DEVELOPMENT OF METHODS FOR THE SIMULTANEOUS VISUALIZATION OF NEUTRAL SUGARS AND EITHER SIALIC ACID AND ITS SIDE CHAIN O-ACYL VARIANTS OR O-SULPHATE ESTER BASED ON THE SELECTIVE PERIODATE OXIDATION OF SIALIC ACID

by

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ABSTRACT:

The objective of this study was to establish conditions for the selective periodate oxidation of sialic acid, and then use these conditions to develop a series of general methods for the simultaneous visualization of "neutral sugars" (ie. hexose, 6-deoxyhexose and N-acetylhexosamine) and sialic acid and its side chain O-acyl substituted variants, or O-sulphate ester.

Investigations of selective conditions for the oxidation of sialic acids demonstrated that oxidation for one hour at 4°C with 0.4 mM periodic acid in approximately 1M hydrochloric acid (PA*) oxidized all available sialic acid residues of both the sialo and sialosulphoglycoproteins of human and rat colon and the sialoglycoproteins of rat sublingual gland. These conditions produced no visible Schiff staining of either neutral macromolecules or vicinal diols located on the "neutral sugars" of sialo and sialosulphoglycoproteins, and did not result in the extraction of epithelial glycoproteins or in the de-O-acylation of side chain substituted sialic acid residues. Therefore, PA* can be used as a specific reagent for the selective oxidation of sialic acids.

Studies of the mechanism of oxidation with PA* showed that the lack of PAS reactivity of "neutral sugars" was not due to the production of Schiff unreactive hemiacetals or hemialdals. It is possible that the selective oxidation of sialic acids with PA* results from an increase in the oxidation rate of sialic acid residues together with a decrease in the oxidation rate of "neutral sugars".

Based upon this method for the selective oxidation of sialic acid residues (PA*), five new methods have been devised for the simultaneous visualization of "neutral sugars" and either sialic acid and it side chain O-acyl

derivatives or O-sulphate ester. The first of these is the selective periodate oxidation-borohydride reduction-saponification-selective periodate oxidation-thionin Schiff-saponification-borohydride reduction-periodic acid-Schiff (PA*/Bh/KOH/PA*/T/KOH/Bh/PAS) technique, in which sialic acids with O-acyl substituents at C7, C8, or C9 (or which have two or three side chain O-acyl substituents) stain blue while "neutral sugars" with periodate sensitive vicinal diols stain magenta. In the second method, the saponification-selective periodate oxidation-thionin Schiff-saponification-borohydride reduction-periodic acid-Schiff (KOH/PA*/T/KOH/Bh/PAS) method all sialic acids stain blue while "neutral sugars" stain magenta. In the third method, the selective periodate oxidation-thionin Schiff-borohydride reduction-periodic acid-Schiff-saponification (PA*/T/Bh/PAS/KOH) method, sialic acids without side chain substituents or which have an O-acyl substituent at C7 stain blue while "neutral sugars" stain magenta. In the fourth method, the saponification-selective periodate oxidation-borohydride reduction-alcian blue pH 1.0-periodic acid-Schiff (KOH/PA*/Bh/AB1.0/PAS) technique, O-sulphate esters stain aquamarine blue while "neutral sugars" stain magenta. In all of these techniques, mixtures of the components stain in various shades of purple. In the fifth and final method, the saponification-selective periodate oxidation-borohydride reduction-periodic acid-Schiff (KOH/PA*/Bh/PAS) technique, selective identification of "neutral sugars" in macromolecules which also contain sialic acids can be achieved.

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LIST OF ABBREVIATIONS

Ac	=	Acetyl group (CH_3 - C = O)
AB1.0	=	Alcian blue 8GX at pH = 1.0
AB2.5	=	Alcian blue 8GX at pH = 2.5
Bh	=	Borohydride reduction
Co	=	Sialic acids without side chain substituents
Cx	=	Sialic acids with side chain substituents. The numbers 7, 8 and 9 (in place of x) refer to which carbon of sialic acid contains the acetyl group.
DNPH-TDMBF	=	2,4 dinitrophenylhydrazine 3,3'-dimethoxybenzidene fluoroborate procedure. The product of this reaction is a formazan (Fig. 6).
KOH	=	Saponification. A solution of 0.5% potassium hydroxide (w/v) dissolved in 70% ethanol (v/v) is used to remove esters from sugar residues.
N/A	=	Not applicable
"neutral sugars"	=	Refers to hexose, 6-deoxyhexose and N-acetyl hexosamine sugars
%	=	Per cent
PA	=	Oxidation with 40 mM periodic acid at room temperature; the subscripts 2, 3 60 refer to the time period of oxidation in minutes.

List of Abbreviations (cont'd.)

PA*	=	Periodate oxidation in 0.4 mM periodic acid dissolved in approximately 1M HCl for 1 hr. at 40°C (selective periodate oxidation)
PA/Bh/KOH	=	periodate oxidation-borohydride reduction-saponification technique used to confine oxidizable vicinal diols to C7 and C8 substituted sialic acids. In this procedure PA was performed for 2 hours.
PAPS	=	Periodate oxidation-phenylhydrazine-Schiff procedure
S	=	Pararosaniline Schiff reagent
SA	=	Sialic acids
T	=	Thionin Schiff reagent

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One ought every day at least, to hear a little song, read a good poem, see a fine picture and, if it were possible, to speak a few reasonable words.

Goethe

from "Wilhelm Meister's Apprenticeship"

Book V, Chapter 1

Intelligence is quickness to apprehend as distinct from ability which is capacity to act wisely on the thing apprehended.

Whitehead

from "Adventures of Ideas" pg. 135

INTRODUCTION

In order to understand the underlying mechanisms involved in a disease process, a knowledge of the normal molecular events within a cell or bodily system is required. There are two approaches by which such knowledge can be obtained - either chemical, or histochemical, with each approach having both advantages and disadvantages. Chemical procedures have the advantage in that they allow for the identification of small molecules and permit the determination of the structure of macromolecules. In addition, chemical methods tend to be both specific and quantitative, and the mechanism of the reaction used is frequently understood. They do not, however, allow for the localization of molecules, and large quantities of samples are often required before precise identification and quantitation can be obtained. Further, for most procedures, only a few samples can be examined concurrently. In contrast, histochemical methods have the disadvantage in that small molecules are difficult to identify as they tend to be removed during fixation and processing, and structural, quantitative, and mechanistic studies are difficult to carry out. Histochemistry has the advantage, however, in that chemical events can be localized at the histological level, a characteristic unique to histochemistry. The sensitivity of histochemical methods is such that a direct visual correlation can be obtained between structure and function such that individual cellular metabolic activities can be readily recognized. In addition, histochemical methods require a minimal amount of tissue, many sections can be studied simultaneously, and the functional heterogeneity at individual cell levels can be visualized. For example, small foci of change can be seen whereas such a change cannot be detected by chemical procedures (Reid et al., 1985a). Further, it is possible to perform retrospective studies

as the tissues are preserved for long periods of time. The sensitivity of histochemical reactions can be illustrated with the following example: if one considers the average rat colon to be approximately 15 cms long, there are 30,000 5 um sections per colon. When epithelial cells are isolated from rat colon, approximately 4 mg of purified glycoprotein is obtained. Such glycoprotein contains approximately 15% by weight sialic acid, i.e. 0.6 mg sialic acid (Reid et al. 1975, 1977). Therefore, there are

$$\frac{0.6 \times 1000 \times 1000}{30{,}000} = 20 \text{ nanograms SA/section}$$

If it is assumed that there are 100 epithelial cells per transverse section, then 0.2 nanograms/cell of SA can be detected histochemically. Currently, 0.2 ug/tube of SA can be detected chemically. This represents a difference in sensitivity of 1000 times.

Histochemical techniques have been applied to the study of many diseased systems, in the diagnosis of certain leukemias and lymphomas, and for the determination of the site of origin of a variety of malignancies (Filipe and Lake, 1983). Histochemistry can increase the accuracy of histological diagnosis (Ehsanullah et al. 1982a,b), has been used in the assessment of malignant transformation and plays a role in monitoring a patient's response to therapy (Filipe and Lake, 1983). In addition, histochemical methods have shown that there are changes in the epithelial glycoproteins associated with various colonic diseases such as colonic cancer, ulcerative colitis, and Crohn's disease (Culling et al., 1975, 1977, 1979, 1981; Fakan and Adamocova, 1981; Fenger and Filipe, 1981; Filipe 1979, 1984; Filipe et al., 1980; Franzin et al. 1981, 1983a,b, 1984; Lev et al., 1985; Listinski and Riddell, 1981; Montero

and Segura, 1980; Reid et al., 1980, 1984c,d, 1985a,b; Rhatigan and Saffros, 1979; Spicer, 1965; Sunter et al., 1985).

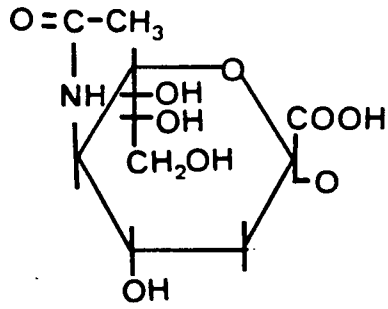
Most histochemical studies of the changes in epithelial glycoproteins associated with colonic disease have been based on methods for the detection of sialic acids, O-acyl sialic acids and O-sulphate ester (Fig. 1). Studies of the "neutral sugars" of these glycoproteins (hexose, 6-deoxyhexose and N-acetyhexosamine), however, have been confined to the use of appropriately labelled lectins (Iannoni et al., 1986; Boland et al., 1982a,b, 1984; Bresalier et al., 1984; Cooper 1980, 1982, 1983; Yonesawa et al., 1982, 1983; Schulte and Spicer, 1983). Although lectin methods are specific, they most commonly detect non- reducing terminal sugar residues and have not been used to determine the relative proportions of anionic groups (sialic acids and sulphate) and "neutral sugars". There existed, therefore, a need for general histochemical methods for the detection of "neutral sugars" and the relative proportions of such sugars and either sialic acids and O-acyl sialic acids or O-sulphate ester. The development of such methods is the subject of this thesis.

There are at least two theoretical approaches for the detection of "neutral sugars". The first involves the use of the periodic acid-phenylhydrazine-Schiff (PAPS) procedure (Spicer 1961) (Fig. 2). In this technique, aldehydes produced by the initial periodate oxidation are condensed with phenylhydrazine. Subsequent treatment with Schiff reagent reverses the blockage of sialic acid monoaldehydes, but not that of "neutral sugar" dialdehydes, resulting in selective staining of sialic acids (Reid et al., 1984a,b). If, therefore, a coloured arylhydrazine can be used in the phenylhydrazine Schiff sequence, or if the hydrazine can be coloured in a subsequent reaction then "neutral sugars" and sialic acids can be visualized simultaneously. Such a procedure has been

Fig. 1. Structure of sugar residues frequently encountered in colonic glycoproteins

Shown is the structure of a) sialic acid without side chain substituents; sialic acid with side chain O-acyl substituents at (b) position C7, (c) position C8, and (d) position C9; (e) O-sulphate ester; (f) hexose; (g) 6-deoxyhexose; and (h) N-acetyl hexosamine.

a.



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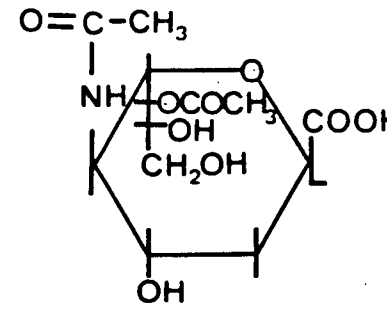

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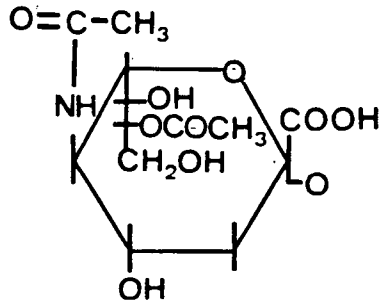

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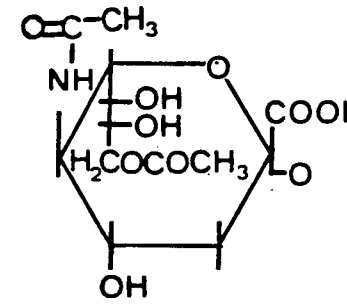

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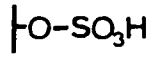

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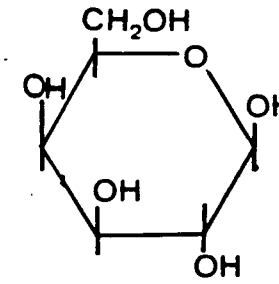

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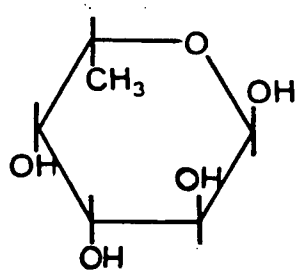


h.

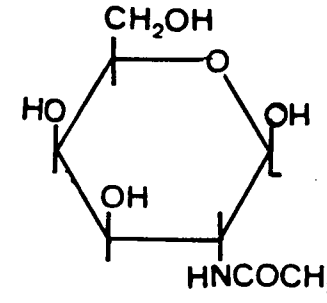
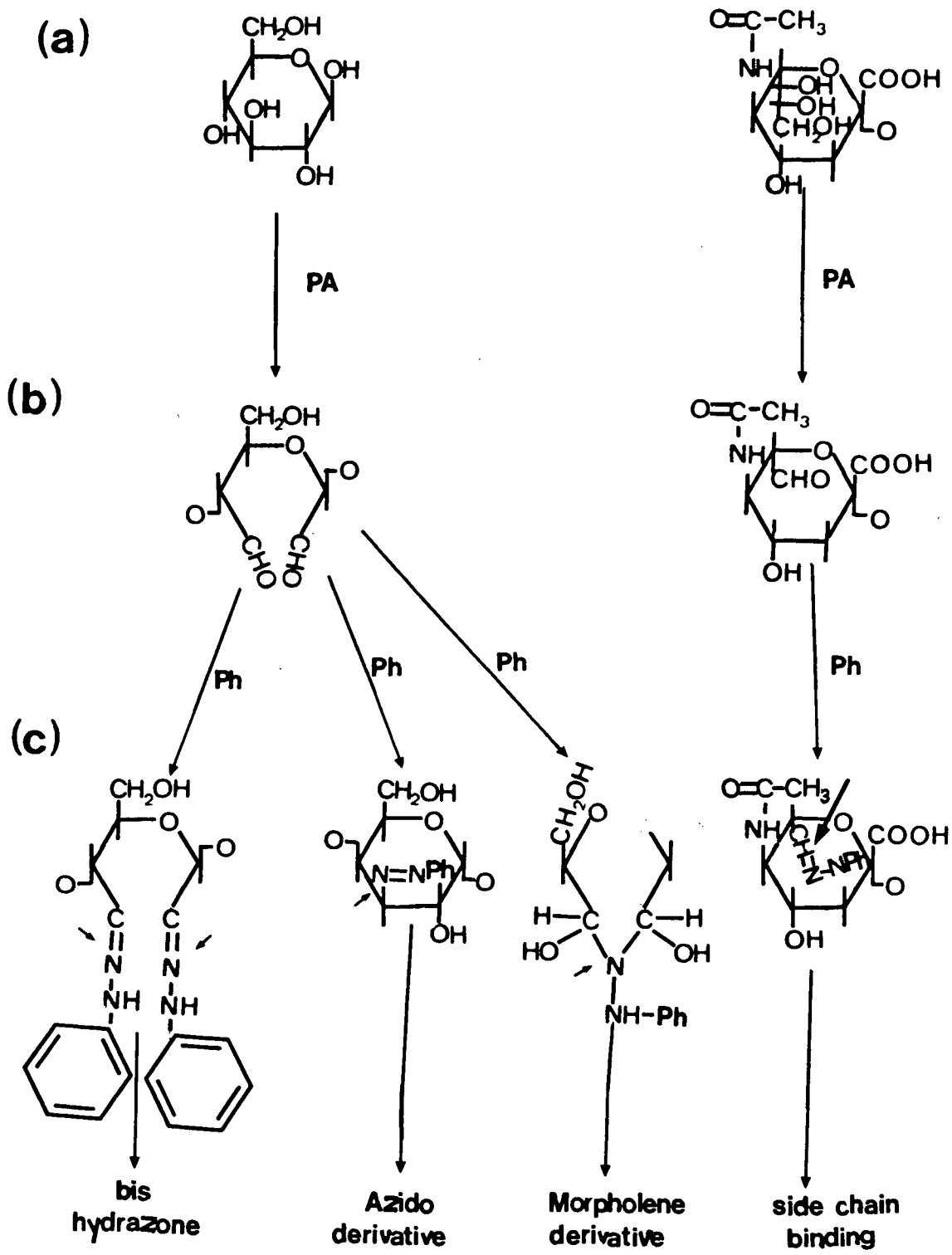


Fig. 2. Diagramatic representation of the mechanism(s) by which the periodic acid-phenylhydrazine-Schiff (PAPS)procedure results in selective detection of sialic acids and the use of PAPS procedures in the simultaneous visualization of sialic acids and "neutral sugars"

Periodate oxidation results in the oxidation of vicinal diols to aldehydes (a-b) which are then condensed with phenylhydrazine (b-c). Subsequent treatment with Schiff reagent reverses the blockage of sialic acid monoaldehydes (large arrow), but not that of "neutral sugar" dialdehydes (small arrow), resulting in selective Schiff staining of sialic acids. If a coloured arylhydrazine is substituted for phenylhydrazine or if the hydrazine can be coloured in a subsequent reaction, then the simultaneous visualization of "neutral sugars" and sialic acids can be obtained.


