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Periodic acid – Schiff – Alcian Blue: a method for the differential staining of glycoproteins

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Summary

The staining mechanism underlying the periodic acid–Schiff (PAS)–Alcian Blue (AB) sequence has been investigated using a variety of glycoprotein-containing tissues from different organs of the monkey, rat and mouse. The results obtained suggest that reactive carbohydrates contain at least three types of chemical end-groups found in neutral and acidic glycoproteins: (1) PA-engendered aldehyde groups coloured magenta by the Schiff reagent; (2) PA-engendered aldehyde groups coloured blue bisulphite-AB; and (3) naturally occurring acidic (carboxyl and/or sulphate) groups coloured blue by AB only. The PAS–AB sequence showed heterogeneity of glycoprotein structures in the conjunctiva and the duodenal goblet cells. Thus, the PAS–AB sequence is not the simple reverse sequence of AB–PAS but has its own definite and unique staining selectivity and can hence be used as a reliable method for the histochemistry of glycoproteins at the light microscope level.

Introduction

Since the combined AB–PAS staining was introduced into the light microscopic histochemistry of glycoconjugates this staining sequence has long been employed as a useful technique for the histochemical characterization of glycoproteins (Mowry, 1963). However, the reverse sequence of the dual staining, the PAS–AB procedure has not widely been used for similar purposes, possibly since its precise staining mechanism is unknown (Margolis, 1959). Thompson (1966) attempted to compare the results obtained by the original and reverse sequences of the dual staining and recognized that both sequences give rise to nearly the same results and preferred the original sequence as a reliable method of choice. However, this author's preference was not based upon theoretical experimentation. Johannes and Klessen (1984) have recently attempted to elucidate the mechanism underlying the reverse sequence of the dual staining and have suggested that the results obtained by the reverse sequence represent a secondary basophilia. According to the results of a previous histochemical experimentation using the rat and mouse conjunctival goblet cells (Yamabayashi & Tsukahara, 1987), however, the reverse sequence of the dual staining (PAS–AB) appeared to be of particular histochemical significance. In view of such conflicting concepts on the histochemical significance of the PAS–AB sequence, in the present study, attempts have been made to elucidate the precise mechanism underlying the particular sequence of the dual staining in a series of glycoprotein-containing tissues from various organs of certain mammalian species. The results obtained in the present study have shown that the PAS–AB sequence has definite and unique staining selectivities and is hence a promising means of histochemically characterizing glycoproteins in light microscopy.

Materials and methods

Male Japanese monkeys (*Macaca Fuscata*, 4 years-old, body weight 6–8 Kg), male Wistar rats (4–5 weeks-old, body weight 120 g) and male ddY mice (10-weeks-old, body weight 30 g) were used. Prior to killing, the monkeys, were anaesthetized by intramuscular injection of 5 mg/Kg of ketamine hydrochloride (Sankyo Pharmaceutical, Tokyo, Japan). All the animals whatever the species, were killed by intravenous injection of an overdose of pentobarbital (Abott Laboratories, North Chicago, Illinois, USA). Various glycoprotein-containing organs were dissected out, e.g. eye, duodenum and colon (ascending and descending colons and rectum). In the course of the removal of these organs, the eyes and associated conjunctival tissues were enucleated from the orbits. Tissue pieces from all the organs were immediately fixed in 0.1M phosphate-buffered 4% paraformaldehyde solution (pH 7.4) for 16–24 h at room temperature. To fix eye balls effectively, they were injected several times with

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fixative and then cut into halves along the meridional equator. After fixation, all the tissue pieces were dehydrated in a series of graded ethanols, cleared in xylene and embedded in Paraplast (Monoject Scientific Co., St. Louis, USA). From the Paraplast-embedded tissue blocks, serial sections of thickness 3 μm were cut on a sliding microtome, affixed on glass slides without adhesives, dewaxed in xylene, rehydrated through graded ethanols and then subjected to the following histochemical staining and experimental procedures.

