

**HISTOCHEMICAL ANALYSIS OF MUCINS IN EPITHELIAL
NEOPLASMS OF GASTROINTESTINAL TRACT**

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INTRODUCTION:

Mucosubstances or mucins as they are popularly called, represent an extremely heterogenous group of carbohydrates, which have a ubiquitous distribution in the glandular and mesenchymal tissues in the mammals. Biochemical and histochemical study of epithelial mucins in health and disease has been a subject of many publications. The subject is fascinating but so vast that only some aspects of mucin histochemistry in few human diseases will be dealt with in this work.

Mucins secreted in the various portions of the gastrointestinal tract have different functions to perform, of which protecting the delicate mucous membrane is the most important. Once the mucin barrier is disturbed or destroyed a variety of disease processes may be induced in the organs like stomach, gall bladder, intestines etc. Variations in the composition of mucosubstances have been demonstrated in the gastric contents of patients with chronic gastritis, pernicious anemia and gastric carcinoma. Similarly, significant changes in the chemistry of mucins have been reported in ulcerative colitis, Crohn's disease, polyps and carcinomas of large intestines. There are three basic varieties of mucin secreted in the normal

gastrointestinal tract, namely: neutral mucins [predominantly in stomach], sialomucins [predominantly in small bowel] and sulfomucins [predominantly in colon and rectum]. In the epithelial neoplasms of these organs, the mucin composition is often indifferent to that encountered normally. Moreover, the histologically identifiable precancerous lesions and even histologically normal appearing mucosa display qualitative and quantitative changes in the mucin structure. Since the fifties, a variety of histochemical methods have been developed, which can distinguish different types of mucins. In the recent times, immunohistochemical methods have also been described. However, these require expensive monoclonal antibodies specific for the type of mucin, whereas mucin histochemistry is more readily available and much cheaper. It is known that biochemical alterations often precede the appearance of precancerous lesions, which are routinely identified on light microscopy. Thus, in the field of gastrointestinal malignancy, which account for one of the commonest forms of cancers in humans, application of mucin histochemistry may throw more light on the subject and at practical level may allow early detection of malignant potential at a given site. The facility of mucin histochemistry can be further extended to identify the site of origin of a mucin secreting cancer, in cases presenting with metastatic lesion in lymphnode, lung, liver, bone, brain etc.

AIMS AND OBJECTIVES

- 1] To carry out a battery of histochemical stains to study mucin pattern in neoplasms of stomach and large bowel.
- 2] To assess the value of mucin histochemistry in identifying precancerous lesions of gastric and colonic mucosa.
- 3] To try to differentiate mucin secreting adenocarcinomas of lower gastrointestinal tract from those of other organs, especially in cases of metastatic adenocarcinoma with unknown primary.

REVIEW OF LITERATURE

MUCIN HISTOCHEMISTRY:

A thorough understanding of the biochemical properties and classification of mucins is necessary to appreciate the application of histochemistry in this field. There is, even today, a considerable confusion over the matter of terminology.

History:

The original term mucin was introduced by an American named Carpenter in 1846 ¹. Much later the term changed to 'mucins', as the complex nature of these substances became evident. Over the years, other terms evolved and these include mucopolysaccharides, glycosaminoglycans and mucosubstances ². Reid and Clamp introduced the term glyco-conjugates as a general name to replace the earlier ones and further subdivided the group into proteoglycans and glycoproteins ³. In simple terms, the mucins are hexosamine containing polysaccharides covalently bound to varying amounts of proteins. Spicer et al ² recommended that mucosubstances demonstrable in tissue sections should be named according to their localization and whether they are: neutral mucosubstances, mucopolysaccharides, sulfomucins or

sialomucins. Customarily, the mucosubstances in connective tissues are designated mucopolysaccharides; those in the epithelia are labelled mucins.

The study of mucins began in the middle of the nineteenth century. Mucin from cartilage was termed chondroitin sulphate by Fischer and Boedeker. Palmer and Meyer, in the thirties, isolated hyaluronic acid. Earlier staining techniques involved use of iodine or metachromatic substances. Carmine for glycogen was described by Best in 1806 and PASstain by McManus in 1946. Steedman invented alcian blue and Gomori discovered aldehyde fuchsin in 1950. To Spicer goes the credit of demonstrating the use of high iron diamine to differentiate different mucosubstances. ⁴

In this dissertation, the term mucin or mucosubstance will be used synonymously and will apply to epithelial mucins.

CLASSIFICATION OF MUCOSUBSTANCES ¹ :

NEUTRAL MUCINS: There are no subdivisions in this group.

ACID MUCINS :

Strongly Sulphated

a] connective tissue

b] epithelial

Weakly Sulphated

a) enzyme labile [N-acetyl form]

b) enzyme resistant [N-acetyl-O-acetyl form]

Sulphated Sialomucin .

STAINING METHODS & THEIR INTERPRETATION ⁶

Method	Interpretation
Diastase PAS	Magenta : neutral mucins some sialomucins Negative : sulphomucins O-acetyl sialomucin
PB-KOH-PAS	Magenta : O-acetyl sialomucin Negative : all other mucins
Alcian Blue [pH 2.5] PAS	Blue : sulphomucin O-acetyl sialomucin Purple: acid & neutral mucins Magenta: neutral mucins

HID-AB [pH 2.5]

Brown : sulphomucins

Blue : sialomucins

MUCIN HISTOCHEMISTRY IN LESIONS OF STOMACH

Normal Gastric Mucosa:

In 1968, Goldman and Ming ⁶ published their study of mucins in normal and neoplastic gastrointestinal epithelium. The study demonstrated that most of the normal gastric mucosal cells in man contain neutral mucin only. The mucin in the surface and foveolar cells has been categorized as a fucomucin, which apparently represents the major component of mucous barrier to autodigestion. In the deeper foveolar cells, acid mucins can be demonstrated, which are of non-sulphated variety i.e sialomucins.