(a)
PA
PA
(b)
Ph
Ph
Ph
Ph
(c)
bis hydrazone
Azido derivative
Morpholene derivative
side chain binding

developed by Park et al. (1987b) using the coloured hydrazine 2,4-dinitrophenylhydrazine.

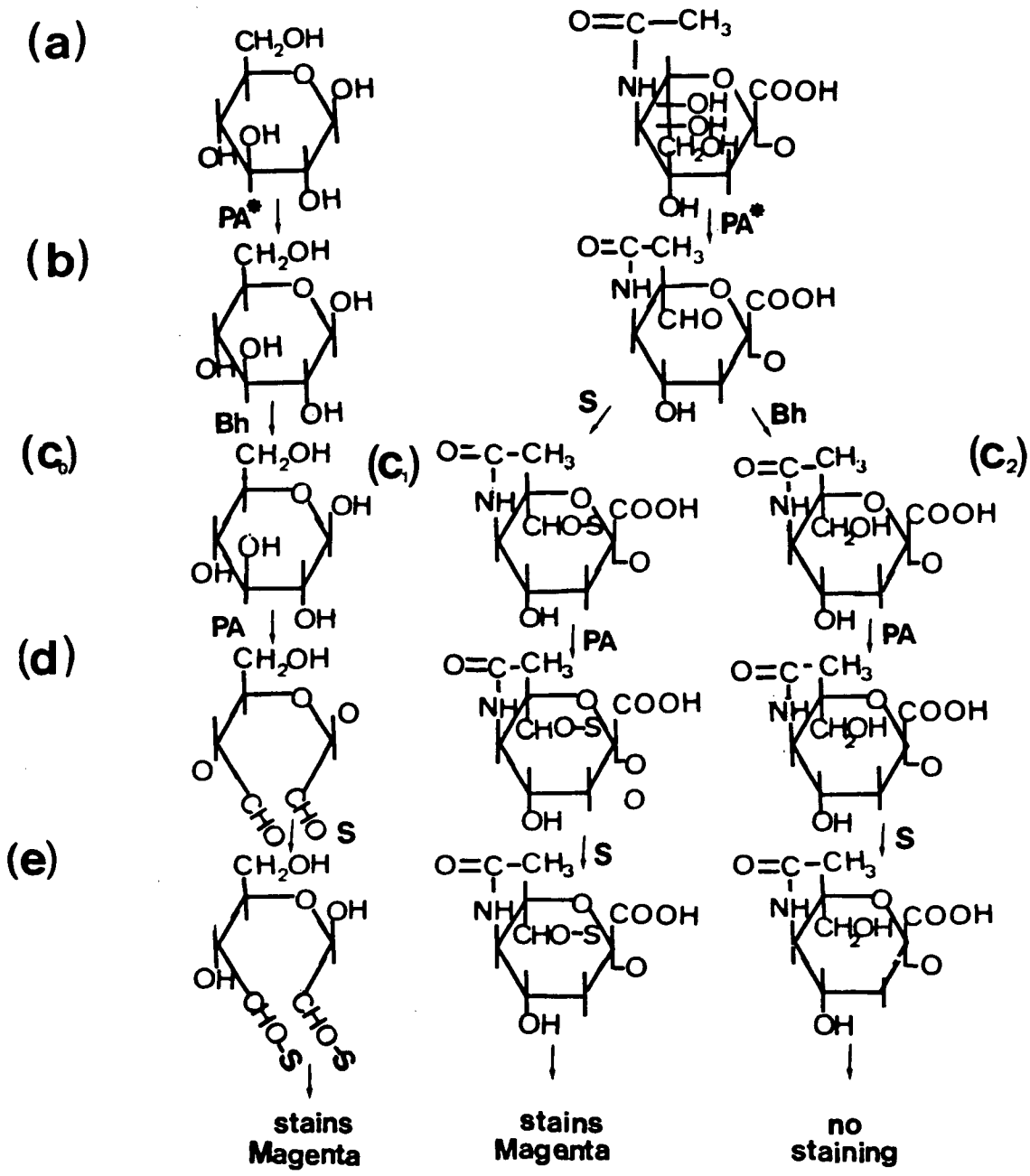
The second approach involves the use of "selective oxidation" in which two step-wise periodate oxidations are employed (Fig. 3). The first oxidation must be carried out under reaction conditions in which all sialic acids oxidize without significant oxidation of other carbohydrate residues. The sialic acids are then either stained with Schiff or converted to Schiff unreactive alcohols with sodium borohydride. The second oxidation is then performed under conditions in which "neutral sugars" oxidize (Volz et al., 1987a,b).

Previous attempts at developing "selective oxidation" conditions were based on the use of dilute periodic acid (Klessen, 1978; Roberts, 1977; Veh et al., 1979; Weber et al., 1975). Due to steric factors, the rate of oxidation of vicinal diols varies, with open chain diols (such as sialic acids) oxidizing at a faster rate than ring cis diols which in turn oxidize at a faster rate than ring trans diols (Culling and Reid, 1977; Suttajit, 1970; McLean et al., 1971). From this it was concluded that if limited quantities of periodate were used, only sialic acid diols would be oxidized. The periodic acid-Schiff (PAS) reaction could then be used to selectively detect sialic acids. It is difficult, however, to estimate the amount of sialic acid in a given tissue section and therefore to determine the amount of periodate required for the oxidation. Further, the above studies did not establish whether or not all sialic acids present in the tissue had oxidized under the conditions used, and no studies were made to determine what effect the pH or ionic strength of the periodate solution would have on the oxidation rate of sialic acids and/or "neutral sugars".

In preliminary studies, Volz et al. (1986) demonstrated that maximal PAS staining of sialic acid residues with minimal staining of other carbohydrate

Fig. 3. A proposed selective oxidation mechanism for the simultaneous visualization of "neutral sugars" and sialic acid residues

The first oxidation is carried out under conditions in which all sialic acids oxidize without significant oxidation of other carbohydrate residues (PA*) (a-b). The sialic acids are then either stained by a process yielding covalent bonds (b-c_1) or converted to Schiff unreactive alcohols by reduction with sodium borohydride (b-c_0 or b-c_2). The second oxidation is then performed under conditions which oxidize "neutral sugars" (c-d). The resultant aldehydes are then stained with Schiff reagent (d-e).


(a)
(b)
(c0)
(c1)
(c2)
(d)
(e)
PA*
Bh
PA
S
stains Magenta
stains Magenta
no staining

residues could best be obtained by oxidation with either aqueous solutions of 40 mM periodic acid for 2 minutes at 4°C or with 4.0 mM periodic acid for 2 minutes at room temperature (Illustration IA). Specific PAS staining of sialic acid residues could be obtained with other concentrations of periodic acid, but maximal staining of sialic acids could not be obtained without significant staining of "neutral" carbohydrate residues. In addition, oxidation of sialic acid residues with very dilute solutions of periodic acid (0.04 mM and 0.004 mM) was extremely slow, requiring 48-72 hours to reach completion. Under such conditions neutral carbohydrate residues, such as those present in liver glycogen and the neutral glycoproteins of human stomach, oxidized significantly. However, when oxidation was performed with very dilute solutions of periodic acid in either 0.125N sulphuric acid (Illustration II) or 1M sodium chloride, the rate of oxidation of sialic acid residues was increased significantly. Parallel studies demonstrated that the oxidation of liver glycogen and stomach mucin was unaffected when the reaction was carried out with 0.04 mM periodic acid in 1M NaCl but was apparently decreased when 0.125 N sulphuric acid was used as the solvent (Illustration III).

Scott and Harbinson (1968, 1969; and Scott and Dorling, 1969) showed that the slow oxidation rate of the 2-3 vicinal diols of the uronic acid residues of glycosaminoglycans was due to the electrostatic field surrounding these molecules which repels the periodate ion, thereby inhibiting or retarding oxidation, an effect that can be overcome by increasing the ionic strength of the oxidant solution (Fig. 4). If a similar repulsion effect occurs when sialic acids are oxidized under conditions of low periodic acid concentration, then the reduction in the sialic acid oxidation rate may be such that the oxidation of other carbohydrate residues becomes significant. It becomes,

therefore, impossible to use very dilute periodic acid for the selective oxidation of sialic acid residues. However, in concentrated periodate, the dual effect of lowering the pH and increasing the ionic strength of the solution would be expected to suppress the ionization of the sialic acid carboxyl groups leading to a faster and more selective oxidation of such residues. It appeared possible, therefore, that manipulation of the oxidation conditions could result in a complete oxidation of sialic acid residues without a significant oxidation of other carbohydrate residues.

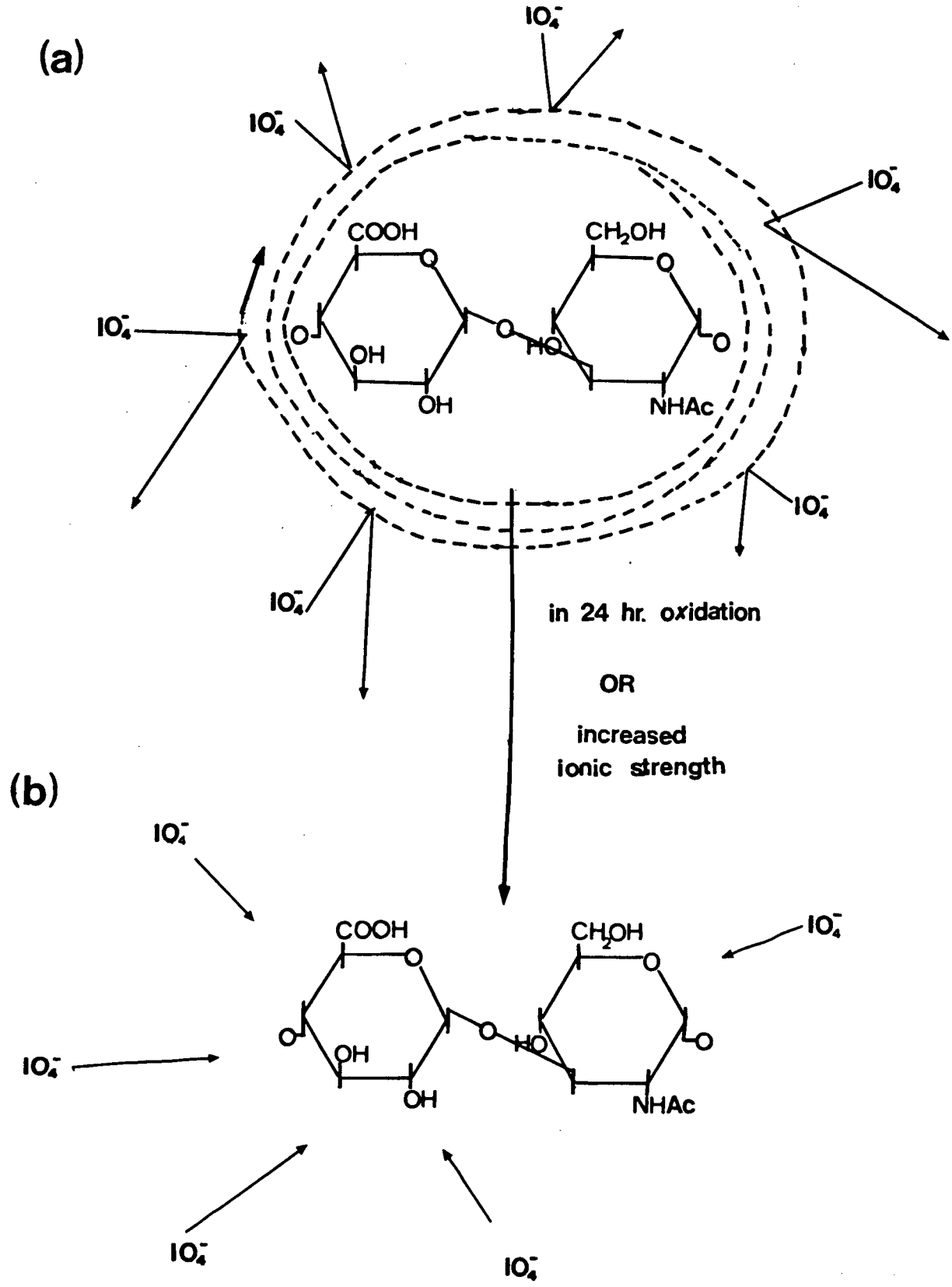
The objectives of this thesis were, therefore, to investigate the following questions:

1. Is the increased oxidation rate in 0.125N sulphuric acid due to a lowering of the pH or to an increase in the ionic strength?
2. Would the use of 1M sodium chloride or a lower pH improve the selectivity of the periodate oxidation of sialic acids, regardless of the periodic acid concentration used?
3. Does the presence of sulphate esters in glycoproteins affect the rate of sialic acid oxidation (possibly by altering the electrostatic field in the microenvironment of the sialic acid molecule)?
4. Assuming that it was possible to selectively oxidize all sialic acids, then could such conditions be used to develop general methods for the detection of "neutral sugars" and the simultaneous visualization of these sugars and either sialic acid and its side chain O-acyl variants+ or sulphate esters.

+ In the following account, sialic acids without side chain O-acyl substituents are referred to as C0 and sialic acids with O-acyl substituents at positions 7, 8 and 9 are designated C7, C8 and C9 respectively. For histochemical purposes the C8 class of sialic acids includes those with two- (C7C8, C7C9, C8C9) and three- (C7C8C9) side chain O-acyl substituents.