A. Staining procedures:

(1) Periodic acid–Schiff (PAS) (after McManus, 1948).
   a) Oxidise for 5 min in 0.5% aqueous periodic acid.
   b) Wash in 3 changes of distilled water for 5 min each.
   c) Immerse in Schiff's reagent for 15 min.
   d) Wash in 3 changes of sulphurous acid baths for 2 min each.
   e) Wash in 3 changes of distilled water for 5 min each.

(2) Alcian Blue (AB), pH 1.0 (Lev & Spicer, 1964)
(3) AB pH 2.5 (Spicer *et al.*, 1967)
(4) High iron diamine (HID) (Spicer, 1965).
(5) AB pH 2.5–PAS (after Mowry, 1963), using the PAS procedure mentioned above.
(6) PAS–AB pH 2.5, the reverse of the previous sequence.

In addition to the full PAS–AB procedure, some sections were reacted for individual components of the PAS reagents such as periodic acid (PA), sodium bisulphite, hydrochloric acid (HCl) and a medium containing both HCl and bisulphite. PA or Schiff was by the same method that was used in the full PAS procedure. The bisulphite procedure was performed with 0.5% sodium bisulphite for 15 min 0.07 N hydrochloric acid was used for 15 min.

(7) HID–AB pH 2.5 (Spicer *et al.*, 1967).

Modifying reactions

In combination with the staining procedure above described, a series of selective methods were performed for experimentations. (The formulae of the combinations are shown in Tables in Results.)

B–1. Chemical modifications:

(i) Aldehyde detection with sodium bisulphite (BI). Sections were incubated in 0.5% sodium bisulphite solution for 15 min at room temperature.

(ii) Aldehyde reduction with sodium borohydride (BH) (a modification of Lillie & Pizzolato, 1972). Sections were immersed in 0.1% sodium borohydride in 1% bisodium hydrogen phosphate (pH 9.2) for 90 seconds at room temperature. The sodium borohydride solution was employed at 4 h following preparation.

(iii) Active and mild methylations (Spicer, 1960; Spicer *et al.*, 1967) (AM and MM). Sections were incubated in 0.1 N hydrochloric acid in methanol for 4 h at 60 (AM) or 37 (MM)° C.

B–2. Digestion with sialidase (Spicer & Warren, 1960) (Slase). Sections were incubated in 0.01 M phosphate buffer (pH 6.8) containing 1 U/ml of neuraminidase from *Arthrobacter ureafaciens* (Marukinshoyu Co. Ltd., Osaka, Japan) for 4 h at 37° C. As a control procedure for the enzyme digestion experiments, some sections were immersed in the buffer solution without enzyme under identical conditions of duration and temperature.

Results

Of all the glycoprotein-containing tissues involved in the three kinds of organs from three mammalian species, nine kinds of tissues were selected for microscopic observation and these were grouped into three categories; those which are known to be (1) sialoglycoprotein-, (2) sulphated glycoprotein- and (3) neutral glycoprotein-containing (Tables 1, 2 and 3). Some tissues of them were done according to the reports of Spicer (1960), Spicer *et al.* (1967) and Spicer *et al.* (1978). The term 'sulphated glycoprotein' means the glycoprotein which contains sulphated glycoprotein residue. Therefore, it includes sulphated sialoglycoproteins.

The effects of the reagents used in the PAS reaction on AB staining of different types of glycoproteins are summarized in Table 1. As is apparent in the table, all the sialoglycoprotein-containing histologic structures were coloured a vivid blue following differently combined components of PAS reagent-AB sequences. In contrast, the enzymatic removal of sialic acid residues from these glycoprotein-containing histologic structures resulted uniformly in negative reactions, except for those that had undergone PA–BI–AB or PA–HCl:BI–AB sequence following digestion with the enzyme, in which a vivid blue colouration persisted. All the sulphated-glycoprotein-containing histologic structures were found to be coloured a vivid blue following every type of combined staining sequences, and these results were never altered by digestion with sialidase prior to staining. In contrast, all the neutral glycoprotein-containing structures were found to exhibit negative reactions following the combined staining sequences, except for those subjected to either PA–BI–AB or PA–HCl:BI–AB, which showed intensely blue colouration. In the neutral glycoprotein-containing structures, digestion with sialidase prior to the staining sequences failed to induce any notable changes in the staining results obtained.

