a 1:5 dilution of Lillie’s). Wash in water, and blue with a drop or two of 20% Na₂CO₃ in 200 c.c. water.
   (b) Stain 2 to 4 min in Weigert’s acid iron haematoxylin: decolourize 10 sec in Pal’s bleach diluted 1:5 with distilled water. Wash 4 min in running water.
   (c) Stain 6 min in Weigert’s acid iron haematoxylin, wash 4 min in running water, counterstain picric acid solution. Other counterstains may be used.
8. Dehydrate (and differentiate) in two changes each of 95% and 100% alcohol. Clear in one change of alcohol xylene mixture (1:1) and two of xylene. Mount in suitable resin, such as polystyrene, HSR, permount, ester gum, clarite, Depex, or the like.

D. Hotchkiss (1948) (Archives of Biochemistry)

Fixation may be in usual fixatives; mercury salts, if used, are removed with iodine; formaldehyde, if used, is removed by thorough washing. If glycogen, or other easily soluble polysaccharide, is to be demonstrated, fixation and washing should be in alcoholic or other fluids that do not dissolve this substance. Approximately 70% alcohol has been used successfully for washing in such cases, and the standard procedure
suggested here calls for this environment up to the stage at which fuchsin-sulphite is used. Whenever such precautions are unnecessary, aqueous solutions may be used.

After bringing the section or smear into alcohol:

(a) leave 5 min at room temperature in periodic acid solution A;
(b) flood with 70% alcohol, transfer to reducing rinse, leave 5 min;
(c) flood with 70% alcohol, leave 15–45 min in fuchsin-sulphite;
(d) wash 2–3 times with SO₂-water as for Feulgen staining, dehydrate and mount as usual;
(e) counterstaining (if desired): If staining is mainly to show polysaccharides, counterstain with a basic dye. Malachite green in dilute aqueous solution (about 2 mg/100 c.c.) has been satisfactory for some preparations. This will tend to stain the nucleic acids, which are not affected by the periodate-fuchsin. If staining is intended for mucin or acid polysaccharides, it is presumably better to counterstain with an acid dye; and
(f) control sections are carried through the same process, eliminating step (a).

RESULTS: Glycogen, starch and mucin are coloured red to purple with about the same intensity as in the PAS reaction. Basement membrane, reticulin of the splenic pulp and other materials stain much less intensely, perhaps as a result of the reducing rinse which can destroy some aldehyde groups.

E. Spot Tests

1. Hotchkiss (1948): Periodate-Fuchsin (Archives of Biochemistry)

Solutions containing approximately 1 mg of various preparations are placed in a spot plate and treated for 5 min with one drop of periodic acid. Towards these aqueous solutions, the fresh alcoholic solution A is fully as vigorous as solution B and the drops are of a more convenient size. Because the periodic acid (and acetate buffer) is not removed, an appropriate small excess of dilute hydrochloric acid is added immediately before the reducing rinse. A periodic acid blank reduced in this way should give no colouration with Schiff's reagent, and also not interfere with colour development when a drop of very dilute formaldehyde is added.

Satisfactory proportions are:

(a) Water or polysaccharide solution, 0·05–0·2 c.c.
(b) Periodic acid solution A, 0·025 c.c. (or 1 drop). Leave 5 min.
PERIODATE OXIDATION TECHNIQUES

(c) N/10 hydrochloric acid, 0·05 c.c. (or 1 drop).
(d) Reducing rinse (alcoholic), 0·1 c.c. (or 4 drops). Mix.
(e) Fuchsin-sulphite reagent 0·05 c.c. (or one drop). Leave 15 min.

Similar proportions of the aqueous solutions may be used, making allowance for larger drop size.

2. McManus and Hoch-Ligeti (1952) (Lab. Invest. 1)

Solutions of various sugars and materials to be tested were made up in distilled water. One part of a sugar solution was mixed with one part of periodic acid, shaken to mix, and left for 5 min at room temperature. At the end of this time five parts of Schiff’s reagent were added to the sugar-periodic-acid mixture. No immediate colour developed as a rule, but after 5 min slight colouration began to appear and this was usually fully developed in 10 or 15 min. Various non-carbohydrate materials were tested, including principally amino acids and non-organic materials. These were uniformly negative under the conditions of this test-tube method, while all the sugars and known carbohydrates-containing compounds were positive.

Hoogwinkel and Smits (1957) have developed another spot test using periodic acid. The publication should be consulted for details. It is of interest that their samples of hyaluronic acid and chondroitin sulphuric acid were negative with their spot test.

IV. CRITIQUE

A cytochemical reaction or method is valid when it is both predictable and provable. The predictability is usually based upon the experience of organic chemists and biochemists. The task of proof depends upon the use of the methods again of classical chemistry, refined and altered for the study of minute amounts of materials in microscopic sections. For the long-term outlook, it is necessary to demonstrate that the process under consideration, in this case periodate oxidation, conforms to expected results with known carbohydrates, and that other structures in tissue which are acted upon in similar fashion are, or are not, behaving in a pattern which conforms to that expected of material containing 1,2-glycol groups, largely carbohydrates. This to my mind is the crux of the usefulness of periodate oxidation in cytochemistry.