Fig 4. Effect of the electrostatic field surrounding molecules on periodate oxidation

Scott and Harbinson (1968,1969; Scott and Dorling, 1969) showed that the slow oxidation rate of the 2-3 vicinal diols of uronic acid residues of glycosaminoglycans was due to the electrostatic field surrounding these molecules which repels the periodate ion, thereby inhibiting or retarding oxidation (a). This effect can be overcome by increasing the ionic strength of the oxidant solution (b).


(a)
IO_4^-
COOH
CH_2OH
O
OH
HO
NHAc
in 24 hr. oxidation
OR
increased
ionic strength
(b)

MATERIALS AND METHODS

I. Materials:

A. Tissues

Specimens of Spraque-Dawley rat liver, salivary gland complex (including the sublingual, submandibular, and parotid glands), and terminal ileum and colon obtained at autopsy immediately following death by ether anesthesia, and surgical specimens of human colon were fixed in 10% formalin calcium for at least 7 days. After fixation, specimens of rat colon and terminal ileum were prepared as a single "Swiss Roll" (Park et al., 1987a). The tissues were then processed through ethanol and xylene and embedded in paraplast. Sections of 5 um thickness were cut with an E. Leitz Wetzlar Type 1212 model microtome, mounted on clean slides using chrome-alum gelatin as adhesive (Kiernan, 1981), and then treated overnight at 60°C in an atmosphere of formalin vapour.

B. Chemicals

The product colour index number and supplier of staining dyes used in this study are as listed below:

Dye	Color Index Number	Supplier
Alcian Blue 8GX	74240	Gurr Chemicals
Pararosaniline hydrochloride	42500	Fisher Scientific Company
Thionin	52000	Fisher Scientific Company

II. Histochemical Procedures

1) Sections were brought to water by treating successively with the following solutions for the times specified.

xylene	5 min.
xylene	5 min.
100% alcohol	3 min.
100% alcohol	3 min.
95% alcohol	3 min.
70% alcohol	3 min.
water	rinse

2) Pararosaniline (S) and thionin (T) Schiff reagents were prepared by the method of Barger and DeLamater (1948).

3) Sodium borohydride (Bh) reduction was performed with the procedure of Lillie and Pizzolato (1972).

4) Saponification (KOH) was carried out using 0.5% (w/v) potassium hydroxide dissolved in 70% (v/v) ethanol for 15 minutes at room temperature (Culling et al., 1974).

5) Periodate oxidation

i) In investigations of selective conditions for the oxidation of sialic acids, periodate oxidation was performed for different time periods (some or all of 2,5,10,20 and 60 minutes and 1 and 2 hours) at either 4°C or room temperature using different concentrations of periodic

acid (0.004mM, 0.04mM, 0.4mM, 4.0mM and 40.0mM) in each of the following solvents: distilled water, 1M sodium chloride or 1M hydrochloric acid.

ii) In studies of the mechanism of selective oxidation of sialic acid, and in the development of methods for the simultaneous visualization of "neutral sugars" and either sialic acid and its side chain O-acyl variants or O-sulphate ester, periodate oxidation was performed with either 1% (w/v) aqueous periodic acid at room temperature, or with 0.4mM periodic acid in approximately 1M hydrochloric acid at 4°C. The latter was prepared by mixing equal volumes of stock solution of 0.8mM aqueous periodic acid and 2M hydrochloric acid (HCl). The 2M HCl was prepared by diluting concentrated HCl (1 volume) with distilled water (4 volumes). These stock solutions were stored at 4°C.

6) Selective periodate oxidation (PA*) was performed as follows.

i) Bring sections to water.

ii) Cool sections to 4°C and treat with a pre-cooled solution of 0.4mM periodic acid dissolved in approximately 1M hydrochloric acid.

iii) Wash sections in running water for 10 minutes at 4°C.

7) Periodic Acid Schiff reaction (PAS). Sections were oxidized in 1% (w/v) aqueous periodic acid for one hour at room temperature, washed in running water for 10 minutes, and stained for 60 minutes with pararosaniline Schiff reagent. The staining intensity was visually assessed on the following

scale: 0 = no staining, tr = trace, 1 = weak, 2 = moderate, 3 = strong and 4 = maximum.

8) Periodic Acid-Borohydride Reduction-Saponification (PA/Bh/KOH) method (Reid et al., 1973; Culling et al., 1974). In this procedure, treatment with 1% (w/v) periodic acid for 2 hours at room temperature oxidizes vicinal diols to aldehydes which are then reduced with sodium borohydride (Bh) to primary alcohols. Following saponification, only sialic acids with O-acyl substituents at C7, C8 or C9 (or which have 2 or 3 side chain O-acyl substituents) are PAS-positive.

9) Saponification-Periodic acid oxidation-Borohydride reduction sequence (KOH/PA_3/Bh). In this procedure, acyl groups are removed by saponification. Oxidation with 1% (w/v) periodic acid for 3 minutes at room temperature (PA_3) then converts all sialic acids to the corresponding C7 aldehydes (Volz et al., 1986) which are then reduced to primary alcohols with sodium borohydride. Positive PAS staining in KOH/PA_3/Bh treated sections is therefore confined to vicinal diols on sugar residues other than sialic acid (Volz et al., 1986).

10) Alcian blue staining - was performed with alcian blue at either pH 1.0 or pH 2.5 as described by Culling (1974).

11) 2,4-Dinitrophenylhydrazine - Tetrazotized 3,3'-dimethoxybenzidene fluoroborate procedure (DNPH-TDMBF). In this procedure periodate derived aldehydes are blocked by treatment with a saturated solution of 2,4-

dinitrophenylhydrazine (DNPH) in 1M HCl at 4°C for 2 hours (Pearse 1968). Formazans are then generated by treatment of the sections with a freshly prepared solution of tetrazotized 3,3'-dimethoxybenzidene fluoroborate (TDMBF) in 25% (v/v) aqueous pyridine for 3 minutes at room temperature (Stoward 1967a,b).

III. Methods based on selective oxidation of sialic acid

These methods are outlined in Table I (Page 20).

A. Method 1. Simultaneous visualization of "neutral sugars" and sialic acids with O-acyl substituents at positions C7, C8, and C9, the selective periodate oxidation-borohyride reduction-saponification-selective periodate oxidation-thionin Schiff-saponification-borohydride reduction-periodic acid-Schiff (PA*/Bh/KOH/PA*/T/KOH/Bh/PAS) procedure

1) Bring sections to water.
2) Cool sections to 4°C and oxidize in a precooled solution of 0.4mM periodic acid in approximately 1M HCl for 1 hour at 4°C.
3) Wash in running water for 10 minutes at 4°C.
4) Reduce with 0.1% (w/v) sodium borohydride in 1% (w/v) dibasic sodium phosphate (anhydrous) for 20 minutes at room temperature.
5) Wash in running water for 10 minutes at room temperature.
6) Rinse in 70% (v/v) ethanol.

Table I

Outline of methods for the simultaneous visualization of "neutral sugars" and either sialic acid and its side chain O-acyl variants or O-sulphate ester

x = step performed; - = step omitted

a) Washing steps have not been included in Table.

Selective periodate oxidation (PA*) = 0.4 mM periodic acid in approximately 1M hydrochloric acid for one hour at 4°C

Borohydride reduction (Bh) = 0.1% sodium borohydride in 1% (anhydrous) dibasic sodium phosphate for 20 minutes at room temperature

Saponification (KOH) = 0.5% potassium hydroxide in 70% ethanol 15 min at room temperature

Thionin Schiff (T) = Thionin Schiff for 2 hr at room temperature

Alcian blue pH 1.0 (AB 1.0) = 0.3% Alcian blue 8GX in 0.1 M hydrochloric acid for 30 min. at room temperature (Culling 1974)

Periodic Acid - Schiff (PAS) = oxidation with 1% periodic acid for 1 hr at room temperature followed by pararosaniline Schiff for 1 hr at room temperature

		Method[a]				
		1	2	3	4	5
Selective Periodate Oxidation	(PA*)	X	-	-	-	-
Borohydride Reduction	(Bh)	X	-	-	-	-
Saponification	(KOH)	X	X	-	X	X
Selective Periodate Oxidation	(PA*)	X	X	X	X	X
Thionin Schiff	(T)	X	X	X	-	-
Saponification	(KOH)	X	X	-	-	-
Borohydride Reduction	(Bh)	X	X	X	X	X
Alcian Blue pH 1.0	(AB)	-	-	-	X	-
Periodic Acid Schiff	(PAS)	X	X	X	X	X
Saponification	(KOH)	-	-	X	-	-

7) Saponify with 0.5% (w/v) potassium hydroxide in 70% (v/v) ethanol for 15 minutes at room temperature.

8) Wash in running water for 10 minutes at room temperature.

9) Cool to 4°C and again oxidize in pre-cooled 0.4mM periodic acid dissolved in approximately 1M hydrochloric acid for 1 hour at 4°C.

10) Wash in running water for 10 minutes at 4°C.

11) Stain with freshly prepared thionin Schiff reagent for 2 hours at room temperature.

12) Wash in running water for 10 minutes at room temperature.

13) Rinse in 70% (v/v) ethanol.

14) Saponify with 0.5% (w/v) potassium hydroxide in 70% (v/v) ethanol for 15 minutes at room temperature.

15) Wash in running water for 10 minutes.

16) Reduce with 0.1% (w/v) sodium borohydride in 1% (w/v) dibasic sodium phosphate (anhydrous) for 20 minutes at room temperature.

17) Wash in running water for 10 minutes at room temperature.

18) Oxidize in 1% (w/v) aqueous periodic acid for 1 hour at room temperature.

19) Wash in running water for 10 minutes.

20) Stain with freshly prepared pararosaniline Schiff reagent for 1 hour at room temperature.

21) Wash in running water for 10 minutes.

22) Dehydrate (through series of 95%, 100% alcohol), clear in xylene and mount with permount.

Staining Results

In this procedure, sialic acids with O-acyl substituents at positions C7, C8, or C9 (and sialic acids containing two or three side chain O-acyl substituents) stain blue, while "neutral sugars" with oxidizable vicinal diols stain magenta. Mixtures of these components stain in various shades of purple.

B. Method 2: Simultaneous visualization of "neutral sugars" and total sialic acid, the saponification-selective periodate oxidation-thionin Schiff-saponification-borohydride reduction-periodic acid-Schiff (KOH/PA*/T/KOH/Bh/PAS) procedure

1) Bring sections to water.

2) Perform steps 6-22 of Method 1.

Staining Results

In this procedure all sialic acids stain blue, "neutral sugars" (with oxidizable vicinal diols) stain magenta, and mixtures stain in various shades of purple.

C. Method 3: Simultaneous visualization of "neutral sugars" and sialic acids without side chain substituents or which have O-acyl substituents at C7, the selective periodate oxidation-thionin Schiff-borohydride reduction-periodic acid-Schiff-saponification (PA*/T/Bh/PAS/KOH) procedure.

1) Bring sections to water.

2) Perform steps 9-12, 16-21, 13-15, and 22 of Method 1 (in this order).

Staining results

In this procedure sialic acids without O-acyl side chain substituents or with a substituent located at position C7 stain blue, "neutral sugars" (with oxidizable vicinal diols) stain magenta, and mixtures stain in various shades of purple.

D. Method 4: Simultaneous visualization of "neutral sugars" and O-sulphate esters, the saponification-selective periodate oxidation-borohydride reduction-Alcian blue pH 1.0-periodic acid-Schiff (KOH/PA*/Bh/AB1.0/PAS) procedure.

1) Bring sections to water.
2) Perform steps 6-10 and 16-17 of Method 1.
3) Stain with 0.3% (w/v) Alcian blue 8GX in 0.1M HCl pH 1.0 for 30 minutes at room temperature.
4) Rinse briefly with 0.1M HCl pH 1.0.
5) Wash in running water for 10 minutes.
6) Perform steps 18-22 of Method 1.

Staining results

"Neutral sugars" (with oxidizable vicinal diols) stain magenta; O-sulphate esters stain aqua and mixtures stain in various shades of purple.

E. Method 5: Selective staining of "neutral sugars" the saponification-selective periodate oxidation-borohydride reduction-periodic acid-Schiff (KOH/PA*/Bh/PAS) procedure.

1) Bring section to water.

2) Perform steps 6-10 and 16-22 of Method 1.

Staining results

"Neutral sugars" stain magenta.

F. Control Methods

When performing Methods 1-5, controls are required to ensure proper functioning of the method. These are as follows:

1) Sections of rat liver and parotid gland treated with either the PA*/S or the PA*/Bh/PA*/S sequences should be unstained as they contain no sialic acid, and "neutral sugars" should not oxidize under conditions of PA*. Sections of rat liver and parotid glands should stain only magenta with Methods 1-5.

2) When treated with the PA/Bh/KOH/PA*/S sequence, sections of human colon containing 8-O-acyl sialic acids should stain intensely while similar tissues exposed to the sequence PA/Bh/KOH/PA*/Bh/PA*/S should be unstained, an indication that all sialic acid is oxidized under conditions of PA*.

RESULTS AND DISCUSSION

I. Establishment of conditions for the selective oxidation of sialic acid

The first objective of this study was to develop a method for the selective detection of sialic acids in tissues based upon the previous studies of Volz et al. (1986). To accomplish this, conditions were sought in which sialic acids oxidized maximally but other carbohydrate residues did not oxidize significantly. Two parameters were investigated simultaneously.

a) To determine whether the increased oxidation rate in 0.125N sulphuric acid (Volz et al., 1986) was due to a lowering of the pH or was due to an increase in ionic strength, oxidations were performed in 1M HCl and 1M NaCl as solvents. These provided solutions similar in ionic strength, but differing in pH.

b) To determine whether the oxidation rate of sialic acid is affected by the presence or absence of sulphate esters, comparative studies were carried out with the tissues shown in Table II (Page 27). These tissues were selected because they provided examples of neutral polysaccharides alone, sialoglycoproteins, and sialosulphoglycoproteins, and their histochemical staining patterns had been established.

Untreated sections of rat liver, and PA/Bh/KOH pretreated sections of rat salivary gland complex and rat colon were oxidized at 4°C with 40mM, 4.0mM, 0.4mM, 0.04mM and 0.004mM periodic acid in one of water, 1M sodium chloride, or 1M hydrochloric acid for each of 2, 5 and 60 minutes and 24 and 48 hours, stained with Schiff reagent, and the intensity of staining assessed. The results of this experiment confirmed that 40mM periodate in water for 2 minutes at 4°C and 4.0mM periodate in water for 2 minutes at room temperature were selective for the oxidation of sialic acids. Further, selective oxidation

Table II

Location and histochemical characteristics of the carbohydrate containing macromolecules studied during the investigation of methods for the selective oxidation of sialic acids

Tissue	Macromolecule examined	Histochemical characteristics
Rat Liver	Glycogen	Neutral PAS reactive macromolecule
Rat Salivary Glands		
Parotid	Acinar cell granules	"Neutral" PAS reactive macromolecule (Munhoz, 1971: Schackleford and Klapper, 1962; Spicer and Duvenci, 1964; Simson et al., 1973; Park et al., published)
Sublingual	Acinar cell glycoprotein	Sialoglycoprotein containing side chain substituted sialic acids (Park et al., unpublished)
Rat Colon	Epithelial glycoproteins	
Proximal(lower half of crypts)		Sialoglycoproteins containing side chain O-acylated sialic acids but little or no sulphate ester (Park et al., unpublished)
Distal		Sialosulphoglycoproteins containing side chain O-acylated sialic acids (Park et al., unpublished)
Human Colon	Epithelial glycoproteins	Sialosulphoglycoproteins containing side chain O-acylated sialic acids (Filipe, 1979; Culling et al., 1981)

was also obtained with 0.4m<u>M</u> periodate in 1<u>M</u> HCl for 1 hour at 4°C (Illustration Ib). This latter set of conditions appeared to be the most selective since the staining of rat liver and rat parotid gland (containing neutral carbohydrate molecules) was reduced under these conditions when compared to that obtained when either water or 1<u>M</u> NaCl were used as the solvent at the same periodate concentration (Table III and Illustration III). The different results obtained with 1<u>M</u> NaCl and 1<u>M</u> HCl, therefore, can be attributed to a lowering of pH rather than to an increase in ionic strength.