Intestinal Metaplasia [IM] :

Intestinal metaplasia in gastric mucosa is usually a highly differentiated epithelium resembling small intestine as indicated by mucin and enzyme histochemistry and ultrastructural studies ^{7,8}. Intestinal metaplasia is generally considered a premalignant lesion ⁹. Morson ¹⁰ reported 5 cases of gastric carcinoma which were morphologically speculated to arise from metaplastic epithelium. Since ¹¹ Lauren divided gastric carcinoma into diffuse and intestinal types, comparative studies of the two types have

been actively carried out. These demonstrated morphologic and histochemical evidences that the intestinal type of carcinoma was closely associated with intestinal metaplasia but the diffuse type was not. However, the common occurrence and its presence in benign and malignant conditions, limits the value of intestinal metaplasia as indicator of gastric cancer risk. It is now appreciated that all cases of intestinal metaplasia do not possess malignant potential. Some of the Japanese workers, using histochemical techniques, described two types of IM : complete and incomplete ^{12,13}. There are many excellent articles dealing with the study of intestinal metaplasia by mucin histochemistry and relation of the subtypes to development of overt malignancy ^{14,15,16,17,18}.

IM Type I [complete] This type of intestinal metaplasia resembles small intestinal mucosa, although villi are rarely well developed and the ratio of goblet cells to absorptive cells is variable. Mucin histochemistry resembles that of small intestine with N-acetyl predominating. O-acetyl sialomucin is in small quantity and sulphomucin rarely present.

IM Type IIa [incomplete] This comprises goblet and columnar mucous cells, the former secreting N-acetyl sialomucin and occasionally sulphomucin but unlike in Type I **NO** O-acetyl sialomucin is present.

IM Type IIb [incomplete] The goblet cells are again bordered by columnar cells instead of absorptive cells. However, the columnar cells secrete marked sulphomucins and can be distinguished only by special stains from adjacent intestinal goblet cells, which produce sialomucin. On routine H & E stain this epithelium resembles hyperplastic colonic mucosa. Hence the term colonic metaplasia is often used. Following is the mucous content of these three types of intestinal mucosa:

Type I : N-acetyl sialomucin predominate [minimal O-acetyl sialomucin]

Type IIa : neutral mucins + N-acetyl sialomucin

Type IIb : sulphomucins + neutral mucins + N-acetyl sialomucin

All workers in the field have observed that intestinal metaplasia of incomplete type [IIb] often precedes intestinal type of gastric adenocarcinoma.

Huang et al ¹⁸ studied 115 gastric biopsies by mucin histochemistry and found that incidence of sulphomucin colonic intestinal metaplasia [Type IIb] was much higher in gastric carcinoma than in benign lesions.

Iida and Kusuma ¹² studied 34 resected specimens of gastric carcinoma and revealed that Type IIb intestinal metaplasia was associated with dysplasia and well differentiated adenocarcinomas of smaller size.

Filipe et al ¹⁷ carried out a joint prospective long term study of gastric biopsies to find out the incidence, distribution and premalignant status of all types of intestinal metaplasia. Type I accounted for 73% of all 267 biopsies showing IM and was associated with gastric ulcer in 70% and chronic gastritis 76%. Type III or incomplete sulphomucin secreting intestinal metaplasia accounted for only 9.8% of all IM biopsies but was associated with large number of gastric carcinomas. The authors suggested that intestinal metaplasia types may have different malignant potential and their identification may be useful in screening patients for early detection of cancer.

Gastric Adenoma:

Gastric adenoma, comprising about 10% of all gastric polyps, has been implicated in the genesis of gastric adenocarcinoma and is widely believed to be a precancerous condition ^{19,20,21}. Gastric adenoma is histogenetically related to intestinal metaplasia and presence of sulphomucin has been reported in gastric adenomas. ^{22,23}

Kolodziejczyk et al ²⁴ carried out a long term follow-up study of gastric adenoma by immunohistochemical and histochemical methods and concluded that mucin histochemistry is of value in assessing the malignant potential of adenomas. Whereas, immunoexpression of c-erbB-2, bel-2, CA 19-9 and CEA have only limited value.

MUCIN HISTOCHEMISTRY IN LESIONS OF LARGE INTESTINE

Filipe has been closely identified with mucin histochemistry of gastrointestinal tract and published [1969] a very comprehensive account of value of histochemical reactions for mucosubstances in the diagnosis of certain pathological conditions of the colon and rectum ²⁵. She described that normal colonic mucosa contained predominantly sulphated mucosubstances in the lower third to whole crypt. Non-sulphated acid mucins usually resided in upper part of the crypt. Small amount of neutral mucin was found in the surface epithelium and in the upper crypt. This basic knowledge came very handy in interpreting mucin changes in diseased mucosa. At the edge of the tumor, histologically normal appearing colonic mucosa revealed gradual decrease of sulphated mucin, which then was substituted by a non-sulphated product. This was termed the "transitional" mucosa. Finally, in the "transitional" mucosa close to the tumor all goblet cells mainly contained non-sulphated mucins [sialomucin]. Chemical studies confirmed this latter observation. ²⁶

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Filipe ²⁷ subjected 121 rectal biopsies from 99 patients with colorectal carcinoma to high iron-diamine Alcian blue [HID-AB] technique, in which sulphomucins stain brownish-black and sialomucins blue. The histologically normal mucosa adjacent to carcinoma ["transitional" mucosa] revealed an increase in sialomucins [blue] and decrease or absence of sulphomucins in 60 to 90% of the cases depending upon the type of tumor. Similar changes were seen around adenomatous and papillary tumors. The metaplastic polyps did not show this change in mucin pattern. The author recommended the HID-AB stain, because of its specificity, consistent results, and easy technique for routine use in the diagnosis of premalignant changes.