Periodic acid oxidation of tissue does make a number of substances colourable by Schiff’s reagent, whose carbohydrate nature and 1,2-glycol content is well known. Most conspicuous of these materials are mucin, glycogen, ground substance of cartilage, starch granules and plant cell
walls. Other structures and substances whose carbohydrate nature was suspected but not proven include colloid of the pituitary stalk and thyroid, and some basophile (? mucoprotein-gonadotrophin) cells of the anterior hypophysis. Another group of materials include substances whose carbohydrate content was not suspected, as basement membrane, some or all reticulin fibrils, fibrin and "fibrinoid", ceroid, melanin, etc. The last group of materials present the stumbling blocks to the easy cytochemical acceptance of material colouring with Schiff's reagent after periodic acid being carbohydrate, i.e. consisting of, or containing, 1,2-glycol groups, probably carbohydrate.

A number of accessory data need to be used for the determination of the nature of materials colouring with Schiff's reagent after periodic acid oxidation. These are derived from a variety of sources, chief among which are:

(A) Relative data concerning periodate oxidation.
(B) Comparison with other oxidation procedures.
(C) Blocking, reversible, or irreversible, of periodate oxidation.
(D) Identification of Schiff colouring material after periodate oxidation as containing aldehyde.
(E) Identification of material colouring with PAS as of certain chemical constitution.
(F) Removal of PAS-positive material by enzymes.

It will be seen that (A) through (C) above concern the materials acted upon by periodate, particularly in regard to the linkages involved, (D) as studying the mechanism of Schiff's colouring, and (E) and (F) as attempts at the identification of PAS-positive materials by other means, admittedly less precise. These will be discussed in turn.

A. Relative Data concerning Periodate Oxidation

The concentration of periodic acid in a free state influences the rapidity with which periodate oxidation of a-glycols and their substituents occur. Excess of free periodic acid was associated with slow and incomplete reaction with hydroxylysine and serine (Van Slyke, Hiller and MacFadyen, 1941). a-Glycols, hydroxylaldehydes, hydroxyketones and diketones are oxidized fastest in a slightly acid (pH 3-5) medium (Fleury and Courtois, 1950), while a-amino alcohols, amino aldehydes, amino ketones and diamines require a neutral or slightly alkaline (pH 7-8) medium for optimal oxidation rate (Fleury, Courtois and Grandchamp, 1949). Glycol cleavage occurs more readily at higher concentrations of periodate (Moe, Miller and Buckley, 1951). Dyer (1956) points out that "simple and un-
hindered a-glycol and a-amino alcohol groupings will be nearly completely oxidized in 5–10 min at their pH of optimum reaction, if a moderate excess of periodate is present. Usually somewhat longer periods of time are required for the oxidation of a-hydroxyaldehydes, a-hydroxyketones, a-ketoaldehydes, a-diketones and the corresponding amino derivatives”.

The concentration, molarity, pH, solvent and duration are given for a number of periodate oxidation mixtures in Table II taken from Lillie (1954). Dyer (1956) points out that the concentration of periodate usually used for glycol cleavage is 0·01–0·1 M.

<table>
<thead>
<tr>
<th>Author</th>
<th>Date</th>
<th>Source of HIO₄</th>
<th>Concentration</th>
<th>Molarity</th>
<th>pH</th>
<th>Solvent</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>McManus</td>
<td>1946</td>
<td>H₄IO₆</td>
<td>0·5%</td>
<td>0·022</td>
<td>2·1</td>
<td>Distilled water</td>
<td>5</td>
</tr>
<tr>
<td>Lillie</td>
<td>1947</td>
<td>NaIO₅</td>
<td>1·0%</td>
<td>0·036</td>
<td>1·6</td>
<td>0·5% aq. HNO₃</td>
<td>10</td>
</tr>
<tr>
<td>Hotchkiss</td>
<td>1948</td>
<td>H₄IO₆</td>
<td>0·8%</td>
<td>0·035</td>
<td>2·5</td>
<td>0·02 M Na acet., aq.</td>
<td>5</td>
</tr>
<tr>
<td>Hotchkiss</td>
<td>1948</td>
<td>H₅IO₆</td>
<td>0·8%</td>
<td>0·035</td>
<td>2·4</td>
<td>0·02 M Na acet., 70% alc.</td>
<td>5</td>
</tr>
<tr>
<td>Lillie</td>
<td>1949</td>
<td>KIO₄</td>
<td>0·8%</td>
<td>0·035</td>
<td>1·9</td>
<td>0·3% aq. HNO₃</td>
<td>10</td>
</tr>
<tr>
<td>Lillie</td>
<td>1950</td>
<td>KIO₄</td>
<td>0·69%</td>
<td>0·030</td>
<td>1·9</td>
<td>0·3% aq. HNO₃</td>
<td>10</td>
</tr>
<tr>
<td>Mowry</td>
<td>1952</td>
<td>H₅IO₄</td>
<td>1·0%</td>
<td>0·044</td>
<td>---</td>
<td>90% alcohol</td>
<td>120</td>
</tr>
</tbody>
</table>

* After using this solution for some months, it was found that it tended to deposit a few crystals at 20–25°C temperatures, therefore the 1950 modification was made.

It will be seen that the oxidation time, molarity and pH favour a-glycol oxidation in preference to substituent groups capable of oxidation. The fortunate combination of these factors probably explains the ease with which known carbohydrates, rich in 1,2-glycol groups, are coloured with the PAS reaction.

McManus (1956) has summarized much of the data which favour the histochemical reaction of the periodic acid Schiff (PAS) reaction being restricted to 1,2-glycols.
B. Comparison with Other Oxidants

Bauer (1933) introduced one-hour oxidation in 5% aqueous chromic acid as a preliminary to colouring glycogen with Schiff's reagent. He specifically stated that mucin was not coloured by this technique, but subsequent reports by a number of workers (Wallraff and Beckert, 1939, Bignardi, 1940, Dempsey et al., 1947) showed that mucin, thyroid colloid and other substances are coloured also. It was pointed out by Hotchkiss (1948), and subsequently by Lillie (1951), that chromic acid destroyed aldehyde groups formed from glycol oxidation. Potassium permanganate used as a histochemical oxidant of carbohydrates by Casella (1942), and by Lillie (1947), appears to have the same defect according to Lillie (1951).