Finally, the rate of oxidation of sialic acid residues was not affected by changes in the pH of the solvent used whether the tissue contained sulphate (rat colon) or was unsulphated (rat sublingual gland). The rate of oxidation of sialic acid is, therefore, independent of the presence or absence of O-sulphate ester in the glycoprotein.

In conclusion, it appears that the oxidation rate of sialic acid in tissues is dependent on the pH of the oxidation solution, the concentration of periodic acid used, and is independent of the presence or absence of sulphate in the glycoprotein.

Table III

The effect of pH on the oxidation of neutral macromolecules. The oxidation of neutral macromolecules with 0.4 mM periodic acid (as demonstrated by the intensity of Schiff staining) is greater when water (aq) or 1M NaCl is used as solvent in place of 1M HCl. The staining intensity was graded on the following scale:

0 = no staining, tr = trace, 1+ = weak, 2+ = moderate, 3+ = strong, 4+ = maximum.

TIME (hours)	Rat Liver		Rat Parotid Gland	
	1MNaCl	1M HCl	1MNaCl	1M HCl
1	2+	tr	1+	tr
24	3+	1+	2+	tr
48	4+	1+	2+	tr

II. Investigation of oxidation with 0.4mM Periodic acid in approximately 1M HCl

A. Specificity

In the previous study it was shown that treatment with 0.4mM periodic acid in 1M HCl at 4°C for one hour (PA*) resulted in the selective oxidation of all sialic acids. To further investigate the selectivity of the PA* technique, a series of control experiments was performed to demonstrate that under conditions of PA*, all sialic acids were oxidized, oxidation of "neutral sugars" was insignificant, and glycoproteins were not extracted or de-O-acylated. These experiments are summarized in Table IV (Page 33).

To study the oxidation of sialic acid residues with the PA* technique, sections of rat ileum, colon, salivary glands, and human colon were treated with the PA/Bh/KOH sequence to confine vicinal diols to sialic acid residues. The sections were then treated with each of 1% periodic acid for 1 hour at room temperature (PA/Bh/KOH/PA_{60}/S), PA* (PA/Bh/KOH/PA*/S) or with the PA* procedure followed by oxidation with 1% periodic acid for 1 hour at room temperature (PA/Bh/KOH/PA*/PA_{60}/S). No difference in the staining intensity of these three treatments could be detected (Illustration IV), suggesting that PA* oxidized all sialic acid residues. In a second set of experiments, sections of human colon treated with the PA/Bh/KOH/PA*/S sequence yielded a strong (4+) Schiff reaction. However, when serial sections were reduced with borohydride after the PA* step and then further oxidized with PA* (PA/Bh/KOH/PA*/Bh/PA*/S) or with 1% periodic acid for 1 hour at room temperature (PA/Bh/KOH/PA*/Bh/PA_{60}/S), no Schiff staining was obtained. This indicated that when only sialic acids were present, they are completely oxidized under PA* conditions (Illustration V).

Table IV

Evidence for the selectivity of periodate oxidation with 0.4 mM periodic acid in 1M hydrochloric acid at 4°C for one hour

The nature of the macromolecules studied in these tissue sites is summarized in Table II (Page 27).

PA_3 and PA_{60} = oxidation with 1% (w/v) periodic acid at room temperature for 3 and 60 minutes, respectively

PA* = oxidation with 0.4 mM periodic acid in approximately 1M hydrochloric acid at 4°C for one hour

KOH = saponification

Bh = borohydride reduction (Lillie and Pizzolato 1972)

S = treatment with pararosaniline Schiff

AB 2.5 and AB 1.0 = staining with alcian blue 8GX at pH 2.5 and pH 1.0, respectively (Culling 1974)

0 = no staining

4+ = strong staining

Tissue	Procedure and Results	Interpretation
Rat colon and sub-lingual gland and human colon	PA/Bh/KOH/PA_{60}/S = 4+ PA/Bh/KOH/PA*/S = 4+ PA/Bh/KOH/PA*/PA_{60}/S = 4+	The PA/Bh/KOH sequence confines vicinal diols to sialic acid residues. Subsequent oxidation with either PA_{60} or PA* gives Schiff staining of the same intensity and following borohydride reduction of PA* engendered aldehydes, PA* produces no further Schiff reactivity. Therefore, PA* oxidized all sialic acids.
Human colon	PA/Bh/KOH/PA*/S = 4+ PA/Bh/KOH/PA*/Bh/PA*/S = 0 PA/Bh/KOH/PA*/Bh/PA_{60}/S = 0	
Rat liver and parotid gland	PA*/S = 0	Oxidation with PA* produces no visible Schiff with neutral macromolecules.
Rat colon	KOH/PA_3/Bh/PA_{60}/S = 4+ KOH/PA_3/Bh/PA*/PA_{60}/S =4+ KOH/PA_3/Bh/PA*/S = 0	Following removal of O-acyl esters with KOH, the PA_3/Bh sequence confines vicinal diols to "neutral sugars" in sialo- and sialosulphoglycoproteins. Absence of Schiff staining following PA* but strong staining after PA_{60} indicates PA* does not produce Schiff reactive sites from "neutral sugars".
Rat colon and sub-lingual gland human colon	KOH/PA*/S = 4+ KOH/PA*/Bh/PA*/S = 0 KOH/PA*/Bh/PA_{60}/S = 4+	PA* oxidizes all sialic acids but does not produce Schiff positive sites with "neutral sugars".
Rat colon and sub-lingual gland	AB 2.5 = 4+ PA/Bh/KOH/AB2.5 = 4+ PA/Bh/KOH/1M HCl, 4°C, 1 h = 4+	PA* or 1 M HCl, 4°C, 1 hour does not extract epithelial glycoproteins.
Rat colon and human colon	AB1.0 = 4+ KOH/PA^+/Bh/AB1.0 = 4+	
Rat colon and sub-lingual gland and human colon	PA/Bh/PA_{60}/S = 0 PA/Bh/1M HCl, 4°C, 1 h/PA_{60}/S=0	O-Acyl esters are stable to 1 M HCl at 4°C for 1 hour.

To study the effect of PA* on "neutral sugars", tissues containing macromolecules with only "neutral sugars", such as rat liver and parotid gland, were subjected to PA* followed by Schiff. Oxidation under these conditions produced no significant staining of either liver glycogen (Illustration VIa) or the PAS positive granules of rat parotid gland. PAS reactivity was produced, however, when oxidation with 0.4mM periodic acid in 1M HCl at 4°C was performed for 24 hours (Illustration VIb) or when the routine PAS procedure was employed (Illustration VIc). These results indicate that PA* does not produce significant Schiff staining of "neutral sugars".

The oxidation of the "neutral sugars" of sialo- and sialosulphoglycoproteins was investigated in sections of rat ileum-colon which were pretreated with the KOH/PA_3/Bh sequence to confine vicinal diols to "neutral sugar" residues (Volz et al., 1986). Subsequent oxidation of these sections with PA* (KOH/PA_3/Bh/PA*) produced no Schiff reactivity (Illustration VIIa). In contrast, oxidation with either 1% periodic acid for 1 hour at room temperature (KOH/PA_3/Bh/PA_{60}), or with PA* followed by 1% periodic acid for 1 hour at room temperature (KOH/PA_3/Bh/PA*/PA_{60}), resulted in similar strong (4+) PAS staining, indicating that PAS-reactive "neutral sugar" residues are present and available for oxidation, but do not oxidize and then stain under conditions of PA*.

To investigate the oxidation of sialic acid in the presence of the "neutral sugars" of sialo- and sulphosialoglycoproteins, sections of rat ileum-colon and salivary gland complex and human colon were first saponified to remove O-acyl esters. Oxidation with PA* (KOH/PA*/S) then resulted in strong (4^+) Schiff reactivity. If, however, saponification and PA* treatment were followed by

reduction with borohydride, further oxidation with PA* (KOH/PA*/Bh/PA*/S) followed by Schiff, produced no Schiff reactivity. When, however, the second oxidation step was carried out using 1% periodic acid for 1 hour at room temperature (KOH/PA*/Bh/PA_{60}), a strong Schiff reaction resulted. This indicated that PA* oxidized all sialic acids regardless of the presence of either "neutral sugars" or O-sulphate ester in the glycoprotein.

Next, a number of experiments were conducted to investigate any potential non-specific effects which may occur under PA* conditions. The conditions of PA* are acidic, therefore the extraction of glycoproteins from the tissues may occur. To eliminate this possibility, PA/Bh/KOH treated sections of rat ileum-colon and salivary gland were incubated in PA* or 1M HCl at 4°C for 1 hour and were then stained with Alcian blue pH 2.5 (a stain which detects sulphate and carboxyl groups). The Alcian blue pH 2.5 staining of these sections did not differ from that of untreated control sections (Alcian blue pH 2.5). Further, the Alcian blue pH 1.0 (stains sulphate groups) staining of rat ileum-colon and human colon was unaffected by the KOH/PA*/Bh sequence (Illustration VIII). These data indicate that PA* does not extract epithelial glycoproteins.

To determine whether PA* de-O-acetylates sialic acids, sections of rat ileum-colon and salivary gland complex and human colon were treated with the PA/Bh sequence to remove all oxidizable vicinal diols. Sections were then incubated with 1M HCl for 1 hour at 4° C (Illustration IX). These sections were not PAS-reactive, indicating that such conditions do not de-O-acetylate sialic acids. Finally, to determine whether conditions of PA* invoke a Feulgen reaction, sections of rat liver, ileum-colon, salivary gland complex and human

colon were incubated with either 1M HCl for 1 hour at 4°C or with PA* (Illustration X). A minimal Schiff staining was obtained. However, when sections were incubated in PA* for 24 or 48 hours, strong Feulgen staining was observed. The Feulgen reaction, however, would not be expected to interfere with sialic acid detection in the PA*/S procedure.

B. Mechanistic Studies of PA* Conditions

In the previous section it was demonstrated that PA* did not form Schiff positive products with "neutral sugars". However, whether "neutral sugars" oxidize in PA* and form Schiff-negative products such as hemialdals or hemiacetals, or whether they either do not oxidize or do not produce a sufficient quantity of aldehydes to produce a visible Schiff reaction, was not determined. To investigate this, sections of rat liver, ileum-colon and salivary gland complex were subjected to the KOH/PA_3/Bh technique to confine vicinal diols to "neutral sugars", and were then oxidized with one of PA* (KOH/PA_3/Bh/PA*) (Fig. 5B), 1% periodic acid for 1 hour at room temperature (KOH/PA/Bh/PA_{60}) or both PA* and PA_{60} (KOH/PA/Bh/PA/PA_{60}) (Fig. 5A). The sections were then treated with either Schiff reagent or with 2,4-dinitrophenylhydrazine (DNPH) followed by tetrazotized 3,3'-dimethoxybenzidine fluoroborate (TDMBF) (Stoward, 1967b) (Fig. 5A and 5B). Sections exposed to the KOH/PA_3/Bh/PA_{60} and KOH/PA_3/Bh/PA*/PA_{60} were strongly Schiff-positive and yielded formazans on treatment with DNPH and TDMBF (Fig. 5A). However, sections exposed to the KOH/PA_3/Bh/PA* sequence were Schiff-unreactive and showed only traces of formazan with the DNPH/TDMBF sequence (Fig. 5B). These data imply that PA* does not produce hemiacetals or hemialdals (Illustration XIa & b).

Fig. 5A and 5B. Flow diagram of the investigation into the mechanism by which "neutral sugars" react following treatment with PA* and Schiff reagent

Sections of rat liver, ileum-colon and salivary glands were subjected to the KOH/PA_3/Bh technique to confine vicinal diols to "neutral sugars", and were oxidized with either 1% periodic acid for 1 hour at room temperature, or both PA* and PA_{60} (Fig. 5A) or PA* (Fig. 5B). Sections were then treated with either Schiff reagent or 2,4-dinitrophenylhydrazine (DNPH) followed by tetrazotized 3,3-dimethoxybenzidine fluoroborate (TDMBF) (Stoward, 1967b). Sections treated with PA or the PA*/PA combination were strongly Schiff positive and yielded formazans on treatment with DNPH and TDMBF (Fig. 5A). Sections treated with PA*, however, were Schiff-unreactive and showed only traces of formazan with the DNPH/TDMBF sequence (Fig. 5B). These data imply that PA* does not produce hemiacetals or hemialdals.