To determine the nature of alcianophilia obtained by the PAS–AB sequence, the effects of various experimental procedures upon the reactions for acidic groupings in different glycoproteins were tested and the significant results obtained are illustrated in Table 2.

As is noted in Table 2, every sialoglyprotein-containing histologic structure was found to stain in vivid blue with the HID–AB sequence (Fig. 1a). The

Table 1. The effects of separation of PAS reagents into their components upon the AB reactions of different types of glycprooteins.

Staining procedures	*Sialoglycoproteins*			*Sulphated glycoproteins*			*Neutral glycoproteins*		
	Monkey conj. gob.	*Monkey duodenum gob.*	*Mouse conj. gob.*	*Monkey colon gob.*	*Rat duodenum gob.*	*Mouse colon gob.*	*Monkey Brunner gld. aci.*	*Rat Brunner gld. aic.*	*Mouse Brunner gld. aci.*
Schiff–AB	3B	3B	3B	3B	3B	3B	0	0	0
HCl–AB	3B	3B	3B	3B	3B	3B	0	0	0
BI–AB	3B	3B	3B	3B	3B	3B	0	0	0
PA–AB	3B	3B	3B	3B	3B	3B	0	0	0
PA–HCl–AB	3B	3B	3B	3B	3B	3B	0	0	0
PA–BI–AB	3B	3B	3B	3B	3B	3B	3B	3B	2–3B
PA–HCl:BI–AB	3B	3B	3B	3B	3B	3B	3B	3B	3B
Slase–Schiff–AB	0	0	0	3B	3B	3B	0	0	0
Slase–HCl–AB	0	0	0	3B	3B	3B	0	0	0
Slase–BI–AB	0	0	0	3B	3B	3B	0	0	0
Slase–PA–AB	0	0	0	3B	3B	2B	0	0	0
Slase–PA–HCl–AB	0	0	0	3B	3B	3B	0	0	0
Slase–PA–BI–AB	2–3B	3B	3B	3B	3B	2–3B	2–3B	3B	2–3B
Slase–PA–HCl:BI–AB	3B	3B	3B	3B	3B	3B	3B	3B	3B

Explanations of tables. Numbers indicate the staining intensity on a subjectively estimated scale from 0 (unreactive) to 3 (most reactive).

Abbreviations: conj. = conjunctiva, gob. = goblet cell, Brunner gld aci. = Brunner's gland acini, AB = alcian blue (pH 2.5), PA = periodic acid, HCl = 0.05 N hydrochloric acid, BI = sodium bisulphite, HCl:BI = medium containing both HCl and sodium bisulphite, Slase = sialidase, HID = high iron diamine, MM = mild methylation, AM = active methylaltion, BH = borohydride, B = blue, DBr = dark brown, M = magenta, BP = blue purple, V = violet.

Table 2. The effects of various experimental procedures upon the reactions for acidic groupings in different glycoproteins.

Staining procedures	*Sialoglycoproteins*			*Sulphated glycoproteins*			*Neutral glycoproteins*		
	Monkey conj. gob.	*Monkey duodenum gob.*	*Mouse conj. gob.*	*Monkey colon gob.*	*Rat duodenum gob.*	*Mouse colon gob.*	*Monkey Brunner gld. aci.*	*Rat Brunner gld. aci.*	*Mouse Brunner gld. aci.*
AB (1.0)	0–1B	0	0	3B	3B	3B	0	0	0
HID–AB	3B	3B	3B	3DBr-B	3DBr–B	3DBr–B	0	0	0
HID	0–1DBr	0	0	3DBr	3DBr	3DBr	0	0	0
PA–BI–HID	3DBr	3DBr	3DBr	3DBr	3DBr	3DBr	3DBr	3DBr	3DBr
PA–BI–HID–AB	3DBr	2DBr	3DBr	3DBr	3DBr	2DBr	3DBr	3DBr	3DBr
PA–BI–MM–HID–AB	3DBr	2DBr	2DBr	3DBr	2DBr	2DBr	2DBr–B	1–2DBr	0
PA–BI–AM–HID–AB	0	0	0	0	0	0	0	0	0
Slase–HID–AB	2DBr	3DBr	3DBr	3DBr	3DBr	2DBr	0	0	0
Slase–HID	0	0	0	3DBr	3DBr	3DBr	0	0	0
Slase–PA–BI–HID	3DBr	3DBr	2DBr	3DBr	3DBr	3DBr	3DBr	3DBr	3DBr
Slase–PA–BI–HID–AB	3DBr	2DBr	2DBr	3DBr	3DBr	3DBr	3DBr	3DBr	2DBr
Slase–PA–BI–MM–HID–AB	2DBr	2DBr	2DBr	2DBr	2DBr	2DBr	2DBr	2DBr	0
Slase–PA–BI–AM–HID–AB	0	0	0	0	0	0	0	0	0