Filipe & Branfoot in 1974 ²⁸ further studied the abnormal patterns of mucous secretion in apparently normal mucosa of large intestine with carcinoma. They showed increase in sialomucins in patches of mucosa at a distance from frank tumor and also revealed that increase in the amount of sialomucin secretion proportional to the degree of invasion according to Duke's classification.

Culling et al ²⁹ developed a new histochemical technique to differentiate mucin secreted by adenocarcinomas of lower gastrointestinal tract from that secreted by adenocarcinomas from other visceral organs. Culling, Reid and Dunn in 1971 ³⁰ showed that in certain defined areas of gastrointestinal tract, there is an increase in PAS reactivity following treatment with potassium hydroxide [KOH], and that this effect is due to the removal of O-acetyl esters from the side chains of sialic acids in epithelial

mucins. In this technique called PB-KOH-PAS, periodate oxidation followed by reduction with sodium borohydride abolished the normal PAS reactivity of the tissue. Subsequent saponification breaks the masking O-acetyl bonds, thus exposing vic glycols, which react with PAS. Thus, only O-acetylated sialomucins will be stained magenta. In this study, the primary tumors of organs other than lower gastrointestinal tract or their metastases were negative for PB-KOH-PAS. This finding has application in determining source of metastatic adenocarcinoma in lung, lymphnode, liver etc, when primary is dormant clinically.

Culling and others in 1977 ³¹ introduced a new method, similar in principle to PB-KOH-PAS, but using periodic acid thionin Schiff before KOH-PAS sequence. Following are the staining characteristic for different conditions of the colon with this new PAT-KOH-PAS:

Normal colon	Red
Benign lesions	Red
All adenocarcinomas	Blue
Poorly & moderately differentiated carcinomas	Blue
Well differentiated carcinomas	Blue-Purple
Malignant villous adenomas	Purple

The change from red to blue colour, according to the authors indicates reduction in the degree of side chain O-acetylation.

It should be noted that the above technique stains normally PAS positive material blue while KOH-PAS reactive tissue components [O-acetylated sialic acids] show as red and mixture purple.

PBT-KOH-PAS : red colour indicates substitution at C7 &or C8

PAT-KOH-PAS : red colour indicates substitution at C8

PAT-KOH-PAS : blue colour indicates substitution at C7 or C9

It was evident that in atypical adenomas blue staining with PAT-KOH-PAS indicated strong evidence of malignant change and this is further supported by negative staining with PB-KOH-PAS. This in chemical terms means that O-acetylated sialic acid are reduced, when malignant change supervenes the adenoma. These observations were further supported by the excellent work of Agawa and Jass in 1990 ³², in which a change in sialic acid, notably loss of O acetyl substitution, was demonstrated in a series of 93 colorectal adenomas that included 14 malignant polyps. These findings are of great significance, since it is accepted that most , if not all, adenocarcinomas of the large bowel arise within pre-existing adenomas. It is known that malignant change in an

adenoma shows important association with size, histological type and grade of epithelial dysplasia, ^{33,34}. Of these factors, grade of epithelial dysplasia is considered to be the most selective risk factor. Indirect evidence in support of this feature now comes from studies of DNA content and identification of mutations by molecular biological methods ^{35, 36}.

The reduction of mucin staining that accompanies increasing epithelial dysplasia is well known and was also shown by Griffioen et al ³⁷, who found a mixture of sulphomucin and sialomucin or slight predominance of sulfomucins in normal colonic mucosa and a shift to sialomucin in colorectal adenomas. It has been noted that HID-AB stain has not disclosed any change in the brown:blue [sulphomucin:sialomucin] ratio in relation to dysplasia. However, loss of O-acetyl sialic acid has been previously demonstrated by Culling et al ³¹. The loss of O-acetyl substitutes may be accompanied by the retained expression of N-acetyl sialic acid. Agawa and Jass ³² showed that qualitative change in the structure of sialic acid [O to N acetyl sialic acid] occurs in tandem with increasing grades of epithelial dysplasia within a series of colorectal adenomas. All malignant adenomas revealed intraluminal mucous stained blue with PAT-KOH-PAS and positive reaction with mPAS [staining N-acetyl sialic acid]. These authors emphasized that an abundant luminal accumulation of altered sialic acid in association with an epithelial lining that was non-goblet, was specific for cancer. Conversely, the presence of unchanged intracellular or intraluminal O-acetyl sialic acid may help exclude a diagnosis of malignancy in equivocal cases. Thus loss of O-acetyl sialic acid is a

sensitive marker for malignant change in an adenoma. Eide et al ³⁸ had also documented partial loss of O-acetyl substitution in moderately dysplastic adenomas and showed that the absence of this substance was highly predictive of severe dysplasia.

MUCIN HISTOCHEMISTRY AT SITES OTHER THAN LOWER GI TRACT

PANCREAS: Matsuzama et al ³⁹ carried out histochemistry for mucins in normal, metaplastic, and hyperplastic ductal epithelium as well as in carcinomas of pancreatic tissue. Thirty five cases of common type of pancreatic adenocarcinoma and nine mucin secreting adenocarcinomas were studied. The cells of mucinous metaplasia and epithelial hyperplasias revealed gastric type of mucins and organoid differentiation simulating gastric pyloric mucosa but never stained for 8-O-acetylated sialomucin. The common type of pancreatic carcinomas produced mucins of gastric and small intestinal type, but rarely 8-O-acetylated sialomucins. Cells of mucin secreting carcinoma produced 8-O-acetylated sialic acid, which is a marker for colonic mucosa.