Fig. 5A

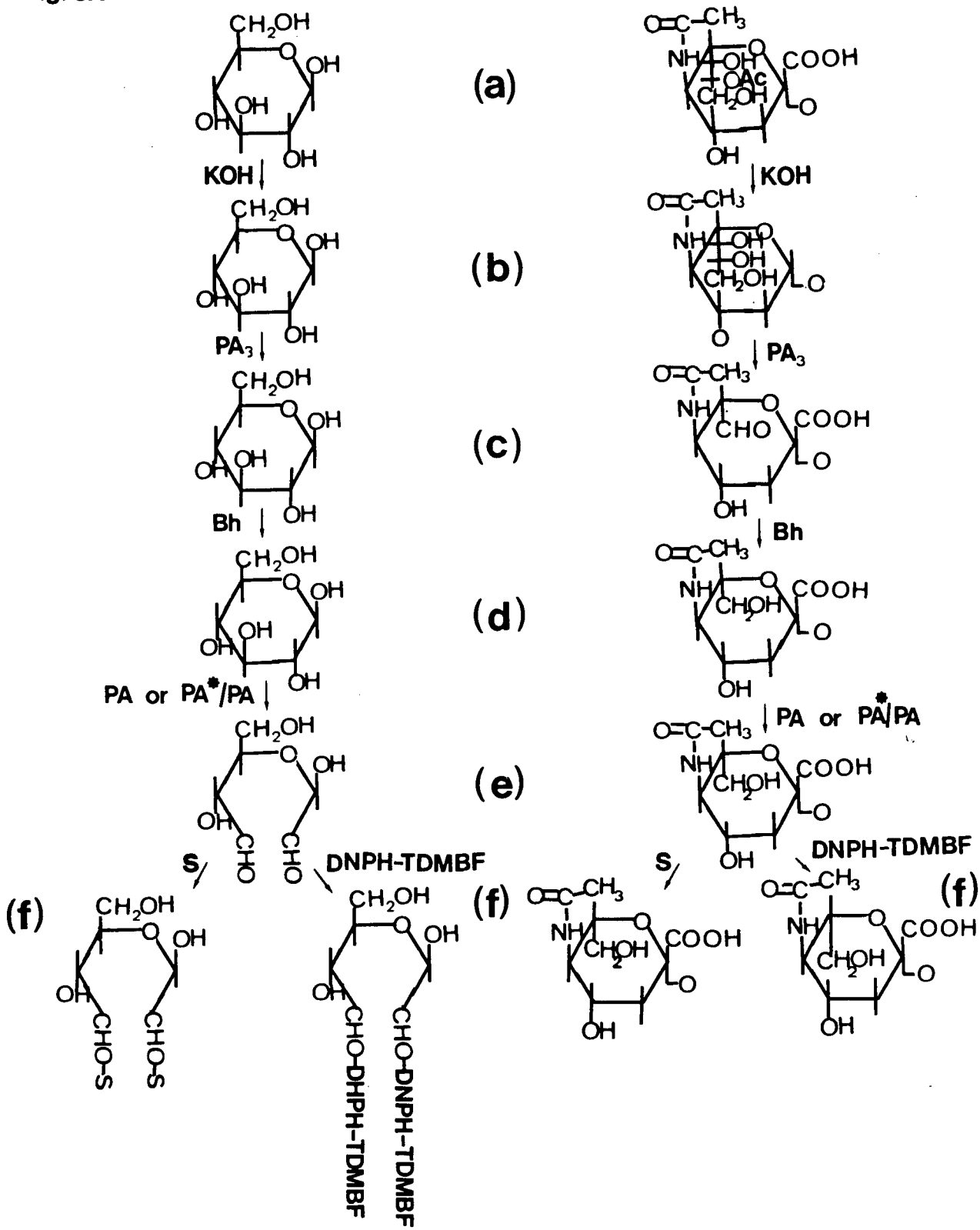

(a)
KOH
(b)
PA3
(c)
Bh
(d)
PA or PA*/PA
(e)
S
DNPH-TDMBF
(f)
CHO-S
CHO-DNPH-TDMBF
COOH
NH
OAc
CHO
CH2OH

Fig. 5B

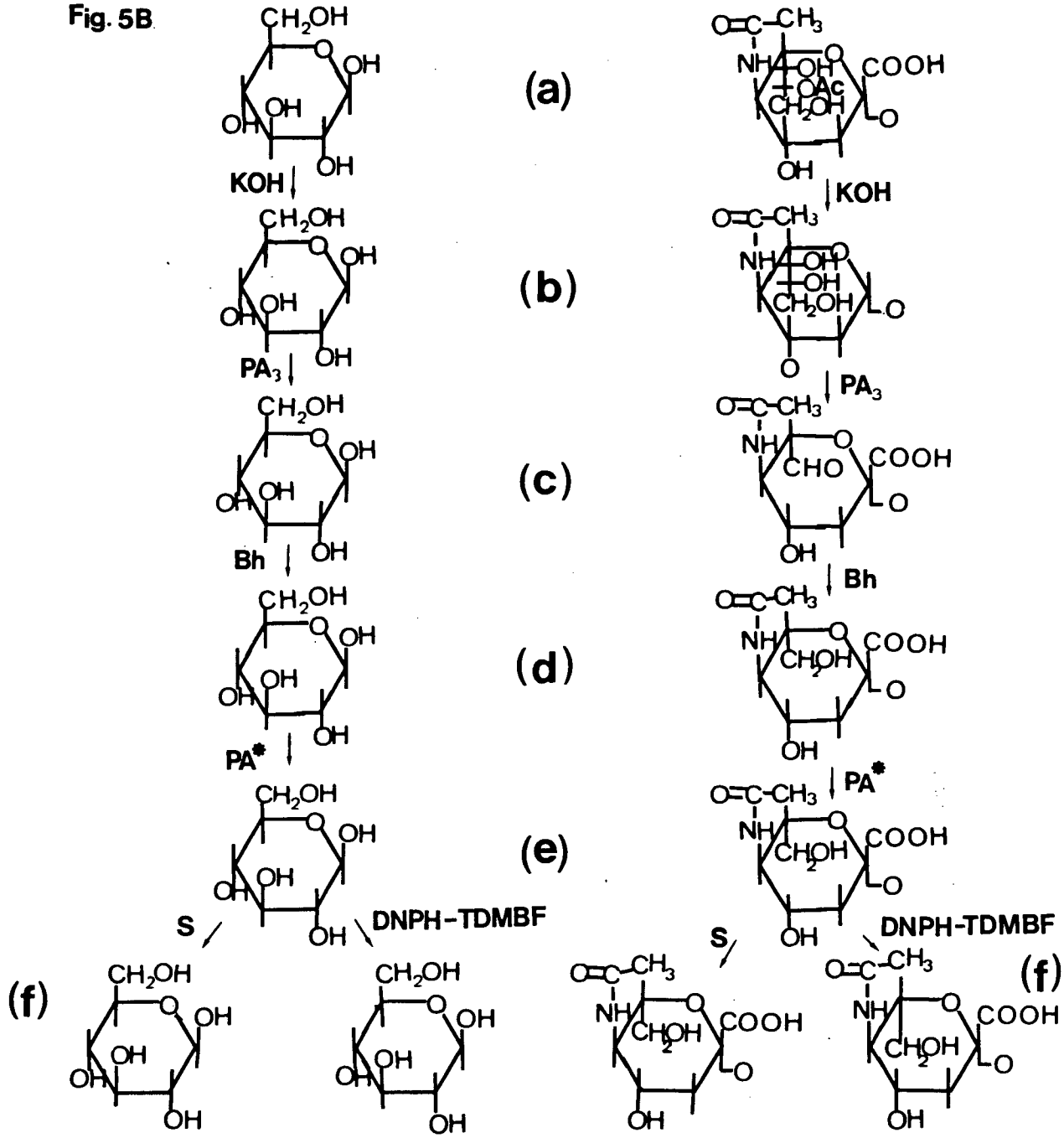
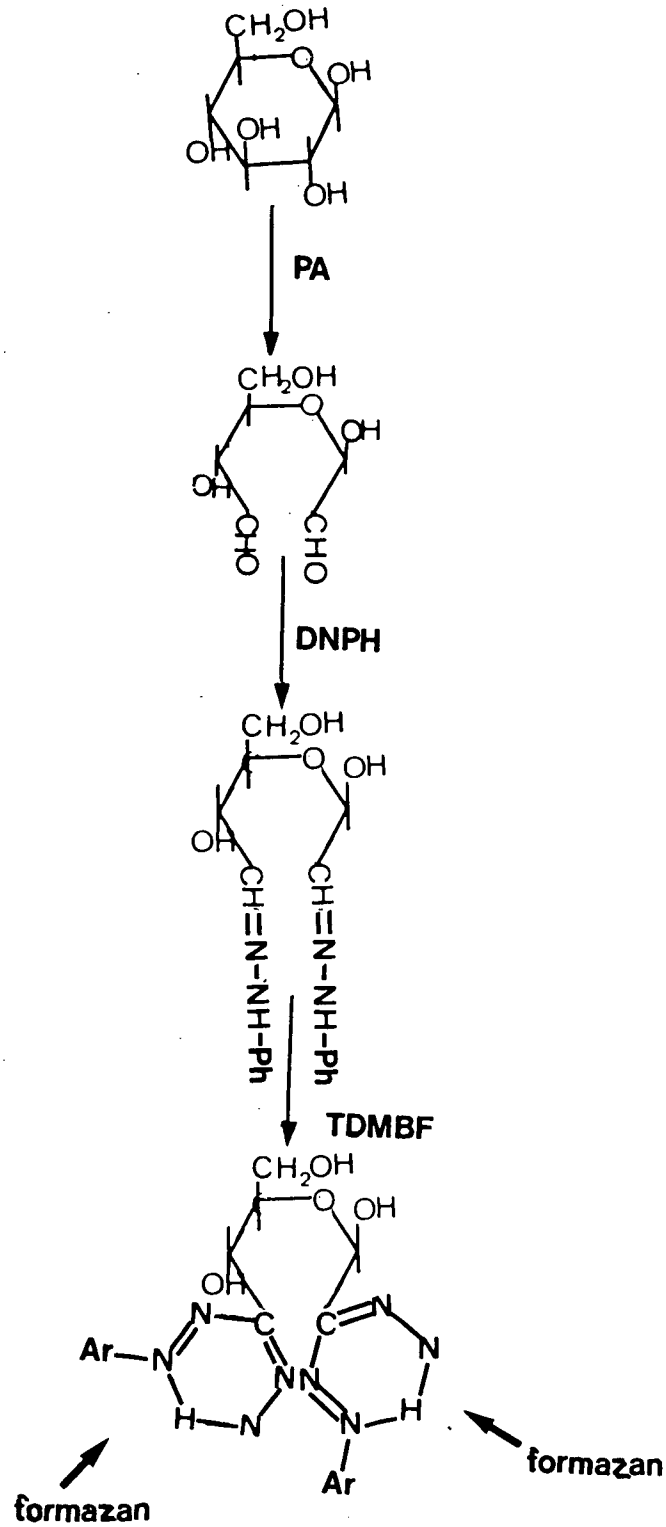


Fig. 6. Illustration of the 2,4-dinitrophenylhydrazine 3,3'-dimethoxybenzidine fluoroborate (DNPH-TDMBF) procedure

Sections of glycoprotein containing tissues (such as rat colon) are oxidized to produce aldehydes (a-b). Sections are then treated with 2,4-dinitrophenylhydrazine (DNPH) which reacts with the aldehydes to form phenylhydrazones (b-c). Next, treatment with tetrazotized 3,3'-dimethoxybenzidine fluoroborate (TDMBF) (c-d) results in the formation of formazans (d).


PA
DNPH
TDMBF
Ar
formazan
formazan
Ar

C. Discussion and Conclusions

In this study it was shown that PA* (0.4 mM periodic acid in 1M HCl at 4°C for 1 hour) completely oxidizes the side chain triol of all sialic acid residues. Under such conditions there was no visible staining of the neutral macromolecules in rat liver and parotid glands, or of the "neutral sugar" residues of either sialo- or sialosulphoglycoproteins. In addition, the reagent did not extract epithelial glycoproteins, de-O-acylate the side chain of sialic acid residues, or produce a significant Feulgen reaction. Therefore, oxidation with 0.4 mM periodic acid in 1M HCl at 4°C in 1 hour results in the selective visualization of total sialic acids and can be used to identify sialic acids with PAS techniques.

The mechanism by which the selective oxidation of sialic acid residues with 0.4 mM periodic acid in 1M HCl occurs has not been fully established. These oxidation conditions did not produce Schiff-positive reactive sites with "neutral sugars". This does not appear to be due to the formation of Schiff unreactive hemiacetals or hemialdals (Guthrie, 1961; Stoward, 1967a,b,c) since such derivatives would be expected to form bis 2,4-dinitrophenylhydrazones and therefore produce formazans on subsequent treatment with tetrazotized 3,3'-dimethoxybenzidine fluoroborate (Mester, 1955, 1958; Chittenden & Guthrie, 1963; Stoward, 1967a). Further, extended oxidation of "neutral sugars" with PA* produced Schiff reactivity. It is probable, therefore, that oxidation of "neutral sugars" with PA* results in too few aldehyde groups to produce a visible reaction product with Schiff reagent.

Volz et al. (1986) suggested that the increased rate of oxidation observed when sialic acid residues are oxidized with very dilute periodic acid in 0.125 N sulphuric acid was due to the suppression of the ionization of the

carboxyl groups of the sialic acid residues. Therefore, the selectivity of the PA* technique is probably a result of an increase in the oxidation rate of sialic acids together with a decrease in the oxidation rate of "neutral sugars". The selective periodate oxidation method for sialic acids (PA*/S), described here, provides a rapid, technically simple substitute for the PAPS reactions, a method currently used to selectively visualize sialic acids.

III. Development of Methods Based on PA* Selectivity

Having established conditions for the selective oxidation of sialic acids which do not significantly oxidize other carbohydrate residues, the potential existed for the development of methods for the simultaneous visualization of "neutral sugars" and either sialic acid and its side chain O-acyl variants or O-sulphate ester. The development of such methods would permit the determination of the types and relative proportions of the various sugar residues in normal tissues and it would therefore be possible to detect changes in the types and ratios of these residues in diseased tissues. The final objective of this study was, therefore, to develop methods for (i) the simultaneous visualization of "neutral sugars" with periodate sensitive vicinal diols (hexose, 6-deoxyhexose and N-acetyl hexosamine) and either sialic acids and their O-acyl side chain variants or O-sulphate ester and (ii) the selective identification of "neutral sugars" in sialic acid-containing tissues.

The five histochemical procedures devised in this study are listed in Table I. Table V shows the results expected when these methods are applied to tissues composed of structural elements including "neutral sugars" with periodate oxidizable vicinal diols, sialic acids without side chain O-acyl substituents or with an O-acyl substituent at C7, C8, or C9 (or which have either two or three side chain O-acyl substituents) and O-sulphate esters.

A. Results of Methods 1-5

Method 1: Method for the Simultaneous Visualization of Sialic Acids with O-acyl substituents at positions C7, C8, and C9 and "Neutral Sugars"

Method 1, the selective periodate oxidation-borohydride reduction-saponification-thionin Schiff-saponification-borohydride reduction-periodic

Table V

Predicted results of the application of the methods outlined in Table 1 to tissue sites containing sialic acids both with and without side chain O-acyl substituents, "neutral sugar" vicinal diols and O-sulphate ester

a) Neutral sugar = hexose, 6-deoxyhexose and N-acetyl hexosamine residues

b) If 9-O-acyl sialic acids oxidize in the initial PA* steps of methods 1 and 3, then they will appear in the CO class of sialic acids.

c) For histochemical purposes 8-O-acyl sialic acids include sialic acids with two -(C7C8, C7C9, C8C9) or three - (C7C8C9) O-acyl substituents.

O = no staining; M = magenta; B = blue; A = aqua. The term aqua is used to distinguish the aquamarine shade imparted by Alcian blue from the grape blue produced following staining with thionin Schiff.

Other abbreviations used are as in Table I.

Results of the application of Methods 1-5 on the staining patterns of the tissues listed here were predicted on the basis of the known composition of these tissues (Table II - Page 27).