See Table 1 for explanation of Tables.

Table 3. The effects of BH treatment interposed between PAS and AB upon the reactions of different glycoproteins.

Staining procedures	*Sialoglycoproteins*			*Sulphated glycoproteins*			*Neutral glycoproteins*		
	Monkey conj. gob.	*Monkey duodenum gob.*	*Mouse conj. gob.*	*Monkey colon gob.*	*Rat duodenum gob.*	*Mouse colon gob.*	*Monkey Brunner gld. aci.*	*Rat Brunner gld. aci.*	*Mouse Brunner gld. aci*
AB	3B	3B	3B	3B	3B	3B	0	0	0
PAS	3M	3M	3M	3M	3M	3M	3M	3M	3M
AB–PAS	3BP	3V	3BP	3BP–V	3BP	3V–BP	3M	3M	2–3M
PAS–AB	3BP–M	3BP	3BP–M	3BP–V	3BP–V	3BP	3BP	3BP	2–3BP
PAS–BH–AB	3BP	2BP	3BP	3BP	2BP–V	3BP–B	2M	2M	2M
Slase–AB	0	0	0	3B	3B	3B	0	0	0
Slase–PAS	3M	3M	3M	3M	3M	3M	3M	3M	2M
Slase–AB–PAS	3M	3M	3M	3BP–V	3V	3BP–V	3M	3M	3M
Slase–PAS–AB	3BP	3BP	3BP	3BP–V	3V	3BP–V	3BP	3BP	3BP
Slase–PAS–BH–AB	2M	2M	2M	3BP	3BP	3BP	3M	3M	2M

See Table 1 for explanation of Tables.

PA–BI sequence prior to HID–AB procedure changed the blue colouration of the structure into a brown one (Fig. 1b). The mild methylation procedure interposed between PA–BI and HID–AB failed to alter the brown colouration of the structure (Fig. 1c), whereas the active methylation procedure interposed similarly abolished the brown colouration completely. Such stainabilities of sialoglyprotein-containing structures were nearly unchanged by digestion with sialidase prior to the staining sequences. Most of sulphated glycoprotein-containing structures were coloured a vivid brown following the HID–AB sequence, and such stainability of the structures were nearly unaffected by various experimental procedures combined with the particular sequence. The only exceptions for this were those structures subjected to either PA–BI–AM–HID–AB or Slase–PA–BI–AM–HID–AB, which exhibited negative reaction and some parts of colon subjected to HID–AB sequence which showed blue staining. All the neutral glucoprotein-containing histologic structures were found to exhibit negative reactions for the staining procedures for acidic groupings, however, the PA–BI sequence prior to these procedures induced a vivid brown colouration of the structures. The MM interposed between PA–BI and HID-AB procedures either suppressed significantly or abolished the brown colouration, whereas the AM interposed similarly abolished it. Digestion with sialidase prior to the staining sequences failed to bring about any alterations in the staining results obtained.