A variety of other oxidants, sodium persulphate (Bignardi, 1940), acetates of manganese (Lhotka, 1953), sodium perbismuthate (Lhotka, 1952) and lead tetra-acetate (Crippa, 1951), have been used as preliminary oxidants to produce colouring of mucins, etc., by Schiff's reagents. It is proposed to describe the features of lead tetra-acetate oxidation in some detail. Suffice it to say that these other 1,2-glycol oxidants produce about the same results in tissue sections with Schiff's reagent as do periodic acid, taking into account the differences in rate, concentration, solvent, etc.

Lead tetra-acetate was pointed out by Criegie (1943, ref. in Jackson, 1944) to behave about the same as periodic acid. Crippa (1951), Shimizu and Kumamoto (1952), Lhotka (1952a) Glegg et al., (1952), Hashim and Acra (1953), and Jordan and McManus (1952), all used lead tetra-acetate as an oxidant to 1,2-glycols in histochemistry. There is a notable decrease in the rate of oxidation of some substances in tissues with lead tetra-acetate, glycogen for one. Glegg et al., (1952) suggest that areas which stain less intensely with Schiff's after periodate than after lead tetra-acetate, e.g. some types of mucus—may contain a-hydroxy acids while areas showing the reverse phenomena, more after periodate than after lead tetra-acetate, contain mainly a-amino alcohol groups, e.g. reticulin fibres, basement membranes. It was the feeling of Jordan and McManus (1952) that the differences in rate of oxidation were explainable upon the solvent used.

Table III describes the composition of various lead tetra-acetate oxidation mixtures.

Staple (1955, 1957) reports that boric acid treatment of reactive sites will block lead tetra-acetate oxidation, but not periodic acid oxidation if conditions of ionic strength are adjusted. The boric acid blockade needs to be carried out, in the case of lead tetra-acetate, before and during the
 action of the oxidant. Staple (personal communication, 1959) emphasizes the role of ionic strength in periodate oxidation as a feature requiring further study.

Graumann (1953) describes using 0·5% lead tetra-acetate in 8 N glacial acetic acid. He was able to colour cartilage ground substance of the ulnar epiphysis of *Cricetus auratus* and the piston cartilage of *Petromycon marinus* with Schiff's reagent, while no colouring was produced by the PAS reaction. The difference he blamed upon every second monosaccharide unit of chondroitin sulphuric acid being an oxyacid, susceptible of lead tetra-acetate oxidation, while only the terminal members contain a-glycol susceptible of periodate oxidation. It is unfortunate that he did not try periodic acid in 8 N acetic acid. Mowry (personal communication) tells me that both Wharton's jelly and ground substance of cartilage are much more Schiff-positive after HIO₄ in glacial acetic than either the regular aqueous PAS or the use of alcoholic periodate before the Schiff's. It may be that selective staining of specific groups with lead tetra-acetate is occurring as Graumann (1953) suggests, but the effect of the solvent on periodate oxidation is very important as the following data tabulated from Mowry (personal communication) suggest.

In summary, it can be said that organic oxidants similar in action to periodic acid produce the same appearances in tissue sections, when followed by Schiff's reagent, as do the routine aqueous periodate methods, allowing for solvent differences, rate, concentration and so on. The field of periodate solvent requirements and optima is a new one which Dr. Robert Mowry is actively exploiting. Theoretically, there is some
comparison possible between the conditions and positions of reactive side chains of tissue constituents in relation to potential chemical changes, and the phases of mono-layer techniques. Solvents which do, or do not, allow the easy access of reagent to reactive site will be expected to alter reaction rate and specificity.

C. BLOCKING, REVERSIBLE OR IRREVERSIBLE, OF PERIODATE OXIDATION

1. Acetylation

Classically, the demonstration of a reaction as involving hydroxyl groups is accomplished by esterification of the hydroxyl groups by acetic anhydride or ketone. McManus and Cason (1950) set up a system of acetic anhydride-pyridine (15:20) which was effective for renal basement membrane in 45 min but which required up to 16-18 hr (Lillie, 1951) for glycogen. This acetylation process was suggested earlier and independently by Gersh (1949) without details of procedure.

The reaction takes place as follows:

\[
\begin{align*}
\text{R} & \quad \text{H} - \text{C} - \text{OH} \\
& \quad \text{H} - \text{C} - \text{OH} \\
& \quad \text{H} - \text{C} - \text{OH} \\
& \quad \text{H} - \text{C} - \text{OH} \\
& \quad \text{R} \\
\end{align*}
\]

Periodic acid oxidation of this structure does not produce any aldehyde as it would be expected to do from the original 1,2-glycol.