Method	Procedure	O-sulphate ester	"Neutral Sugar" vicinal diol a)	Sialic Acid O-acyl side chain substitution		
				None b)	C7	C8 and C9
1	PA*/Bh/KOH/PA*/T/KOH/Bh/PAS	O	M	O	B	B
2	KOH/PA*/T/KOH/Bh/PAS	O	M	B	B	B
3	PA*/T/Bh/PAS/KOH	O	M	B	B	O
4	KOH/PA*/Bh/AB1.O/PAS	A	M	O	O	O
5	KOH/PA*/Bh/PAS	O	M	O	O	O

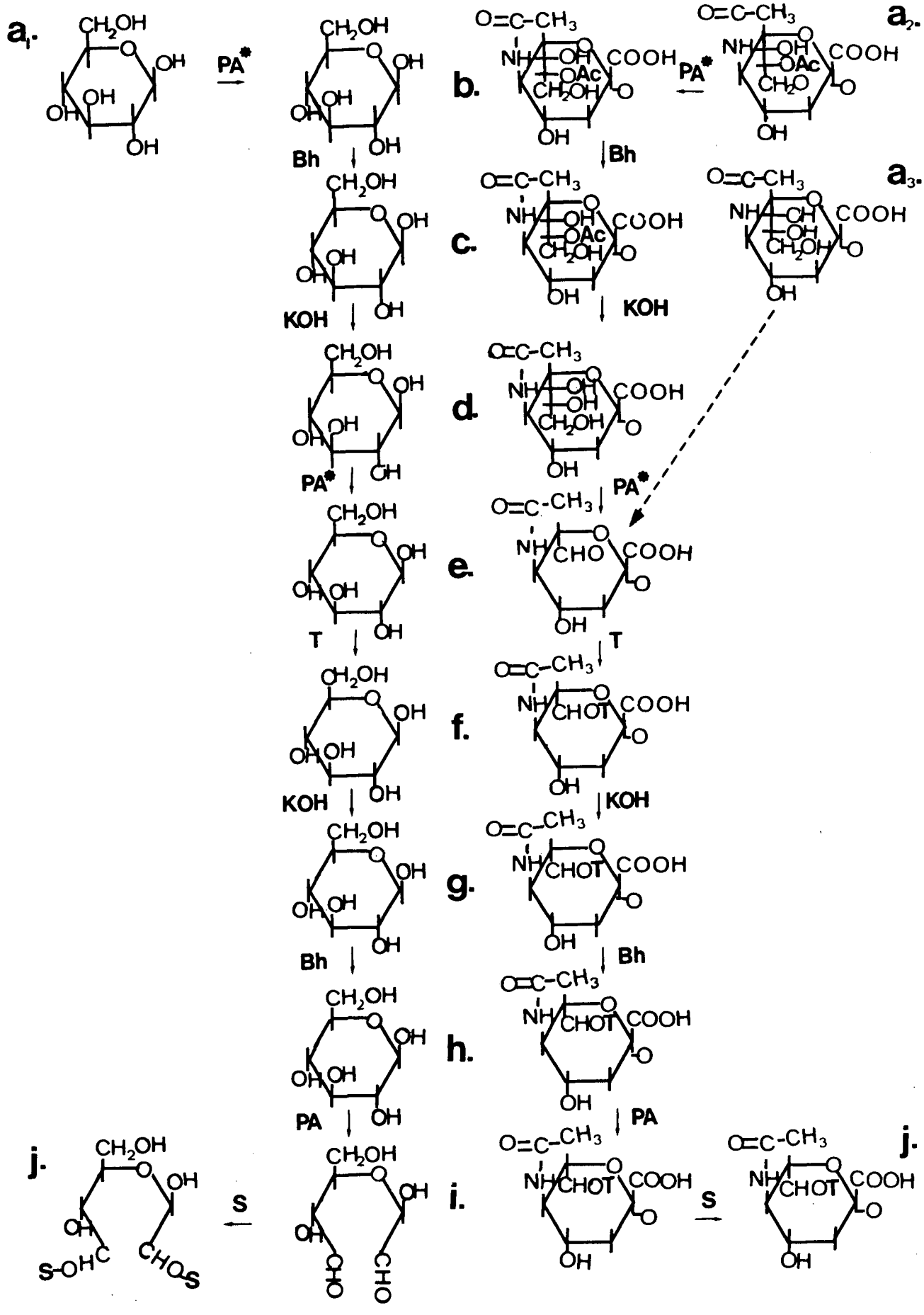
acid-Schiff technique; in the initial PA* oxidation step of this procedure, sialic acids without side chain O-acyl substituents or with an O-acyl substituent at C7 are selectively oxidized to aldehydes. The O-acyl groups of sialic acids with side chain O-acyl substituents at C7, C8 and C9 are then removed (saponification step), rendering them available for oxidation in the second PA* step. The aldehydes produced are then stained blue with thionin Schiff reagent. In the subsequent steps of the sequence, non-specific thionin staining is removed by saponification (KOH), the sections are reduced with sodium borohydride, and then "neutral sugar" vicinal diols are oxidized and stained magenta with the periodic acid Schiff technique. In this method, sialic acids with side chains O-acyl substituents at C7, C8 and C9 stain blue, and "neutral sugar" residues stain magenta (Fig. 9, Illustration XII).

Method 2: Method for the Simultaneous Visualization of Total Sialic Acid Residues and "Neutral Sugars"

In this method, the saponification-selective periodate oxidation-thionin Schiff-saponification-borohydride reduction-periodic acid-Schiff technique, the initial saponification step removes O-acyl esters from all sialic acids. These residues are then oxidized with PA* and subsequently stained blue with thionin Schiff reagent. The "neutral sugars" are then stained magenta with the same KOH/Bh/PAS technique as used in Method 1 (Fig. 9, Illustration XIII).

Fig. 7: The mechanism and expected staining patterns for Method 1, the selective periodate oxidation-borohydride reduction-saponification selective periodate oxidation-thionin Schiff-saponification-borohydride reduction-periodic acid-Schiff (PA*/Bh/KOH/PA*/T/KOH/Bh/PAS) technique, and Method 2, the saponification-selective periodate oxidation-thionin Schiff-saponification-borohydride reduction-periodic acid Schiff (KOH/PA*/T/KOH/Bh/PAS) technique.

In Method 1, in the initial PA* step (a-b), sialic acids without side chain O-acyl substituents or with an O-acyl substituent at C7 are oxidized to aldehydes and reduced to Schiff unreactive primary alcohols with borohydride. They are not, therefore, identified in this procedure. Saponification (c-d) then removes acyl groups including those located on sialic acids at position C7, C8, or C9 (or which had 2 or 3 side chain substituents) which are then available for oxidation (d-e) in the second PA* step (d-e) and are stained with thionin Schiff (e-f). Non-specific thionin Schiff staining is then removed by saponification (f-g), tissues are reduced with borohydride (g-h), and then "neutral sugar" vicinal diols are oxidized with PA (h-i) and stained magenta with Schiff reagent (i-j). In Method 2, the initial saponification step (c-d) results in the oxidation of all sialic acids (d-e) which were then stained with thionin Schiff (e-f) following the initial PA* step (d-e). Non-specific staining of thionin is then removed with KOH (f-g), tissues are reduced with borohydride (g-h) and "neutral sugars" are then oxidized with PA (h-i) and stained magenta with pararosaniline Schiff (i-j).


a1.
PA*
Bh
KOH
PA*
T
KOH
Bh
PA
j.
S
b.
PA*
a2.
Bh
c.
KOH
a3.
d.
PA*
e.
T
f.
KOH
g.
Bh
h.
PA
i.
S
j.

Method 3: Method for the Simultaneous Visualization of Sialic Acids without Side Chain Substituents or with O-Acyl Substituents at C7 and "Neutral Sugar" Residues

In this method, the selective periodate oxidation-thionin Schiff-borohydride reduction-periodic acid-Schiff-saponification technique, sialic acids without side chain substituents or with O-acyl substituents at C7 stain blue with the selective periodate oxidation-thionin Schiff sequence. Following borohydride reduction, "neutral sugars" are stained magenta with the PAS method and non-specific thionin staining is then removed by saponification (Fig. 8, Illustration XIV).

Method 4: Simultaneous Visualization of All Sialic Acids and O-sulphate Esters

In this method, the saponification-selective periodate oxidation-borohydride reduction-Alcian blue pH 1.0-periodic acid-Schiff technique, the initial KOH/PA*/Bh sequence renders all sialic acids periodate unreactive. The O-sulphate esters are then stained aqua with Alcian blue at pH 1.0. The "neutral sugars" are then oxidized and stained magenta with the PAS procedure (Fig. 9, Illustration XV).

Method 5: Method for the Selective Oxidation of "Neutral Sugars"

In this method, the saponification-selective periodate oxidation-borohydride reduction-periodic acid-Schiff technique, all sialic acid residues are rendered Schiff unreactive by the KOH/PA*/Bh sequence. "Neutral sugars" are then stained magenta in the PAS procedure (Fig. 9, Illustration XVI).

Fig. 8: The mechanism and expected staining patterns for Method 3, the selective periodate oxidation-thionin Schiff-borohydride reduction-periodic acid-Schiff-saponification (PA*/T/Bh/PAS/KOH) technique.

Sialic acids without side chain substituents or with O-acyl substituents at C7 stain blue with the selective periodate oxidation-thionin Schiff sequence (a-c). Following borohydride reduction (c-d), "neutral sugars" are oxidized with PA (d-e), stained magenta with pararosaniline Schiff (e-f), and then non-specific thionin staining is removed by saponification (f-g).

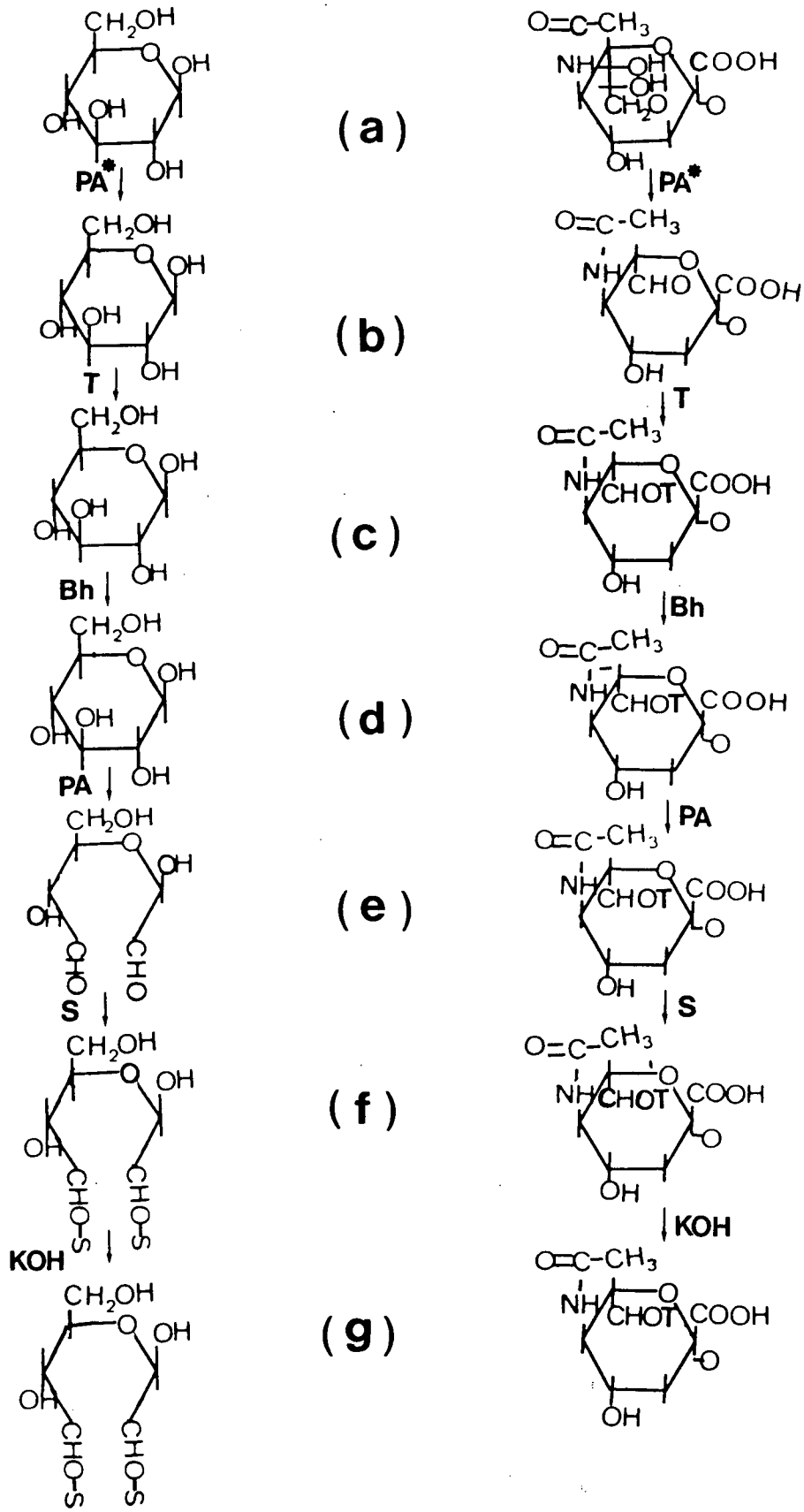
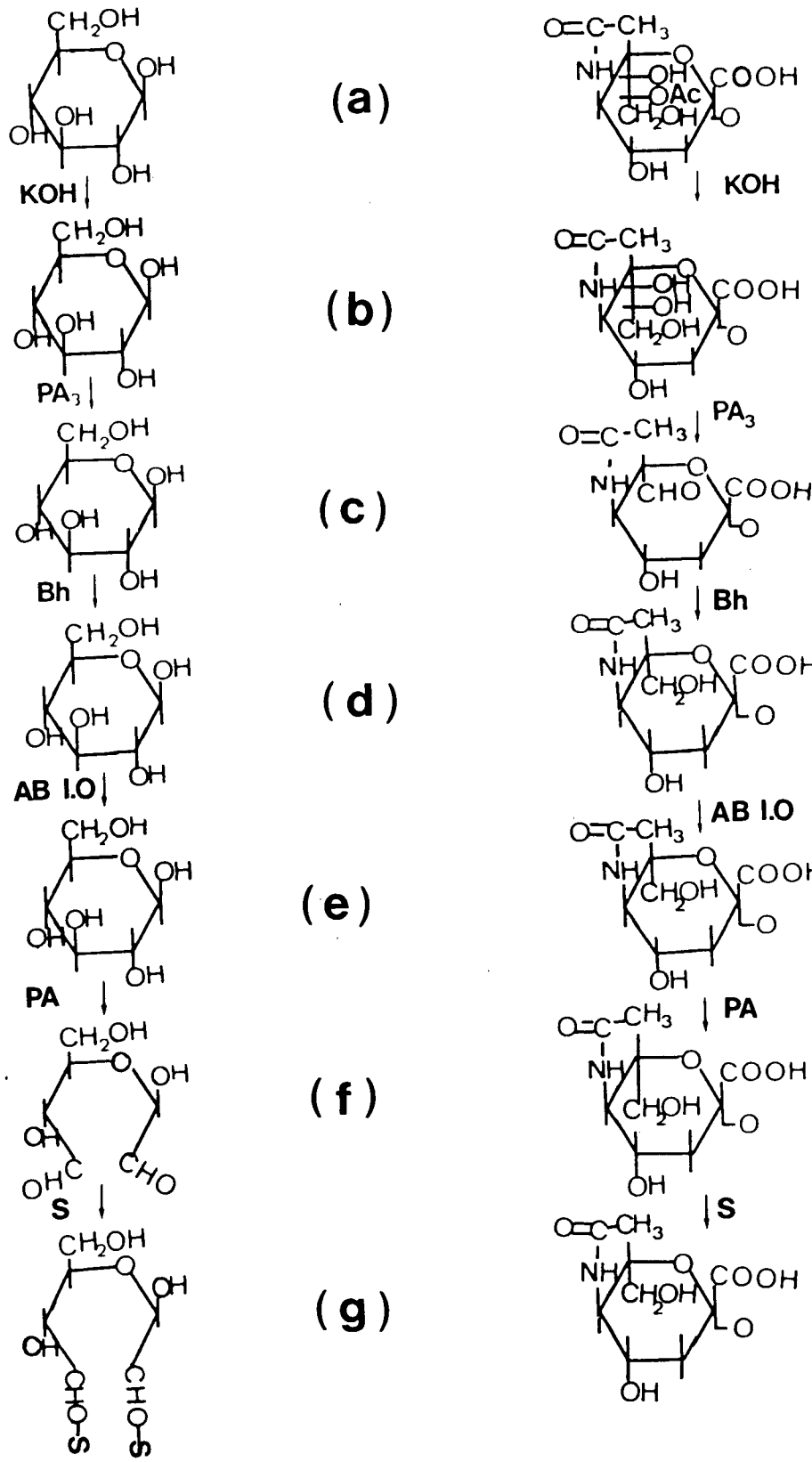

(a)
(b)
(c)
(d)
(e)
(f)
(g)
PA*
T
Bh
PA
S
KOH

Fig. 9: The mechanism and staining patterns expected for Method 4, the saponification-selective periodate oxidation-borohydride reduction-Alcian blue pH 1.0-periodic acid-Schiff (KOH/PA*/Bh/AB1.0/PAS) technique, and Method 5, the saponification-selective periodate oxidation-borohydride reduction-periodic acid-Schiff (KOH/PA*/Bh/PAS) technique.