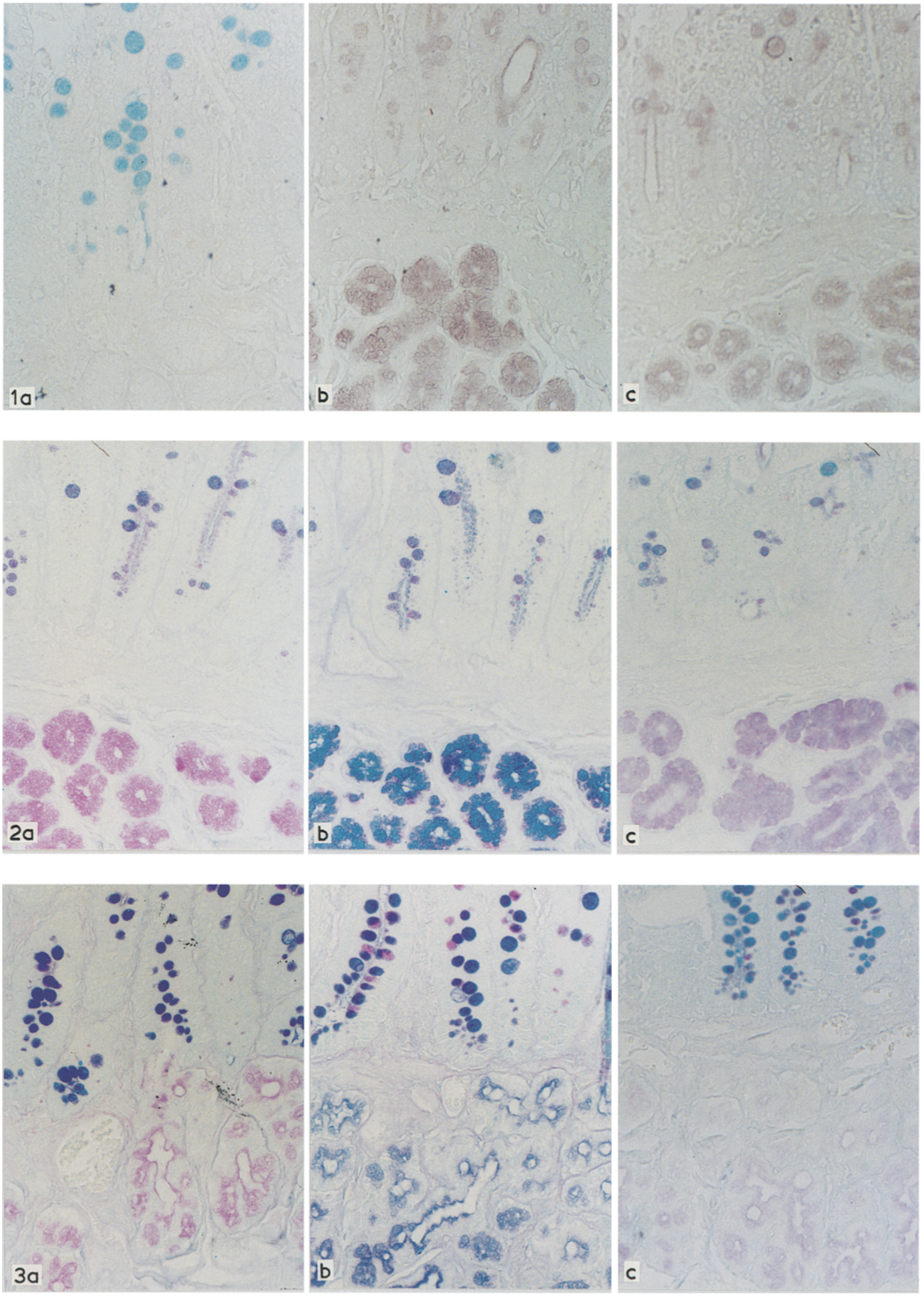
In Table 3, the effects of PAS–AB sequence and the effects of treatment with BH interposed between the PAS and AB procedures upon the reactions of different glycoproteins are summarized.