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### TABLE IV

**Minimum Time for Optimum Colouration, HIO₄, Different Solvents**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Renal basement membrane</th>
<th>Glycogen</th>
<th>Epithelial mucin</th>
<th>Cartilage ground substance</th>
<th>Reagent stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>5-10 min</td>
<td>5-10 min</td>
<td>5-10 min</td>
<td>5-10 min</td>
<td>Months</td>
</tr>
<tr>
<td>Ethanol, abs.</td>
<td>10 min</td>
<td>6-12 hr</td>
<td>2 hr</td>
<td>2 hr</td>
<td>Hours</td>
</tr>
<tr>
<td>Methanol, abs.</td>
<td>10 min</td>
<td>1-2 hr</td>
<td>10-30 min</td>
<td>10-30 min</td>
<td>Days</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>10 min</td>
<td>1-2 hr</td>
<td>10-30 min</td>
<td>10 min&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Months&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>10 min</td>
<td>2 hr</td>
<td>60 min</td>
<td>60 min</td>
<td>Hours</td>
</tr>
</tbody>
</table>

<sup>a</sup> Optimum colouration of glacial acetic solution is deeper than aqueous solution.

<sup>b</sup> Precipitate of iodate after several days, removable.
PERIODATE OXIDATION TECHNIQUES

PROCEDURE

Acetylation:
1. Paraffin sections to water.
   (a) Place sections in: Acetic anhydride, 13 c.c.
       Pyridine, 20 c.c.
       Leave for 45 min at room temperature up to 18 hr.
   (b) Wash tap and distilled water.
2. Periodic acid 0.5% aqueous for 5 min.
3. Following steps as above, regular PAS (p. 178). Negative reaction.

De-Acetylation:
1. Sections to water.
   (a) Place sections in: Acetic anhydride, 13 c.c.
       Pyridine, 20 c.c.
       Leave for 45 min at room temperature.
   (b) Washed distilled water.
   (c) Place in 0.1 N KOH for 45 min at room temperature.
   (d) Wash in tap and distilled water.
2. 0.5% periodic acid, 5 min.
3. Following steps as regular PAS. Positive reaction.

Acetylated sections do not colour with Schiff's reagent after periodic acid oxidation. As Pearse (1953) has pointed out, certain substances are soluble in acetic anhydride-pyridine and are dissolved rather than acetylated. For this reason it is desirable to return the glycol grouping in the sections and proceed with the PAS.

It has been repeatedly reported that since many groups are acetylated besides hydroxyl groups (amine, etc.) the simple blockage of the PAS reaction, or even its reversal by saponification of the acetyl esters to hydroxyl groups, does not suggest any further specificity. It has been noted, however (Wolform et al., 1936), that N-acetyl groups are stable under alkaline conditions which remove O-acetyl groups. The ready removal of the blocking effect of acetylation by saponification with weak alkali is strong evidence for primarily O-acetyl groups being involved.

2. Sulphation

Sulphation was discovered accidentally (McManus and Mowry, 1952) in an attempt at caramelizing carbohydrate materials in tissue sections with concentrated sulphuric acid. The PAS reactivity of many substances disappeared. Subsequently it was found that basophilia acquired by many substances after sulphuric acid treatment was associated with
the new appearance of metachromasia (Kramer and Windrum, 1953). These workers decided (Kramer and Windrum, 1954) that this effect of sulphuric acid treatment was due to the formation of sulphuric acid esters. A number of additional methods for sulphation have been described by them.

Not all of the methods used to induce sulphation yield the same results. Glycogen is sulphated by some but not all. While nearly all PAS-positive structures become basophilic, only certain structures are distinctly metachromatic. For example, collagen is strongly basophilic but not clearly metachromatic after sulphation.

This process has been under study by Dr. Robert Mowry at the University of Alabama Medical Center, Birmingham, Alabama. The following paragraph is derived from unpublished material which he has been good enough to make available.

"Variations in PAS positivity in dextran have a direct relationship with sulfation and vary inversely with metachromasia, i.e., maximally sulfated dextran is PAS-negative and metachromatic while non-sulfated dextran is strongly PAS-positive and not metachromatic. Intermediate degrees of sulfation, metachromasia and PAS-positivity can be produced, (Mowry, 1954). A comparable range of histochemical properties is seen in the ground substance of cartilage under various conditions, and may signify varying degrees of sulfation of chondroitin.

In tissue sections, the PAS positivity of Brunner gland mucin could be abolished by 30–60 seconds in concentrated sulphuric acid, but metachromasia appeared in the previously non-metachromatic mucin. Dr. Mowry has developed a new method for sulfation which is somewhat less drastic than sulfuric acid. Sections are placed in a mixture of equal parts of sulfuric acid and anhydrous diethyl ether. One hour suffices for most materials, e.g., mucin, renal basement membrane, collagen—to become PAS-negative. Glycogen is less constant in the sulfation produced by diethyl ether sulfuric acid methods, resembling the oxidation effect in various solvents described previously."

The sulphation process appears to be a true esterification of hydroxyl groups principally, but because of the drastic effect of the sulphuric acid, it is quite probable that other structural alterations are being produced and probably other groups being sulphated. It would be desirable to desulphate the sections with a hope of returning the hydroxyl groups. This has been difficult to do in the past. It should be possible by a method used for the production of cellulose acetate. According to Malm et al.,
PERIODATE OXIDATION TECHNIQUES

(1946), cellulose is first combined with sulphuric acid as the acid sulphate and finally the sulphate replaced by acetyl groups. A similar process for tissues is now being attempted.

Both acetylation-deacetylation and sulphation have in common the removal of the free 1,2-glycol groups from availability for periodic acid oxidation, the former process including the return of the reactive linkages. There is the certainty that both "blocking" processes are not specific for the hydroxyl groups, but there is the additive information of destruction of PAS-positivity by hydroxyl-active reagents in carbohydrates like glycogen and mucin, as well as the materials in which the basis for PAS-positivity is less clear.