In Method 4, the initial saponification step (a-b) removes O-acyl groups from vicinal diols, resulting in oxidation of all tissue sialic acids with PA* (b-c). These sialic acids are then reduced to Schiff unreactive primary alcohols with borohydride (c-d). Sulphate esters are stained aqua with Alcian blue pH 1.0 (d-e) and "neutral sugars" are then oxidized with 1% periodic acid (e-f) and stained magenta with Schiff (f-g). "Neutral sugars", therefore, stain magenta and sulphate esters stain aqua. Method 5 is similar to Method 4 except that the Alcian blue pH 1.0 step (d-e) is omitted. This method therefore results in magenta staining of "neutral sugars".


(a)
KOH
KOH
(b)
PA3
PA3
(c)
Bh
Bh
(d)
AB I.O
AB I.O
(e)
PA
PA
(f)
S
S
(g)

B. Specificity of Methods 1-5

The specificity of these sequences was examined using the experiments outlined in Table VI.

The order of application of thionin and pararosaniline Schiff reagents (thionin first, pararosaniline second) in Methods 1,2, and 3 was based upon past studies of double Schiff procedures (Van Duijn, 1956; Culling et al., 1976; Reid et al., 1984c) and was necessary to prevent the replacement of one reagent by the other. In contrast to previous investigations (Reid et al., 1984c), it was found in initial studies that it was not necessary to prepare thionin Schiff reagent by the method of Van Duijn (1956) as the faster method of Barger and DeLamater (1948) was satisfactory. In addition, use of this reagent diminished the time required for maximum staining with thionin Schiff from 4 hours (Reid et al., 1984c) to only 2 hours.

A KOH/Bh sequence was used following the thionin Schiff step of Methods 1 and 2 (Tables I and V). In previous studies (Reid et al., 1984c), it was shown that the saponification step was required to remove non-specific thionin staining of nuclei as well as for the de-O-acylation of sialic acids, and that the borohydride reduction step was necessary to prevent anomalous pararosaniline Schiff staining. In this study a number of controls were carried out to establish the specificity of the KOH/Bh sequence used in Methods 1-3. When a borohydride reduction step was inserted between the final periodic acid and Schiff steps of Methods 1 and 2, (PA*/Bh/KOH/PA*/T/KOH/Bh/ PA/Bh/S or KOH/PA*/T/KOH/Bh/PA/Bh/S) to prevent staining with pararosaniline Schiff, only blue staining (thionin) was obtained (Illustration XVII). This indicated that there was no exchange of Schiff reagents in these procedures.

Table VI: Methods for the verification of the specificity of Methods 1 to 5.

The nature of the macromolecules studied in these tissue sites in summarized in Table II (Page 27).

PA_3 and PA_{60} = oxidation with 1% (w/v) periodic acid at room temperature for 3 and 60 minutes, respectively

PA* = oxidation with 0.4 mM periodic acid in approximately 1M hydrochloric acid at 4°C for one hour

KOH = saponification

Bh = borohydride reduction (Lillie and Pizzolato, 1972)

S = treatment with pararosaniline Schiff

AB 2.5 and AB 1.0 = staining with Alcian blue at pH 2.5 and pH 1.0, respectively (Culling, 1974)

0 = no staining

4+ = strong staining

Purpose	Method	Experimental Sequence	Results	Interpretation
To determine if one Schiff reagent is replaced by another	1	PA*/Bh/KOH/PA*/T/KOH/Bh/PA/Bh/S	Thionin staining only	Insertion of the borohydride sequence step between the final periodic acid and Schiff steps of Methods 1 and 2 will prevent staining with Schiff. Since no pink (only blue) staining was observed, no exchange of Schiff reagents occurs in these procedures.
	2	KOH/PA*/T/KOH/Bh/PA/Bh/S		
	3	PA*/T/Bh/PA/Bh/S/KOH	Thionin staining only	When a borohydride reduction step was placed between the PA_{60} and Schiff steps, thereby preventing staining with pararosaniline Schiff, only thionin staining occurred, illustrating that no exchange of Schiff reagents occurs, and that moving the position of the KOH step does not affect staining results.

Purpose	Method	Experimental Sequence	Results	Interpretation
To demonstrate that no non-specific staining with thionin Schiff occurs	1	PA*/Bh/KOH/PA*/Bh/T/KOH/Bh/PA/S	Magenta staining only	No non-specific thionin Schiff staining occurs
	2	KOH/PA*/Bh/T/KOH/Bh/PA/S		
	3	PA*/Bh/T/Bh/PA/S/KOH		
To determine if the resulting staining patterns in Methods 1-5 reflect those expected on the basis of the known composition of the various macromolecules in the tissues (Table V) (Page 46)	1	PA*/Bh/KOH/PA*/T/KOH/Bh/PA/S	See Table V (Page 46)	Staining patterns obtained with these methods were as predicted on the basis of the known composition of these tissues. These methods represent, therefore, valid methods for the detection of sialic acid and its O-acyl substituents and O-sulphate esters and "neutral sugars".
	2	KOH/PA*/Bh/T/KOH/Bh/PA/S		
	3	PA*/T/Bh/PA/S/KOH		
	4	KOH/PA*/Bh/AB1.0/PAS		
	5	KOH/PA*/Bh/PA/S		

The KOH/Bh sequence could not be used in Method 3 (PA*/T/Bh/PAS/KOH), however, since the saponification (KOH) step would de-esterify 7- and 9-O-acyl sialic acids which would then be identified as "neutral sugars" in the subsequent PAS step. As a result, the saponification step was placed after the final pararosaniline Schiff treatment. The feasibility of moving the placement of the KOH step was ascertained by demonstrating that, when a borohydride reduction step was placed between the PA_{60} and Schiff steps of Method 3 (PA*/T/Bh/PA_{60}/Bh/S/KOH/), only thionin Schiff staining was obtained.

To demonstrate that there was no non-specific staining with thionin Schiff (Methods 1, 2 and 3), a borohydride reduction step was placed between the periodate oxidation and thionin Schiff steps. Only magenta staining was obtained in these control studies (Illustration XIX) ,indicating that non-specific staining with thionin Schiff had not occurred.

Finally, in all five methods the final PAS procedure was performed under conditions which resulted in maximal staining of rat liver glycogen and the neutral macromolecules of parotid gland acinar cell granules.

The specificity of the methods was also investigated by comparing the staining patterns obtained when they were applied to sections of rat liver, salivary gland complexes and colon and human colon to that predicted on the basis of the known composition of the various macromolecules in the tissues (Table II). As would be expected, liver glycogen and the neutral macromolecules of the acinar cells of the rat parotid gland, which do not contain sialo-, sialosulpho-, or sulphated glycoproteins, stained only magenta with all methods. With Methods 4 ("neutral sugars" vs sulphate) and 5 ("neutral sugars" only), the rat sublingual gland, terminal ileum and crypt bases of the proximal colon of the rat, which contain only sialoglycoproteins

and "neutral sugars" but no sulphate, stained magenta. In contrast, the heavily sulphated epithelial sialoglycoproteins of human colon and rat distal colon, which also contain "neutral sugars", stained in various shades of purple with Method 4 but magenta with Method 5.

In Method 2, the glycoproteins of the rat ileum-colon and sublingual glands and human colon, which contain varying proportions of sialic acids and "neutral sugars", stained in various shades of purple. The acinar cells of rat submandibular glands, however [which contain only traces of sialic acid as shown by staining with the high iron diamine-Alcian blue pH 2.5 technique (Spicer, 1965)], stain only magenta with Method 2.

In Method 1 (C7, C8 and C9 SA vs "neutral sugars"), tissues containing large quantities of these sialic acids such as the rat sublingual gland (Park et al., 1987 unpublished), human colon (Culling et al., 1981) and the bases of the crypts of the rat proximal colon (Reid et al., 1973) stained in varying shades of purple. Tissues which contain less C7, C8, and C9 O-acyl substituted sialic acids, such as the upper halves of the crypts of rat proximal colon (Reid et al., 1973) stained with a redder hue, while tissues containing little or no C7, C8 and C9 substituted sialic acids, such as rat terminal ileum, stained magenta. Finally, sites containing significant quantities of sialic acids without side chain substituents such as rat sublingual gland and rat terminal ileum and colon, stained in various shades of purple with Method 3.

C. Discussion and Conclusions

These studies have resulted in the development of methods for the selective oxidation of sialic acid, the simultaneous visualization of "neutral sugars" and either sialic acid and its side chain O-acyl variants or O-sulphate ester,

and a general method for the identification of "neutral sugars" in the presence of sialic acid. The specificity of these procedures depends upon several factors. Firstly, the selective periodate oxidation procedure (PA*) used in the initial steps of Methods 2-5 (Table I) and for the first two oxidation steps of Method 1 (Table I), must oxidize all available sialic acid vicinal diols without producing a visible Schiff reaction by the oxidation of "neutral sugars". In Methods 2-5, the incomplete oxidation of sialic acid residues will result in their identification as "neutral sugars". In Method I, those sialic acids failing to oxidize in the initial PA* step, will subsequently be identified as 7-, 8- or 9-O-acyl substituted sialic acids, and those that do not oxidize in the second oxidation step will be identified as "neutral sugars". In addition, if a significant oxidation of "neutral sugars" occurs during the initial oxidation steps of Methods 2 and 3, and during the first two oxidation steps of Method 1, these sugars would subsequently be identified as sialic acids. Further, in Methods 4 and 5, such oxidation would result in an underestimate of the quantity of "neutral sugars" present. However, control studies carried out during the investigation of the PA* method demonstrated that the complete oxidation of sialic acids without side chain O-acyl substituents can be achieved using 0.4 mM periodic acid in approximately 1M hydrochloric acid for 1 hour at 4°C. These conditions did not produce visible Schiff staining of either neutral macromolecules or the "neutral sugars" of sialo- and sialosulphoglycoproteins, therefore the initial periodate oxidation steps of Methods 2, 4 and 5 can be considered specific for sialic acids.

The interpretation of the results obtained following the oxidation of O-acyl side chain substituted sialic acids with PA* in Methods 1 and 3 is

more complicated. Chemical studies (Haverkamp et al., 1975; Schauer, 1982; Shukla and Schauer, 1982; Diaz and Varki, 1985) have shown that 9-O-acyl sialic acids oxidize at a much slower rate than sialic acids without side chain O-acyl substituents. However, if 9-O-acyl sialic acids are present in the tissues examined in this study, and if they oxidize under PA* conditions, then the observed thionin Schiff staining in Method 1 would be due to sialic acids with an O-acyl substituent at positions C7 or C8 only. In Method 3, in contrast, the staining of sialic acid would be due to unsubstituted sialic acids or sialic acids with a single O-acyl substituent at position C7. Also, if O-acyl migration (Cheresh and Reisfeld, 1984; Schauer, 1982; Varki and Diaz, 1984; Diaz and Varki, 1985) from position C7 to position C8 or C9 occurs during the initial oxidation step of Method 3, then 7-O-acyl sialic acids would not be identified. Acyl migration would not, however, affect the staining in Method 1. In studies performed on gastrointestinal mucins (Reid et al., 1977, 1978), 9-O-acyl sialic acids were not detected; however, they were demonstrated in bovine submandibular gland (Reid et al., 1978). Veh et al. (1979) have detected 9-O-acyl sialic acids in bovine submandibular glands using conditions which accounted for the slow oxidation of such acids.

Secondly, the periodate oxidation conditions employed in the final PAS step of all five methods must completely oxidize all "neutral sugar" vicinal diols and the Schiff reagent used should react completely with all aldehydes produced. If either of these reactions is incomplete, then the quantity of "neutral sugars" present will be underestimated. The conditions chosen for the final PAS reaction result in maximal staining of neutral macromolecules. The intensity of the PAS reaction is, however, dependent upon the structure of the macromolecule oxidized (Reid & Culling, 1980). It is necessary, therefore, to

consider the possibility that an incomplete PAS reaction can occur. Furthermore, "neutral sugars" containing O-acyl esters upon potential vicinal diols will not be detected in Method 3. Such residues have only been detected thus far in one gastrointestinal mucin, the mucous cells of the distal rat colon (Park et al., 1987, unpublished). O-acetyl "neutral sugars" will not, however, complicate the interpretation of staining patterns seen in Methods 1, 2, 4 and 5, since the saponification step in these methods will remove any O-acyl esters present before the PAS procedure is carried out.

Finally, as demonstrated in control experiments, the product of thionin Schiff reagent and sialic acid monoaldehydes was sufficiently stable to survive the steps following it, and therefore anomalous pararosaniline Schiff staining of sialic acid residues did not occur.

A list of control experiments which should be done concurrently with Methods 1-5 is shown in Table VII.

Table VII: List of Control Methods which should accompany Methods 1-5.

x = step performed

PA* = oxidation with 0.4 mM period acid in 1M hydrochloric acid for 1 hour at 4°C

Bh = reduction with 0.1% (w/v) sodium borohydride in 1% dibasic sodium phosphate (anhydrous) for 15 minutes at room temperature

KOH = saponification with 0.5% potassium hydroxide in 70% ethanol for 15 minutes at room temperature

PA = oxidation with 1% periodic acid for 2 hours at room temperature

S = pararosaniline Schiff for 1 hour at room temperature

T = thionin Schiff reagents for 2 hours at 4°C

Note: In most cases it is only necessary to perform control step Ia of step Ia and b, and not both steps Ia and Ib. If Ia is positive, then Ib should be carried out. If Ib is also positive, then Methods 1-5 are not specific.

Tissue	Procedure	Expected Result	Rationale
Ia) rat liver rat parotid gland	PA*/S	no staining	These tissues do not contain sialic acids and "neutral sugars" - should not oxidize under conditions of PA*
b) rat liver rat parotid gland	PA*/Bh/PA*/S	no staining	any sugar residues oxidized in PA* should be reduced by boro-hydride and therefore be unavailable for subsequent oxidation (PA* second) and staining with Schiff reagent
II) rat liver rat parotid gland	Method 1 PA*/Bh/KOH/PA*/T/KOH/Bh/PAS Method 2 KOH/PA*/T/KOH/Bh/PAS Method 3 PA*/T/Bh/PAS/KOH Method 4 KOH/PA*/Bh/AB1.0/PAS Method 5 KOH/PA*/Bh/PAS	Magenta Magenta Magenta Magenta Magenta	These tissues do not contain sialic acids and therefore should not be oxidized by PA*, but only by PA, resulting in magenta staining
IIIa) human colon	PA/Bh/KOH/PA*/S	intensely magenta	All unsubstituted sialic acids and sialic acid substituted at C7 will be oxidized and reduced by the PA/Bh sequence. C8 substituted sialic acids will then be de-O-acylated by KOH, then oxidized by PA* and stained magenta with Schiff reagent

b) human colon	PA/Bh/KOH/PA*/Bh /PA*/S	no staining	All sialic acids and "neutral sugars" should be oxidized by either the PA or PA* step, and then reduced by the Bh step following each of these oxidations. No sialo- sugars should remain, therefore, to be oxidized in the second PA* step, resulting in lack of staining in tissues treated in this manner.