As is shown in Table 3, all the sialoglycoprotein-containing histologic structures were stained bluish-purple or violet with the AB–PAS procedure. The reverse sequence of the dual staining, the PAS–AB procedure gave rise to similar results. In some conjunctival goblet cells of the monkey and mouse, however, this sequence resulted in an intense magenta staining of sialoglyproteins involved. The enzymatic removal of sialic acid-residues led all the sialoglycoprotein-containing structures reacted with the AB–PAS procedure yielding a vivid magenta colouration. However, the same treatment resulted in an intense blue–purple staining of the structures stained with the reverse sequence, the PAS–AB

Fig. 1. Monkey duodenum. (a) Goblet cells are alcianophilic but acinar cells of Brunner's glands are negative. HID–AB. × 330. (b) Goblet cells stains brown by PA–BI and acinar cells show a positive HID reaction. PA–BI–HID–AB. × 330. (c) Mild methylation reduced slightly the intensity of HID reaction. PA–BI–MM–HID–AB. × 330.

Fig. 2. Monkey duodenum. This figure shows the effects of borohydride. (a) Most Goblet cells are alcianophilic but a few goblet cells show PAS positive. Acinar cells stains magenta. AB–PAS. × 330. (b) Duodenal goblet cells remained to stain in the same colour, bluish-purple, but acinar cells exhibited alcianophilia except a few PAS positive-cells at periphery acinus. PAS–AB. × 330. (c) A magenta colour reappeared in acinar cells and resulted in violet colour. Most goblet cells showed alcianophilia and a few cells show PAS-positive. PAS–BH–AB. × 330.

Fig. 3. Rat duodenum. The staining pattern was similar to that in Fig. 2. (a) AB–PAS. × 330. (b) Some duodenal goblet cells were stained magenta. PAS–AB. × 330. (c) A magenta colour reappeared in acinar cells and resulted in violet colour. But the colouration is weaker than that of Fig. 2c. PAS–BH–AB. × 330.


1a
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procedure. In addition, the BH reduction interposed between the PAS and AB procedures yielded magenta staining of the structures. The AB–PAS sequence was found to stain all the sulphated glycoprotein-containing histologic structures in an intense blue–purple or violet colour (Fig. 3a), and a nearly similar colour of the structures was obtained by the reverse sequence of the dual staining, PAS–AB (Fig. 3b). The BH reduction interposed between the PAS and AB procedures could not induce any notable changes in the staining obtained (Fig. 3c). The AB–PAS sequence stained all the neutral glycoprotein-containing structures in an intense magenta colour (Figs. 2a & 3a). Contrary to this finding, however, the reverse sequence of the dual staining (PAS–AB), gave rise to a vivid blue-purple colour of the structures (Figs. 2b & 3b), which was changed into a magenta colour, if the BH reduction was interposed between the PAS and AB procedures (Figs. 2c & 3c). Digestion with sialidase prior to the AB–PAS, PAS–AB or PAS–BH–AB sequence did not alter the staining from that seen in undigested sections.

Discussion

In the present study, the effects of separation of PAS reagents into their components upon the PAS–AB reaction of sialo- and neutral glycoproteins (Table 1) have proved to be useful for the precise analysis of the true chemical mechanism underlying the dual staining reaction. The enzymatic removal of sialic acid residues from sialoglycoproteins was found to abolish their intense alcianophilia throughout the different combinations of reagents of the PAS–AB procedure, except for the PA–BI–AB or PA–HCl:BI–AB methods. This appears to indicate that the BI step of these procedures could introduce acidic groupings into PA oxidized asialoglycoproteins, since AB is known to bind selectively to such groupings (Pearse, 1985; Spicer, 1960). Furthermore, all the different combinations of reagents of the PAS–AB procedures failed to yield any alcianophilia of neutral glycoproteins, except for the PA–BI–AB and PA–HCl:BI–AB sequences with or without prior sialidase digestion. These findings suggest that the BI step interposed between the PA and AB procedures can induce acidic groupings into neutral glycoproteins.

The effects of various experimental procedures upon the reactions of acidic groupings in sialo- and neutral glycoproteins (Table 2) appear to be useful for ascertaining the type of acidic groupings engendered by the BI step in PA oxidized saccharide moieties. It is generally accepted that in *vicinal* diols of saccharides PA oxidation engenders dialdehyde groups (Pearse, 1985), which are presumed to lead to the formation of sulphonate radicals when is added bisulphite (Puchtler *et al.*, 1961; Puchtler & Sweat, 1964; Morrison & Boyd, 1973; Pizzolato *et al.*, 1975). The validity of such presumption is thought to be substantiated not only by the positive HID reaction of sialo- and neutral glycoproteins after application of the PA–BI sequence but by the suppressive effects of active methylation upon the HID reaction.