D. IDENTIFICATION OF THE MATERIAL COLOURING SCHIFF’S REAGENT AFTER PERIODIC ACID OXIDATION AS ALDEHYDE

Herman and Dempsey (1951) used the Ashbel-Seligman reagents for carbonyl compounds after periodic oxidation, and found the same situations staining as in the PAS reaction. There was, however, some difference in intensity of staining which was unexplained. Glegg et al., (1952b) used aldehyde reagents—benzidine and o-dianisidine—and found no significant change in the histological distribution and intensity of the reaction as compared with Schiff’s reagent, all these aldehyde reagents following periodate oxidation. Lillie (1954) used phenylhydrazine hydrochloride, aniline hydrochloride and neutral potassium cyanide, as agents blocking the aldehyde and preventing Schiff colouring with periodic acid oxidation. Similar blockade appears in Hotchkiss’s (1948) reducing rinse, as shown by McManus (1948), Lillie (1951), McManus and Hoch-Ligeti (1952). Lillie (1954) reports reversal of the bisulphite blockade of Hotchkiss. Exposure to Schiff’s reagent (2–3 hr instead of 15 min of PAS) will overcome sulphite blockade. In this same discussion by Lillie, the various other blockade mechanisms and techniques are described and discussed.

Lillie (personal communication, 1956) has demonstrated some interesting features about blockade of aldehyde by phenylhydrazine, aniline hydrochloride or o-aminophenol hydrochloride following periodate oxidation. Further oxidation of the blocked section produced further aldehyde, suggesting that oxidation was complete in 10 min. Cyanide blockade was reversed by further periodate oxidation on the other hand and new aldehyde, or uncovered aldehyde, could be demonstrated with Schiff’s reagent.

The studies referred to in the previous paragraph have been presented in published form (Lillie, 1956) to which reference should be made.
Staple (1958) has studied factors in aldehyde blockade by hydroxylamine, aniline, phenylhydrazine and other materials. His results vary in some details from Lillie’s (1956) and should be consulted for studies in this interesting area.

Silver complexes. It has been suggested since Mitchell and Wislocki (1944) that aldehyde released by oxidation can be demonstrated by silver complexes. These authors produced aldehyde from glycogen by potassium permanganate (cf. p. 184), and coloured it with ammoniacal silver, followed by formalin reduction. Arzac (1947) also used the same sequence, without knowledge of Gomori’s (1946) criticism of a method for the demonstration of aldehyde using an aldehyde (formalin). At the same time Gomori described a method for glycogen in which the aldehyde was coloured by a methanamine-silver. Lillie (1948) used periodic acid before silver complexes to demonstrate glycogen. Pearse (1953) used two of Arzac and Flores (1949) silver solutions, a lithium silver and a piperazine silver, to colour glycogen after chromic acid (cf. p. 184) oxidation. Mucin was coloured as well. Jones (1953) used periodic acid before methenamine silver to colour basement membrane in the kidney.

It seems obvious that certain ammoniacal silver solutions will colour aldehyde after periodate oxidation, and that satisfactory histological preparations may be so prepared. It can be shown, for example, that Pap’s silver solution after periodic acid oxidation will colour many of the structures shown by the Schiff’s reagent (McManus, unpublished studies, 1947-48). However, other structures are coloured as well and it does not seem reasonable to use a silver method for demonstrating aldehydes. For histology, the matter is not so critical. The chemistry of complex silver salts is even less clear than that of Schiff’s reagent.

Other means of demonstration of the aldehyde have been described using various coloured or colourable compounds. Bennett (1940) used phenylhydrazine, Dempsey and Wislocki (1946) used 2:4 dinitrophenylhydrazine, and Danielli (1949) used azobenzene phenylhydrazine. Camber (1949) and Ashbel and Seligman (1949) substituted 2-hydroxyl 3-naphthoic acid hydroxide, producing a coloured compound by coupling with a diazonium salt. Pearse (1953) reviews these methods, and describes the colouration of the sites normally coloured with Schiff’s reagent after periodate oxidation by Seligman, Gofstein and Rutenberg’s (1949) phenyl-hydrazine-formazan reaction after periodate oxidation. Monné and Slautterbeck (1950) combined phenyl-hydrazine and Schiff’s reagent for colouring materials in the sea-urchin egg after periodate oxidation. Inferences were drawn as to different materials being shown, perhaps rather tenuously.
A less well-founded method for demonstration of aldehyde is that in which basic fuchsin or para-rosaniline is used. DeLamater et al., (1950) had noted that after acid hydrolysis, aldehyde mordanting and basic fuchsin, colouring occurred much like that seen in the Feulgen reaction to which they compared the reaction. These authors recall the nuclear staining by the compound formed by formaldehyde and Schiff's reagent (Choudhouri, 1943) confirmed by Danielli (1947). DeLamater (1948a, b, c) had developed the method for the study of the nuclear cytology of fungi, and noted that without the formaldehyde "mordanting", the basic fuchsin was not firmly fixed.

Arzac (1948) reported staining carbohydrates following periodic acid oxidation by 0.05% basic fuchsin in 3% aqueous phenol containing 5 c.c. of 96% ethanol, followed by sulphite rinses. In 1950, Arzac gave further details of his method, added aniline water as a solvent for the basic fuchsin but later withdrew it, and referred to DeLamater's (1948b) study suggesting that aldehyde reacted with the para-amino groups of the dye molecule. The technique has been called "forming Schiff’s reagent on the slide" (McManus, 1952), and that is precisely what seem to be happening. It may well be that an analysis of Arzac’s colouration of aldehydes will give us information about Schiff’s reagent. In the meantime, caution should be used in considering Arzac's (1950) method a histochemical reaction for aldehydes.