URINARY BLADDER : Glandular metaplasia of intestinal type has been described in urothelium as a consequence of chronic irritation. The metaplastic response is characterized by colonic type of goblet cells producing sulphomucins and O-acetylated

sialomucins. In the absence of chronic infection, replacement of urothelium by intestinal mucosa is rare, but carries high risk for the development of cancer ⁴⁰.

Newbould & McWilliam ⁴¹ applied mucin histochemistry to study vesical adenocarcinoma, intestinal metaplasia and related lesions. Intracellular mucins expressed in five of the 17 cases of cystitis glandularis and in all of eight cases of frank intestinal metaplasia with goblet cells displayed colonic phenotype, with production of O-acetylated sialomucins. A similar profile was expressed by all 6 cases of adenocarcinomas, which included tumors of probable vesical and urachal origin. It was concluded that identification of O-acetylated mucins cannot distinguish between primary bladder tumors and metastases from colonic primary or between carcinomas of vesical and urachal origin.

URETER : Ureterosigmoidostomy has long been used in the treatment of patients with both malignant and non-malignant diseases of the bladder. The development of colonic carcinoma at the site of reimplantation of the ureters has frequently been reported as a late complication of this operation. In cases of uretero-sigmoidostomy, the colonic mucin secretions are greatly altered and in one study ⁴² involving staining of colonic mucosa with hige-iron-diamine and alcian blue method revealed significant increase in proportion sialomucins [alcian blue positive]

OVARY: The mucinous tumors of ovary can be subdivided into intestinal and endocervical types according to the type of mucin secreted. The latter type has mucin composition similar to endocervix with abundant neutral and acidic mucins and a slight predominance of sialomucins over sulphomucins. The intestinal types of tumors showed mucin histochemistry similar to that of gastric mucosa with varying degrees of intestinal metaplasia ⁴³.

IMMUNOHISTOCHEMISTRY IN THE STUDY OF GI MUCINS

This method has not been employed in the present work. However, a brief reference may be made to this subject to understand its potential and future scope. In a recent study of 219 primary gastric cancer, mucin histochemistry and immunohistochemistry using monoclonal antibody to mucin associated sialosyl-Tn antigen was carried out. It was concluded that this was an excellent marker of small intestinal mucins and is indicative of a small intestinal type of differentiation in many cases of gastric cancer ⁴⁴.

Milton et al ⁴⁵ developed a mouse monoclonal antibody following immunization with highly purified human colonic mucin. The antibody called MMM-17 proved to have specificity for O-acetylated sialomucins. The results supported the previous reports of reduced O-acetylation in colonic cancer and neo-formation of this mucin in intestinal metaplasia in the stomach.

MATERIALS AND METHODS

A five year study between 1990 to 1995 was conducted. Adenocarcinomas of stomach and large bowel, polyps of colon and a miscellaneous group of mucin containing neoplasms occurring elsewhere in the body were subjected to mucin histochemistry and following stains were employed:

ALCIAN-BLUE PAS [pH 2.5] : neutral and acid mucins

HID-AB [pH2.5] : acidicsulphated& nonsulphated mucins

PB-KOH-PAS : O-acetylated silomucins

In all, 39 formalin fixed tissue specimens were studied, the paraffin blocks being collected from the files of Surgical Pathology section in a teaching institute.

In each case of gastric and colonic carcinomas, 3 sections were stained by the above methods, in addition to routine PAS and H & E stain. One section was from the tumor, one from the adjacent mucosa and one from an area at a distance from the tumor. In the miscellaneous lot, only the sections bearing the lesion were stained by the various mucin stains.

Following Table lists the number and histological types of cases:

TABLE I

DIAGNOSIS	NO OF CASES
Adenocarcinomas of Stomach	9
Adenocarcinomas of cecum & ascending colon	3
Adenocarcinomas of sigmoid and rectum	6
Adenomatous polyps	5
Hyperplastic polyp	1
Juvenile polyp	1
Villous adenoma	1
TOTAL GASTRO-INTESTINAL	26
Adenoid cystic carcinoma of salivary gland	1
Mucoepidermoid carcinoma of salivary gland	1
Adenocarcinoma of pancreas	1
Adenocarcinoma of gall bladder	1
Bronchiolo-alveolar carcinoma	2
Metastatic carcinoma in lung [colon primary]	1
Metastatic carcinoma in lymphnode [stomach primary]	1
Metastatic ovarian mucinous carcinoma in bladder	1
Colloid carcinoma of breast	1
Myxoid plexiform neurofibroma of foot	1
TOTAL	11
GRAND TOTAL	37

The positivity of mucosubstance by each stain was assessed qualitatively and graded from + to +++++. Intracellular positivity of a mucosubstance was given more importance than positive staining encountered in the extracellular location.

The type of coloured product expected for a positive result is given below.

METHOD FOR ALCIAN BLUE-PAS [2.5 pH]

i] Alcian blue 1 g
acetic acid [pH 2.5] 100 ml

ii] Schiff's reagent

1. Deparaffinize sections and bring to water
2. Alcian blue solution, 5 minutes
3. Wash in water, then in distilled water
4. 1% aqueous periodic acid, 5 minutes
5. Rinse well in distilled water
6. Schiff's reagent, 15 minutes
7. Wash in running tap water, 5-10 minutes
8. Harris hematoxyline counterstain, differentiate and blue
9. Rinse in water
10. Dehydrate, clear and mount

Results: Acid mucins : blue
 Neutral mucins : magenta
 Nuclei : pale blue

METHOD FOR HID-AB [pH 2.5]

Solutions:

N,N-Dimethyl-metaphenylenediamine dihydrochloride	120 mg
N,N-Dimethyl-paraphenylenediamine dihydrochloride	20 mg
Distilled water	50 ml
Ferric chloride [60% BDH solution]	1.4 ml

The reagent to be made freshly.