GENERAL CONCLUSION:

This study was comprised of three parts. The first involved the development of selective conditions for the oxidation of sialic acids, and therefore a selective method for the detection of sialic acids with the PA*/S technique. The second involved an investigation of the mechanism of this selectivity, and the third involved the application of the selective periodate oxidation of sialic acids to the development of general methods for the detection of "neutral sugars" and for the simultaneous visualization of sialic acids and its O-acyl side chain substituents and O-sulphate ester, and "neutral sugars". These methods provide new techniques for the examination of epithelial glycoproteins in normal and diseased tissues.

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Illustration I:

A. Sections of rat colon stained with the PA/Bh/KOH/PA_2/S (Slide Ai), PA/Bh/KOH/PA*/S (Slide Aii) and PA/Bh/KOH/PA_{60}/S (Slide Aiii) procedures. The intensity of the Schiff staining of the colonic goblet cells is similar regardless of the oxidation technique used, indicating that PA* oxidizes all sialic acid diols.

B. Sections of rat liver stained with the PA_2/S, PA*/S and PA_{60}/S procedures. There is little or no staining of glycogen with the PA_2/S and PA*/S techniques (Slides Bi and Bii), but strong staining following treatment with the PA_{60}/S (Slide Biii). This indicates that glycogen does not stain significantly with the PA_2/S and PA*/S methods. Note the slightly greater intensity obtained with the PA_2/S technique, indicating the greater specificity of the PA*/S.

Illustration II: Effect of pH on the rate of periodate oxidation of sialic acid residues. PA/Bh/KOH treated sections of human colon were oxidized in 0.004 mM periodic acid dissolved in water (Slide IIa) or 0.125N sulphuric acid (Slide IIb) for 24 hours, and then stained with Schiff reagent. Note that the intensity of staining in acid solvent (Slide IIb) is greater than that obtained when water was used as solvent.

Illustration III: Effect of pH on the periodate oxidation of "neutral sugars". Sections of rat liver were oxidized in 0.4mM periodic acid at 4°C using either water (Slide IIIa) or 0.125N sulphuric acid (Slide IIIb) as solvent, for 24 hours, then stained with Schiff.

Note: The oxidation of "neutral sugars" is depressed in acid conditions. Oxidation in 1M NaCl (not shown) resulted in a staining pattern similar to that observed when water is used as a solvent.

Illustration IV: Evidence for the specificity of PA* conditions for the selective periodate oxidation of sialic acids - Part A. Sections of rat colon were treated with the PA/Bh/KOH/PA_{60}/S (Slide IVa), PA/Bh/KPH/PA*/S (Slide IVb) and the PA/Bh/KOH/PA*/PA/S (Slide IVc) sequence. Note that no difference in the staining intensity of these treatments is evident, suggesting that PA* oxidized all sialic acid residues.

Illustration V: Evidence for the specificity of PA* conditions for the selective periodate oxidation of sialic acids - Part B. Sections of human colon were treated with the (a) PA/Bh/KOH/PA*/S (Slide Va), (b) PA/Bh/KOH/PA*/Bh/S (Slide Vb) and (c) PA/Bh/KOH/PA*/Bh/PA_{60}/S (Slide Vc) sequences. Treatment with sequence (a) resulted in strong (4+) Schiff staining of the tissue. However, when serial sections were reduced with borohydride after the PA* step and then further oxidized with PA* (sequence b - Slide Vb) or reduced with borohydride and further oxidized with PA_{60} (sequence c - Slide Vc), no Schiff staining was obtained. This indicated that when only sialic acids were present, they are completely oxidized by PA* conditions.

Illustration VI: Effect of PA* conditions on the periodate oxidation of "neutral sugars". Sections of rat liver were treated with (a) PA*/S, (b) periodate oxidation with 0.4 mM periodic acid in 1M HCl at 4°C for 24 hours, followed by Schiff, and (c) the routine PAS procedure. Oxidation with (a) PA*/S produced no significant staining of liver glycogen (Slide VIa). PAS reactivity of glycogen did occur slightly in (b) and intensely with (c). These results indicate that PA* conditions do not result in any significant staining of "neutral sugars". Note the Feulgen staining of nuclei in (b) (See also Illustration X). Such staining is insignificant in (a) or (c). Similar results were obtained with rat parotid gland.

Illustration VII: Effect of PA* conditions on the oxidation of "neutral sugars" in the presence of sialo- and sialosulpho-containing glycoproteins. Sections of rat colon were pre-treated with the KOH/PA_3/Bh sequence to confine vicinal diols to "neutral sugar" residues. Sections were then treated with (a) PA* (KOH/PA_3/Bh/PA*), (b) PA_{60} (KOH/PA_3/Bh/PA_{60}) or (c) PA*/PA_{60} (KOH/PA_3/Bh/PA*/PA) and stained with Schiff reagent. No Schiff reactivity was produced with (a); however, strong (4+) staining was obtained with (b) and (c). This indicates that the PAS reactive "neutral sugar" residues present are available for oxidation, and stain with the PAS. The absence of PA*/S staining indicates that PA* does not produce significant "neutral sugar" staining.

Illustration VIII: To determine if glycoproteins are extracted when treated with PA* conditions.

Sections of rat colon were treated with (c) KOH/PA/Bh/AB1.0 (Slide VIII i) or (b) AB1.0 (Slide VIII ii). No difference in the staining patterns occurred between (a) and (b).

These data indicate that PA* does not extract epithelial glycoproteins.

Illustration IX: To determine if PA* de-O-acetylates sialic acids. Sections of rat colon were treated with the PA/Bh sequence to remove all oxidizable vicinal diols, incubated with 1M HCl for 1 hour at 4°C, and then treated with the PAS technique (PA/Bh/1M HCl 1 hr at 4°C/PAS) (Slide IX). These sections were not PAS reactive, but sections treated with the sequence PA/Bh/1M HCl 1 hr at 4°C KOH/PAS (not shown) stained intensely, indicating that such conditions do not de-O-acetylate sialic acids.

Illustration X: To determine if PA* conditions invoke a Feulgen reaction. Sections of rat colon were incubated with either 1M HCl for 1 hour at 4°C (Slide X) or with PA* (not shown). Minimal Schiff staining of nuclei was obtained. However, when sections were incubated in PA* for 24 hours, a strong Feulgen staining resulted in addition to mucin staining.

Illustration XI. Illustration of results obtained in mechanistic studies of the selectivity of PA*. Sections of rat colon were treated with the KOH/PA_3/Bh sequence to confine vicinal diols to "neutral sugar" residues. Sections were then oxidized with (a) PA* (KOH/PA_3/Bh/PA*) (Slide XIa), (b) PA_{60} (KOH/PA_3/Bh/PA_{60}) (Slide XIb), or (c) PA*PA_{60} (KOH/PA_3/Bh/PA*/PA_{60}) (not shown) and stained with Schiff (not shown) or DNPH-TDMBF (Slides XIa and b). Sections exposed to (b) or (c) (not shown) were strongly Schiff positive and yielded formazans on treatment with DNPH and TDMBF (Slide XIa). Sections exposed to (a), however, were Schiff unreactive and showed only traces of formazan with the DNPH/TDMBF sequence (Slide XIb). These data imply that PA* does not produce hemiacetals or hemialdals.

Illustration XII. Illustration of the selective periodate oxidation-borohydride reduction-saponification-selective periodate oxidation-thionin Schiff-saponification-borohydride reduction-periodic acid-Schiff (PA*/Bh/KOH/PA*/T/KOH/Bh/PAS) technique (Method 1). Sections of rat liver (Slide XIIa), salivary gland complex (Slide XIIb), proximal and distal colon (Slide XIIc and XIId respectively) and human colon (Slide XIIe) were treated with the PA*/Bh/KOH/PA*/T/KOH/Bh/PAS technique. In this method sialic acids with O-acyl substituents at C7, C8 and C9 stain blue, "neutral sugars" stain magenta, and mixtures stain in varying shades of purple. Note that rat liver (Slide XIIa), rat submandibular gland (Slide XIIb) and the neutral macromolecules of the acinar cells of rat parotid gland (not shown) which either do not contain sialoglycoproteins, or only contain traces, stain magenta, while rat sublingual gland (Slide XIIb), human colon (Slide XIIe) and the bases of the crypts of the rat proximal colon (Slide XIIc), which contain large quantities of C7, C8 and C9 sialic acids, stain in various shades of purple. Note that the upper crypts of rat proximal colon, which do not contain much sialic acid, stain magenta.

Illustration XIII. Illustration of the saponification-selective periodate oxidation-thionin Schiff-saponification-borohydride reduction-periodic acid-Schiff (KOH/PA*/T/KOH/Bh/PAS) method (Method 2). Sections of rat liver, salivary gland complex, colon and human colon were treated with the KOH/PA*/T/KOH/Bh/PAS method. In this method all sialic acids stain blue, "neutral sugars" stain magenta, and mixtures stain in varying shades of purple. Tissues that contain no sialic acids or only traces of such residues such as rat liver (staining results similar to XIIa), submandibular gland (Slide XIIIa) and parotid gland (not shown) stain magenta. Tissues which contain varying proportions of sialic acids and "neutral sugars" such as rat colon (Slide XIIIb), sublingual gland (Slide XIIIa) and human colon (Slide XIIIc), stain in varying shades of purple.

Illustration XIV. Illustration of the selective periodate oxidation-thionin Schiff-borohydride reduction-periodic acid-Schiff-saponification (PA*/T/Bh/PAS/KOH) technique (Method 3). Sections of rat liver, salivary gland complex, rat colon and human colon were treated with PA*/T/Bh/PAS/KOH. In this method sialic acids without side chain substituents or with side chain substituents at position C7 stain blue, "neutral sugars" stain magenta, and mixtures stain in varying shades of purple. Tissues containing no sialic acids or only traces of this residue such as rat liver (staining similar to that seen in Slide XIIa), submandibular gland (Slide XIVa) and parotid gland (Slide XIVb), stain magenta, while tissues containing large quantities of sialic acid without side chain substituents such as rat sublingual gland (Slide XIVa) and rat distal colon (Slides XIVc and XIVd respectively) stain in varying shades of purple. Tissues with little unsubstituted sialic acid stain magenta (rat proximal colon (Slide XIVc) and rat distal colon (Slide XIVd)).

Illustration XV. Illustration of the saponification-selective periodate oxidation-borohydride reduction-Alcian blue pH 1.0-periodic acid-Schiff (KOH/PA*/Bh/AB1.0/PAS) procedure (Method 4). Sections of rat liver, salivary gland complex, rat colon and human colon were treated with the KOH/PA*/Bh/AB1.0/PAS technique. In this method, sulphate esters stain aqua and "neutral sugars" stain magenta. Tissues which contain only sialoglycoproteins such as the rat sublingual gland (Slide XVa) and crypt bases of the proximal colon (Slide XVb) and "neutral sugars" (liver (see XIIa), submandibular gland (Slide XVa) and parotid gland (not shown)) but no sulphate, stain magenta. Heavily sulphated epithelial sialoglycoproteins, as are found in human colon (not shown) and rat distal colon (Slide XVc), which also contain "neutral sugars", stain in varying shades of purple.

Illustration XVI. Illustration of the saponification-selective periodate oxidation-borohydride reduction-periodic acid-Schiff (KOH/PA*/Bh/PAS) procedure (Method 5). Sections of rat liver, salivary gland complex, rat colon and human colon were treated with the KOH/PA*/Bh/PAS procedure. In this method "neutral sugars" alone stain magenta. Since all tissues used contain "neutral sugars", they stain in varying intensities of magenta. Shown are rat submandibular and sublingual gland (Slide XVIa), rat proximal (Slide XVIb) and distal (Slide XVIc) colon and human colon (Slide XVId).

Illustration XVII. Evidence that exchange of Schiff reagents does not occur with Methods 1 and 2. Sections of human colon and rat salivary gland complex (containing submandibular and sublingual glands) were treated with a variation of Methods 1 and 2 in which a borohydride reduction step was placed between the final periodic acid and Schiff steps of method 1 (a) PA*/Bh/KOH/PA*/T/ KOH/Bh/PA/Bh/S and Method 2 (b) KOH/PA*/T/KOH/Bh/PA/Bh/S to prevent staining with pararosaniline Schiff. Only blue (thionin) staining was obtained illustrating that no exchange of Schiff reagent occurs in these procedures. A is illustrated with human colon (Slide XVIIa) and (b) with rat submandibular and sublingual glands (Slide XVIIb).

Illustration XVIII. Evidence that the position of the saponification (KOH)/borohydride sequence does not affect its ability to remove non-specific thionin Schiff staining. The KOH/Bh sequence is necessary for the removal of non-specific thionin Schiff staining and to prevent anomalous pararosaniline Schiff staining (Reid et al., 1984b), and is usually placed immediately following the thionin Schiff step (see Methods 1 and 2). This placement is not possible in Method 3 (PA*/T/Bh/PAS/KOH) since the KOH step would de-esterify 7- and 9-O-acyl sialic acids which would then be identified as "neutral sugars". As a result, the KOH step was placed following the pararosaniline Schiff treatment. To demonstrate that moving the position of the KOH step does not affect its ability to remove non-specific thionin Schiff staining, sections of rat colon were treated with a variation of Method 3 in which a borohydride step was placed between the PA_{60} and Schiff steps of Method 3 (PA*/T/Bh/PA_{60}/Bh/S/KOH) (Slide XVIII). Only thionin Schiff staining was obtained, illustrating the KOH step is still functional in its new position.

Illustration XIX. Evidence that non-specific thionin Schiff staining does not occur in Methods 1-3. Sections of human colon were treated with a variation of Methods 1, 2 and 3, in which a borohydride reduction step was placed between the periodate oxidation and thionin Schiff steps - Method 1 (PA*/Bh/KOH/PA*/Bh/T/KOH/Bh/PA/S - see Slide XIX); Method 2 (KOH/PA*/Bh/T/KOH/Bh/PA/S - not shown); and Method 3 ($PA^*/Bh/T/Bh/PA_{60}/S/KOH$ - not shown). Only magenta staining was obtained, indicating that non-specific thionin Schiff staining had not occurred.

List of Publications:

1. Volz, D.E., Reid, P.E., Park, C.M., Owen, D.A., Dunn, W.L. and Ramey, C.W. (1986) Can 'mild' periodate oxidation be used for the specific histochemical identification of sialic acid residues? Histochem. J. 18, 579-582.

2. Volz, D., Reid, P.E., Park, C.M. Owen, D.A. and Dunn, W.L. (1987a) A new method for the selective periodate oxidation of total tissue sialic acids. Histochem. J. in press.

3. Volz, D., Reid, P.E., Park, C.M. Owen, D.A. and Dunn, W.L. (1987b) Histochemical procedures for the simultaneous visualization of "neutral sugars" and either sialic acid and its side chain O-acyl variants or O-sulphate ester I. Methods based upon the selective periodate oxidation of sialic acids. Histochem. J. in press.

4. Park, C.M., Reid, P.E., Owen, D.A., Dunn, W.L. and Volz, D.E. (1987b) Histochemical procedures for the simultaneous visualization of neutral sugars and either sialic acid and its O-acyl variants or O-sulphate ester. II. Methods based upon the periodic acid-phenylhydrazine-Schiff reaction. Histochem. J. 19 in press.

5. Reid, P.E., Volz, D., Park, C.M., Owen, D.A. and Dunn, W.L. (1987) Methods for the identification of side chain O-acyl substituted sialic acids and for the simultaneous visualization of sialic acid, its O-acyl variants and O-sulphate ester. Histochem. J. 19, in press.

6. Park, C.M., Reid, P.E., Owen, D.A., Volz, D. and Dunn, W.L. (1987) Light microscopic histochemical studies of epithelial cell glycoproteins in normal rat colon. Submitted to Histochem. J.

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