Thionine-sulphite complexes have been used in place of Schiff’s reagent by a number of workers. Östergren (1948), Van Duijn et al., (1954), DeLamater et al., (1955) and Van Duijn (1956) have described the use of such mixtures to identify and colour the aldehyde produced by periodate oxidation. Additional Schiff-type reagents have been studied by Kasten (1958).

An accessory confirmation of the aldehyde nature of the material produced by periodic acid oxidation is found in the destruction of aldehyde by Hotchkiss’s Reducing Rinse (p. 180).

The spot test of Hotchkiss made use of the reducing rinse. It had been noted (McManus, 1948) to reduce the intensity of Feulgen staining if interpolated between the acid hydrolysis and the Schiff’s reaction. This action seems to consist of destroying, blocking or making unavailable some of the aldehyde groups which had been produced by the periodic acid oxidation or by the acid hydrolysis. The difficulty of Jeanloz (1950), that cellobiose and methyl L-glucopyranoside while containing 1,2-glycol did not give a positive (Hotchkiss) spot-test, was resolved by the development of a spot-test (McManus and Hoch-Ligeti, 1952) in which no reducing rinse was used, the sulphite of the Schiff's reagent (p. 178) destroying the periodate.
E. IDENTIFICATION OF MATERIAL COLOURING WITH PAS AS OF CERTAIN CHEMICAL CONSTITUTION

The results of the spot-tests of Hotchkiss (1948) and of McManus and Hoch-Ligeti (1952) represent instances in which chemical assay can be done on portions of materials tested by Schiff's reagent after periodate oxidation. Similarly, Glegg et al., (1952b) have used films of various materials, and have confirmed with the periodic acid Schiff technic the intense reactivity of many polysaccharides, mucopolysaccharides and of mucoproteins. They point out that hyaluronic acid and chondroitin sulphuric acid, among carbohydrates, as well as ordinary proteins, gave negative film tests.

Davies (1952) failed to colour hyaluronic acid by a film method PAS technique. Braden (1955) tried to colour acid and neutral mucopolysaccharides using the PAS method of films made with casein-carbohydrate mixtures dried on slides. Acid mucopolysaccharides, except that from dentine, were weakly PAS staining while neutral polysaccharides stained strongly. In passing, Braden found acid mucopolysaccharides such as hyaluronic acid and chondroitin sulphate to be strongly meta-chromatic, recalling the situation with dextran above.

Glegg et al., (1953) investigated by paper chromatography materials from reticulin which contained galactose, mannose, glucose and fucose, believing these to be the sugars responsible for the positive PAS staining of reticulin. The increasing use of the PAS reaction for the study of protein-bound carbohydrates in paper electrophoresis of serum (Koiw and Grönwall, 1952), is particularly useful in human diseases where carbohydrates of the amyloid group are present (Rice, 1954).

Leblond et al., (1957) have studied further the carbohydrates present at sites of PAS staining. Once glycogen is removed, PAS staining material in sections is a carbohydrate-protein complex, according to these authors.

Joel et al., (1956) have found good correlation between the PAS colouration of tissues and the extractable carbohydrates.

F. REMOVAL OF PAS-POSITIVE MATERIALS BY ENZYMES

Diastase or one of the amylases for removal of materials from tissues has been used for a long time (as saliva) as proof of glycogen (Bensley, 1939). The technique of testing is to pair-stain serial sections, one of which has undergone salivary digestion. Material removed by saliva is glycogen if the corresponding material is preserved in the second slide.
and stains appropriately by Best's Carmine, Bauer, PAS, etc. Lillie and Greco (1947) introduced malt diastase solutions as a substitute for saliva, and shortly thereafter Lillie et al., (1949) separated other enzymatic activities out of the diastase, ribonuclease-like and chondroitinase-like.

A similar difficulty exists in the case of the pectinase enzymes—e.g. polygalacturonidase—which McManus and Saunders (1950) found would remove PAS-positive materials (basement membranes, hyalines, mucins, etc.) from acetone fixed tissues. Despite the fact that the pectinase effect, like the diastase effect, could be reversibly blocked by acetylation, suggesting 1,2-glycol participation in the substrate (McManus and Cason, 1951), the enzymes are not pure materials, substrate specific. Even if they were, it might be that a substance other than the material responsible for the stain, but necessary for the structure, is being removed. These features have been dealt with by Danielli (1946).

Eidinger and Ghosh (1956) have studied the effect of mucolytic enzymes including pectinase on the PAS colouration of tissues.

V. ACCESSORY OBSERVATIONS

Hale (1953a, b) notes that certain mucins which colour weakly with the periodic acid-Schiff methods, can be increased in staining ability by exposing them to a solution of sodium hydroxide. More of the susceptible linkages are thought to be made available to periodate oxidation by the manoeuvre, perhaps by protein extraction but not by de-acetylation or de-sulphation. This is an interesting observation which may furnish a means of identification further of PAS-positive materials.

Hale (1955) investigates the role of formalin fixation in reduction of colouring with PAS of rectal mucus, while gastric mucus has shown no such effect. Sodium hydroxide hydrolysis abolishes the formalin effect, perhaps due to polymerization and de-polymerization.

Lhotka (1952c) reports observations on gelatin blocks impregnated with isomers of cyclohexane glycols, and on sections of rat, rabbit and human intestine. cis isomers in the blocks were oxidized at 10-60 sec while trans isomers were oxidized in 30-120 sec. The observations on sections were interpreted as showing cis isomers after 2 min oxidation, while trans isomers were shown after that time. It seems to the reviewer that it is to be remembered that some cis isomers are so oriented that rapid cleavage is possible, but that all cis 1,2-glycol do require longer oxidation. Two minutes' oxidation was recommended in the first histochemical use of periodic acid (McManus, 1946), but 5 min oxidation was found more
satisfactory (McManus, 1948). It may be the difference is on the basis which Lhotka suggests.