1. Deparaffinize section and bring to water
2. Treat the section with the diamine solution for 18-24 hrs
3. Wash well in running water
4. Counterstain with 1% Alcian blue in 3% acetic acid for 5 minutes.
5. Dehydrate, clear and mount.

Results: sulphated mucins : brownish-black
 sialomucins : blue
 nuclei : red

METHOD FOR PB-KOH-PAS:

Solutions:

Sodium Borohydride Solution

Sodium Borohydride	0.1 g
Disodium hydrogen phosphate	1.0 g
Distilled water	100 ml

Saponification

Potassium hydroxide	0.5 g
Ethanol	70 ml
Distilled water	30 ml

1. Deparaffinize sections and bring to water
2. Treat sections with 1% periodic acid for 30 minutes
3. Wash well in distilled water
4. Treat with borohydride solution for 30 minutes
5. Wash in running water and then in 70% alcohol
6. Treat with potassium hydroxide solution for 30 minutes
7. Wash with 70% alcohol and distilled water

8. Treat with 1% periodic acid for 5 minutes
9. Wash in distilled water followed by Schiff's, 15 minutes
10. Wash in running water followed by Harris hematoxyline
11. Dehydrate, clear and mount

Results: O-acetylated Sialomucins : magenta
[sialidase resistant]

Neutral Mucins : negative

OBSERVATIONS & RESULTS

TABLE II **CARCINOMA OF STOMACH**

+ Faintly positive ++Minimally positive +++Moderately positive ++++Strongly positive

CASE	AB-PAS	PB-KOH-PAS	HID-AB
2380-91 IM	++	+++	-
[M.D.] Tumor	-	+	-
1114-95 IM	++	+++	-
[M.D.] Tumor	+	+	++
2698-95 IM	+	+++	+
[M.D.] Tumor	+++	+++	++
1377-95 IM	+++	++	+
[W.D.] Tumor	-	-	-
1963-95 IM	++	+++	+
[P.D.] Tumor	-	-	-
1505-95 IM	+++	+++	-
[P.D.] Tumor	++	++	-
3058-90 IM	++	++	+
[P.D.] Tumor	+++	+++	++++
1144-89 IM	-	-	-
[P.D.] Tumor	-	-	-
309-93 IM	-ve	-ve	-ve
[M.D.] Tumor	-ve	-ve	-ve

The last two cases in this table did not stain with any of the three methods.

In 7 cases, the goblet cells of intestinal metaplasia stained a pleasant blue with AB-PAS stain and in 4 the tumor was also positive [Figs: 1,2 & 5]. Two of these gastric carcinomas were moderately differentiated and two poorly differentiated. In all 7 cases,

the PB-KOH-PAS vividly stained [red] the intestinal metaplasia goblet cells and focal to moderate positivity was encountered in 5 gastric carcinomas, 3 well to moderately differentiated and 2 poorly differentiated [Figs:3,4,6,7,10 & 11]. As to the HID stain, sparse staining was found in intestinal metaplasia in 4 cases while tumor cells were positive in 2 moderately and 1 poorly differentiated carcinomas [Figs: 8,9 & 12].

TABLE III **CARCINOMA OF COLON**

+ Faintly positive ++Minimally positive +++Moderately positive ++++Strongly positive

CASE	AB-PAS	PB-KOH-PAS	HID-AB
1500-95 Tr	++	-	++
[RC,MD] Tu	+	-	-
2068-95 Tr	+++	+++	+
[RC,WD] Tu	-	-	-
892-94 Tr	++	++	+
[RC,WD] Tu	-	+	-
894-95 Tr	++	++	-
[LC,MD] Tu	-	+	-
1004-95 Tr	++	+	-
[LC,PD] Tu	-	+	-
1470-95 Tr	-	+	++
[LC,MD] Tu	-	+	+++
1050-90 Tr	++	+++	+
[LC,PD] Tu	-	+	+
2379-91 Tr	++++	+++	-
[LC,WD] Tu	-	-	+
326-91 Tr	++	-	-
[LC,MD] Tu	+	-	-

RC: right colon LC: left colon Tr: transitional mucosa
Tu: tumor

In the normal sigmoid mucosa, sulphomucin [dark brown] was predominant mucosubstance in the goblet cells [Figs: 13 & 14] while sialomucin [blue] was more predominant in the cecal mucosa [Fig 15]. In the transitional mucosa around the colonic and rectal carcinomas, alcian blue staining [sialomucin] was overwhelmingly positive and sulphomucin hardly seen. This was particularly evident around recto-sigmoid carcinomas [Fig 16]. O acetylated sialomucin was strongly positive in transitional mucosa in 7 instances [Figs 17 & 19] and in 5 of these, sparse sialomucin was present. In one case the tumor contained mucin positivity only in the lumen [Figs:18 & 20]

TABLE IV

COLONIC POLYPS

+ Faintly positive ++Minimally positive +++Moderately positive ++++Strongly positive

CASE	AB-PAS	PB-KOH-PAS	HID-AB
265-95 Adenoma	++	-	+
1501-94 Adenoma	++	+	+
680-95 Adenoma	+++	-	++
3306-90 Adenoma	+++	-	++
1116-95 Adenoma	+	-	-
2826-95 Hyperpla polyp	+++	+	++
1997-95 Juvenile polyp	-	-	+
268-95 Villous adenoma	+++	-	-

All adenomatous polyps had sialomucin and sulphomucin distribution similar to that in normal colon [Figs: 21 & 22]. In 1 adenomatous polyp small quantity of O acetylated sialomucin was detected. In only case of hyperplastic polyp both sialomucin and sulphomucin were present. On the other hand, villous adenoma contained only sialomucin. The juvenile failed to stain normal colonic mucin pattern.