In the course of the present staining of sialo- and neutral glycoproteins with the AB–PAS or PAS–AB sequences (Table 3), asialoglycoproteins were coloured with the former sequence an intense magenta colour, whereas the glycoconjugates being stained with the latter sequence were a vivid blue–purple colour. Such colourations, in particular those with the PAS–AB sequence, can be explained in terms of the concept that BI involved in the PAS reagent can produce sulphonate radicals in saccharide moieties of the glycoproteins.

In view of the known capability of BH to decompose bisulphite–aldehyde compounds (Lillie & Pizzolato, 1972; Morrison & Boyd, 1973; Lillie & Fullmer, 1976) and of the present effects noted, it is apparent that sulphonate groups are formed by reaction of PA-engendered aldehydes with bisulphite. From what has been discussed above, under the proper conditions it is certain that all the aldehyde groups engendered by PA oxidation in saccharide moieties of various glycoproteins do not necessarily react with Schiff reagent (leucofuchsin), and some of Schiff unreactive aldehydes are believed to react with bisulphite involved in the Schiff and rinsing solutions to form sulphonate groups (Morrison & Boyd, 1973), which can then be visualized by following staining procedures for acidic groupings such as AB and HID (Spicer, 1960; Spicer, 1965; Pearse, 1985).

The use of bisulphite compounds in other reported histochemical methods have been known. According to Puchtler *et al.* (1961), Puchtler & Sweat (1964) and Waldrop & Puchtler (1982), the periodic acid–sodium bisulphite–resorcin fuchsin sequence gave a positive reaction in the basement membrane. Moreover, Malinin (1970), Pizzolato *et al.* (1975), Romhányi *et al.* (1975), Fischer & Emödy (1976) and Makovitzky *et al.* (1983) reported using a PA–BI–Toluidine blue sequence. Johannes & Klessen (1984) compared the stainability of AB–PAS and PAS–AB methods and suggested the participation of bisulphite in the PAS–AB sequence.

If both the above concept as to the particular mechanism underlying the PAS–AB staining sequence and the traditionally established specificities of PAS and AB reactions are taken into consideration, at least three types of radicals of glycoconjugates can be visualized by means of the PAS–AB procedure; (1) some PA engendered Schiff-positive aldehydes (coloured magenta), (2) other PA engendered aldehydes react with bisulphite to form alcianophilic sulphonates (coloured blue), and (3) naturally occurring acidic

groupings (sulphate and/or carboxylated) (coloured blue).

There is another problem with the present results of sialo- and sulphated glycoproteins staining. Monkey and mouse conjunctival goblet cells and rat duodenal goblet cell stained blue–purple with the PAS–AB sequence. However, some conjunctival goblet cells and rat duodenal goblets stained magenta. This inconsistency cannot be explained completely by the concept mentioned above. In monkey and mouse conjunctival goblet cells, PAS–AB showed the same staining results as that of neutral glycoproteins after sialidase digestion. It suggests that there are differences in the structures of glycoproteins, especially in the location of sialic acids in glycoprotein structures. From the results of Bayliss and Adams (1976), this phenomenon may be due to the length of the treatment with Schiff reagent but the present study revealed a separation of stainability with the PAS–AB sequence. These inconsistencies will need more study in the future.

According to the results obtained in the present study and the generally accepted concept on the mechanism of PAS and AB reactions; the PAS–AB staining procedure does not yield staining artifacts. It is instead a reliable method involving a definite mechanism which can be subjected to theoretical analyses. In the light of the possible staining mechanism of the PAS–AB procedure, this dual staining can be postulated to be useful for the differentiation of varying structures of saccharide moieties in individual glycoproteins in light microscopy. For fully substantiating the reliability of this particular dual staining method, however, it needs to be applied not only to model carbohydrates of known chemical structures in a light microscopic histochemical system but to a variety of glycoprotein-containing tissue specimens using electron microscopy.

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