Dempsey et al., (1950) observed increased basophilia of tissue proteins after periodate oxidation for 1 hr of a 1% aqueous solution at 37° C. It was observed that greatest basophilia was acquired by regions at high sulphur content—keratinized epidermis and hair—perhaps by the oxidation of sulphide or sulphydryl groups to corresponding sulphonic acids.

Pearse (1951) introduced performic acid as an oxidation agent for the demonstration of phosphatide lipoids and of keratin. It was thought that alamine-beta-sulphinic acid coloured Schiff's reagent after formal oxidation of keratin, while the Schiff reaction was due to aldehyde materials produced from lipoid by performic acid. Lillie (1952) used peracetic acid for the study of ceroid and noted that it coloured hair matrix with Schiff's reagent, and blamed both reactions on ethylamic groups in a lipid present. Lillie and Bangle (1954) did not believe a disulphide bond product such as supposed by Pearse, could give a recolouring of Schiff's reagent. Findlay (1955) was unable to block Schiff's colouring of hair after peracetic acid by aldehyde blocking reagents and other aldehyde reagents, six in all, did not block the Schiff colouring nor colour themselves.

Gomori (1950) introduced aldehyde-fuchsin as a stain for elastic tissue. The relationship of this dye material to Schiff-staining and to aldehyde reactions is obvious. The dye appears to stain a number of metachromatic and/or PAS-positive materials. Included are some of the basophiles of the anterior pituitary. Halmi (1952) and Halmi and Davies (1953) have investigated some of the staining reactions and sites of this interesting and potentially important dye. Benassi and Zini (1951) have described the relationships between metachromasia and PAS positive material in the kidney of several species.

Itikawa and Ogura (1954) studied optimum pH of Schiff's for colouring after periodate oxidation. This was found to be 2.4 while optimum colouring after Feulgen hydrolysis was at pH 3. Ihnuma and Saka (1952) observed colouring of cytoplasmic and nucleolar material, presumably including ribonucleic acid, in the liver of human and a fish, human spleen and lymph nodes, etc. They agree that a positive PAS reaction does not always mean the presence of carbohydrates. The question of 1,2-glycol linkages in ribonucleic acids is a familiar one. After observing PAS-positive material in some human pancreatic cytoplasm, it was no surprise to the reviewer when Allen (1951) reported periodate susceptible linkages in ribonucleic acid.

The duration of periodate oxidation is important. Malaprade (1928) had described destruction of aldehyde by prolonged oxidation, and the
personal communication of Lillie has confirmed the opinion that periodate oxidation in aqueous solutions of known carbohydrates, mucin, glycogen, etc., is complete in 10 min in tissue sections. Dyer (1956) describes dangers of over-oxidation in vitro, and Lhotka (1953) has shown the appearance of Schiff colouration of abnormal situations after prolonged oxidation. It does not seem reasonable to prolong oxidation past 10 min.

Recently the reviewer (unpublished studies) has decreased considerably the amount of periodic acid used in his histochemical technique. While the 0.5% aqueous periodate has a molarity of 0.022 and a pH of 2.1, well within the working optimum of Dyer (1956), it has been found that a 0.25% aqueous solution with corresponding decrease in molarity, still within the active range, and slight elevation of pH, presents the same oxidation effect for intestinal mucin. There seems no reason why 0.25% periodic acid in distilled water should not make an adequate oxidant for the PAS reaction.

Fats may become coloured with Schiff's reagent after periodate oxidation if of the type of glycolipids, as in Morrison and Hack's (1949) study of Gaucher's disease. Wolman (1950) found colouring of unsaturated fats with Schiff's reagent in smears and in sections after periodate acid for 10 min. The reaction was not blocked by acetylation and was thought due to oxidation of double bond carbon-carbon sites to 1,2-glycols. This PAS positivity of unsaturated lipid is still under discussion, but the acetylation procedure and Sudan Black staining should assist in the identification of lipids colouring with the PAS reaction. Wolman (1956) produces further evidence for lipid colouration by the PAS procedure.

Chu et al., (1955) describe in vivo control methods for the study of the PAS procedure.

Summary. These accessory observations represent areas in which investigation and analysis of techniques may prove productive. In some details, there is evidence that periodate oxidation in histochemistry may provide information unavailable or unrecognized by the methods of classical chemistry.

VI. APPENDIX

FURTHER DISCUSSION OF PROCEDURES

There is no point in having a satisfactory cytochemical method for a material if the tissue handling is such that all the substance which it is desired to study is lost before the test is applied. The most obvious example of this loss of material comes from the complete solution of ordinary storage fat, neutral or triglyceride fat, which occurs in the preparation of the paraffin section by the usual methods. A stain for fat, one of the
Sudan dyes, would be negative in the paraffin section of an ordinarily fixed, dehydrated and imbedded material. Osmium tetroxide, on the other hand, can fix neutral fat and carry it through paraffin imbedding, where it shows up in section by the black colour conferred by the reduced osmium.