TABLE V MISCELLANEOUS

CASE	AB-PAS	PB-KOH-PAS	HID-AB
2375-92 Adenoid cystic, salivary	-	- *	-
2720-93 Muco-epi dermoid, parotid	-	-	-
1637-93 Adenoca pancreas	++	+	-
670-95 Adenoca, gall bladder	++	-	-
59-94 Met Adeno lung from colon	-	+++	+
67-92 Bronchio alveolar, lung	-	+ **	- ***
1230-90 Bronchio -alveolar, lung	-	-	-
477-95 Met Adeno node from stomach	++	++	-
583-95 Mucinous carcinoma bladder	-	++	++
745-95 Colloid ca breast	+	-	-
2166-87 Myxoid neurofibroma	-	-	-

* normal salivary acini +ve for PB-KOH

** normal bronchial glands +ve for PB-KOH

*** normal bronchial cartilage +ve for HID-AB

The mucin secreting salivary tumor did not stain by any of the three methods [Fig 24]. The normal salivary tissue contained O acetylated sialomucin [Fig 25: red with PB-KOH]. Adenocarcinomas of pancreas and gall bladder presented only sialomucins positive with AB-PAS stain.

Metastatic adenocarcinoma in lung [primary in colon] metastatic adenocarcinoma in a supraclavicular lymphnode [gastric primary] stained brightly for O acetylated sialomucin [Fig 23]. In two cases of bronchiolo-alveolar carcinomas of lung, all three mucin stains were negative. However, the bronchial cartilage and mucinous cells in the bronchial mucosa, both stained strongly with HID [FigS 26 & 27]. The bronchial glands were strongly positive with PB-KOH stain. Thus, the built in controls were positive, proving the veracity of the stains. The mucin secreting adenocarcinoma of urinary bladder revealed both sialomucin and sulphomucin on HID stain [Fig 28]. In the case of colloid carcinoma of breast HID and PB-KOH stains were negative, while AB-PAS showed purple staining [Fig 29]. In myxoid neurofibroma, all mucin stains were negative.

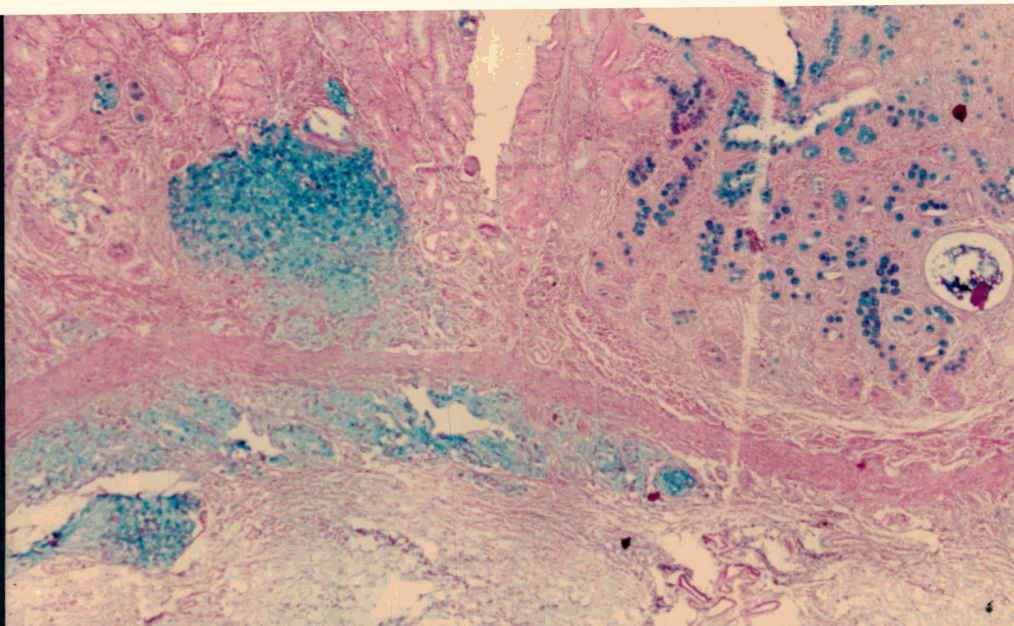


Fig 1. Blue staining goblet cells [left] & mucin pools
[arrows] in carcinomatous foci AB-PAS X 40
CASE 1505-95

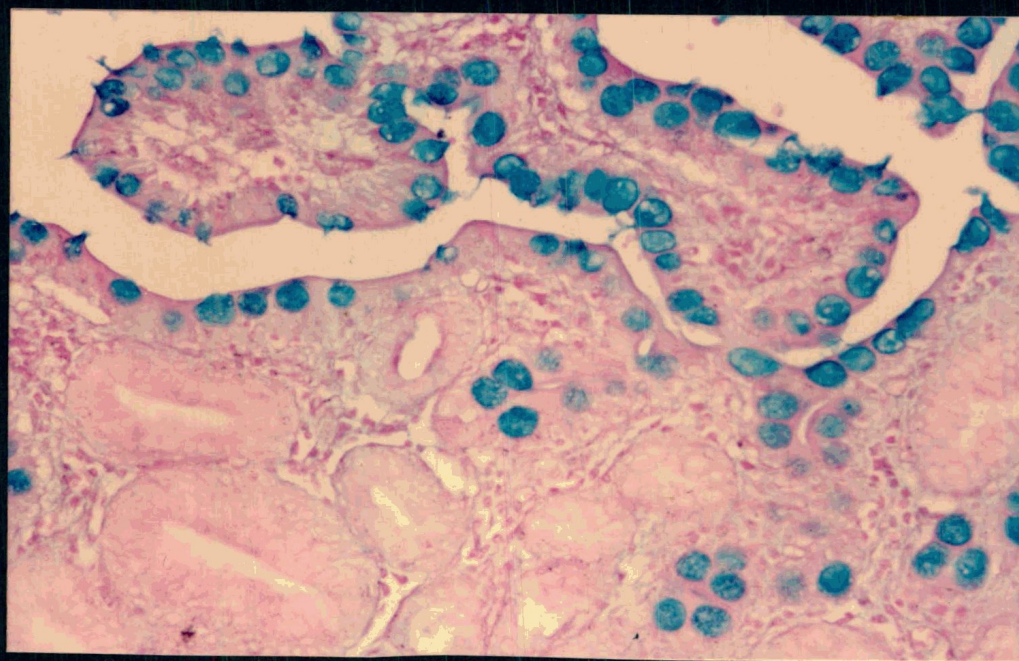


Fig 2. Blue staining goblet cells of intestinal
metaplasia AB-PAS X 20 CASE 1505-95

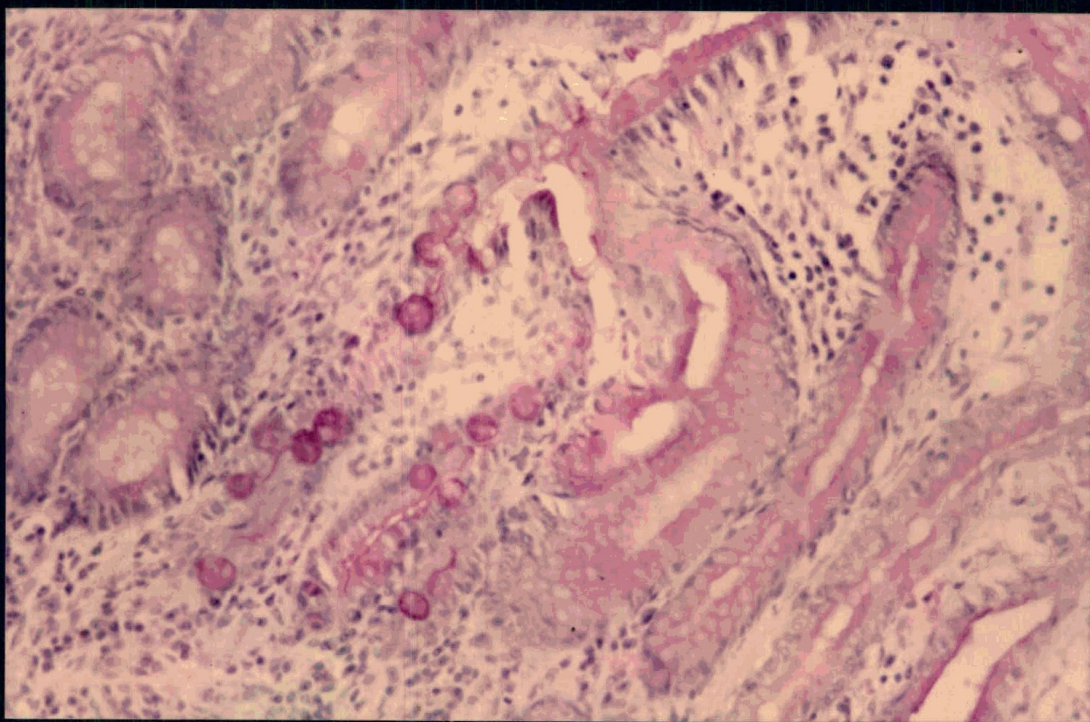


Fig 3. Few red staining goblet cells of intestinal metaplasia PB-KOH-PAS X 200 CASE1505-95