The carbohydrates of animal and plant tissue resemble the neutral fat to some proportion, in that special precautions need to be taken to prevent their loss from tissue handling in the preparation of sections. To begin with, and almost certainly by any methods, the simple sugars are lost by any technique, being soluble in nearly every solution. The more complex sugars, e.g. dextran molecules of molecular weight over 50,000 can be preserved into section (Mowry, personal communication) by avoidance of water. It is possible then to preserve carbohydrates into section by:

1. Avoidance of solvents in tissue handling, e.g. freeze dry or alcohol techniques,
2. Special fixative methods, e.g. Holmgren and Wilander's (1937), subacetate of lead,
3. Certain carbohydrates are so insoluble that solution is difficult or impossible, e.g. Chitin.

Before discussing the more conventional methods of tissue handling, some mention should be made of freeze dry techniques. The sections of glycogen-containing liver which are studied after good freeze dry preparation, are probably as reasonable a facsimile of the living appearance as can be obtained. However, the experience of many workers in the field (and this is shared by this reviewer) without the full-time services of a vacuum specialist or a capable gadgeteer, is that the freeze-dry machines at present on the market and in design are expensive, capricious, exasperating and generally not worth the necessary effort to keep them working; all this apart from the fact that zones of degrees of preservation and morphology are present in the majority of freeze-dry blocks, just like the "Golgi" preparations, from which the investigator would choose an area most conforming to his idea of the real structure. Cytochemistry has by now advanced to the point that a freeze dry apparatus as a badge of guild membership is no longer necessary.

**Fixation.** The classical fixing solution for the water soluble carbohydrates is ethyl alcohol, either absolute or 95%. The cytological disadvantages of this alcohol as a fixative have been outlined by Baker (1950). The other classical fixatives of the carbohydrates have been picric acid mixtures, Rossman's fluid and similar picric acid mixtures. Deane et al., (1947) used an ice-cold picro-alcohol formalin mixture. Lison and
Vokaer (1949) used a similar mixture to which acetic acid was added, the whole kept at -73° by an acetone solid CO$_2$ mixture. Some glycogens are readily preserved by formalinsaline, e.g. cervical squamous epithelium (McManus and Findley, 1949). Liver glycogen in most species and ground substance carbohydrate in all examined require non-aqueous fixatives, preferably left in the cold.

Lillie (1947) used a freezing mixture to freeze eyes immediately on removal, and then placed them in a cold picric acid alcohol mixture, or in cold alcohol, claiming that melting was associated with the penetration of the fixative and obtaining excellent preservation of cellular detail and carbohydrates. I have not used this method but it seems reasonable. For the muco-proteins and muco-polysaccharides, with a high protein content, any of the classical protein precipitant fixatives—Zenker's, Helly's, Regaud's or Maxomov's solutions—are quite adequate.

Fixation by action on the carbohydrate itself probably occurs in Holmgren and Wilander’s (1937) use of sub-acetate of lead, long used as a carbohydrate precipitant in classical chemistry. Dempsey and Wislocki (1946) point out the shortcomings of this as a cellular fixative. Couteaux-Bargeton (1950) has suggested osmium as a fixative for glycogen. McManus and Lupton (unpublished) have used a brief (4–6 hr), fixation in normal saline 50 c.c., bichloride of mercury 10 g, OsO$_4$ 1 g, dissolved in the order given. There is loss rapidly of PAS-positive material, mucin and basement membrane, after 6 hr fixation and blackened myelin sheaths become PAS-positive. This ready appearance of factitious staining diminishes the enthusiasm for OsO$_4$ as a carbohydrate fixative.

Dehydration and imbedding can be carried out in the usual fashions for paraffin or celloidin techniques, although an occasional sample of celloidin will be PAS-positive. If all the mucin or all the glycogen are desired to be preserved, water must be avoided completely, i.e. no washing out after an aqueous fixative, etc.

Sectioning and mounting. Sections when cut can be flattened on the slide with heat and finger pressure, one of the major contributions of the freeze-dry school. Alternatively, they can be floated on to 70% alcohol, which Leach (1938) showed did not harm very delicate mucin or mast cells. The staining can be carried on with the paraffin still on the section, or it can be carried out on the de-paraffinized collodionized slide which has been baked on in the oven, de-paraffinized and coated with collodion as originally recommended by Arnold (1908) for use of Best’s (1906) carmine stain.

Reaction. Difficultly preservable material should be oxidized in alcoholic periodic acid, using 70% to 95% ethyl alcohol. The reaction time
must be lengthened to accomplish the equivalent of 5 min in aqueous periodic, sometimes up to 2 hr or more. In most instances the products of periodate oxidation in tissue—glycogen, mucin, etc.—are less soluble than the original material (Mowry and Millican, 1953) and aqueous Schiff's reagent can be used. When alcoholic Schiff's is felt necessary, it is hard to make since aldehyde-free alcohol is a troublesome preparation and the sulphurous acid of the Schiff's reagent quickly produces aldehyde from the alcohol. A solution usable for a few hours can be made by quickly dumping one part of Schiff's reagent into four parts of 96% alcohol and clearing with charcoal. The sequence is critical.

Dehydration, clearing and cover-slipping can be carried out in the usual fashions. Frozen sections do not allow demonstration of glycogen (McManus, 1948), probably due to water solubility as Pearse (1953) points out. Otherwise they can be handled like paraffin sections, and are preferable when glycolipids are being studied, although many of these will resist dehydration and paraffin imbedding as Gersh (1949), Morrison and Hack (1949) and Black-Schaffer (1949) have shown. Many of the complicating and simplifying procedures in the PAS technique are described and illustrated in McManus and Mowry (1958).

REFERENCES

PERIODATE OXIDATION TECHNIQUES 199

Blakiston, New York.
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Staple, P. H. (1957a). *J. Histochem.* 5, 472.