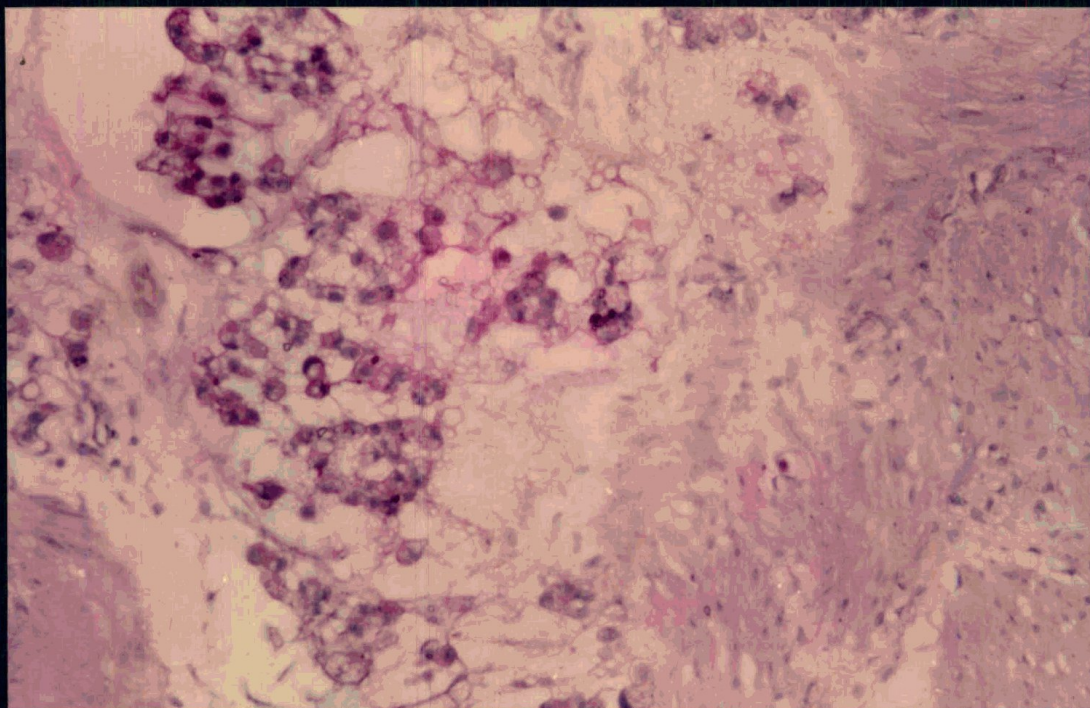


Fig 4. Minimal red staining poorly differentiated carcinoma cells PB-KOH-PAS X 200 CASE 1505-95

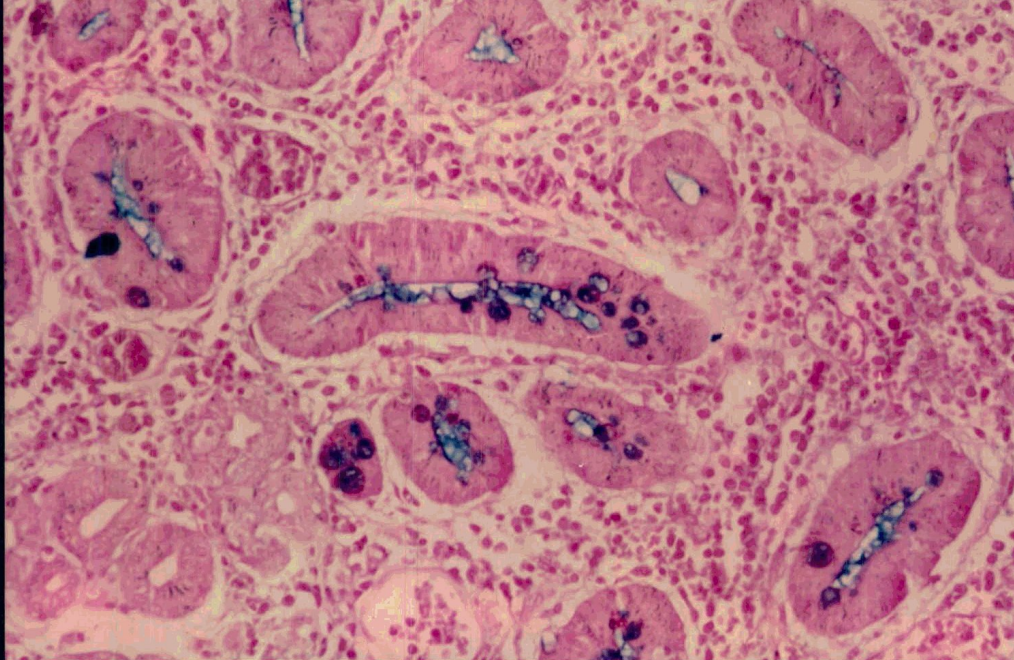


Fig 5. Few blue staining goblet cells of intestinal metaplasia AB-PAS X 200 CASE 1114-95

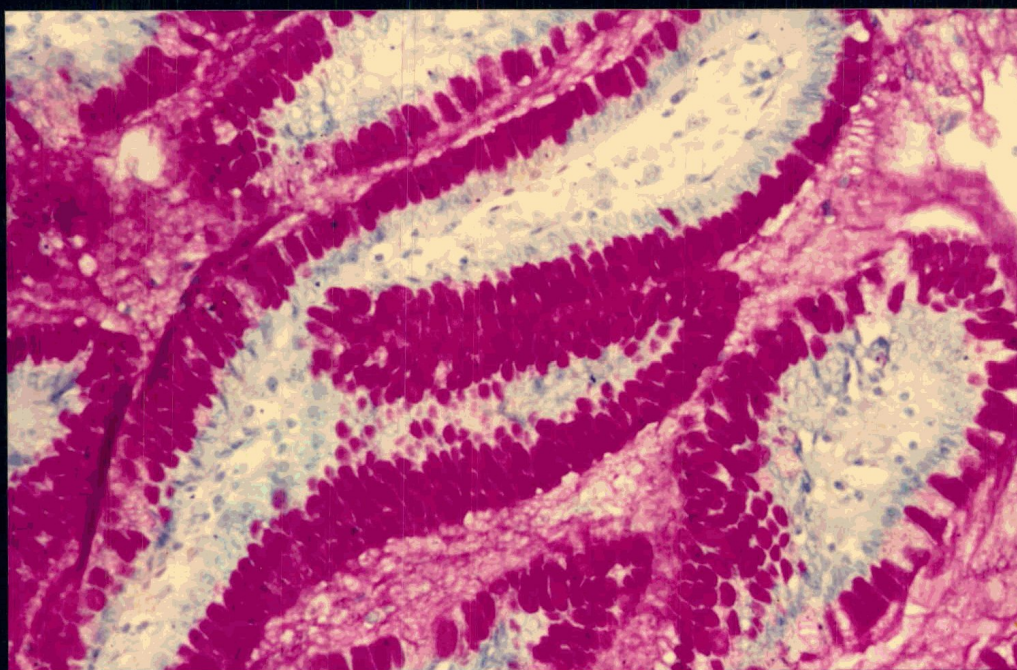


Fig 6. Extensive bright red staining of goblet cells PB-KOH-PAS X 200 CASE 1114-95

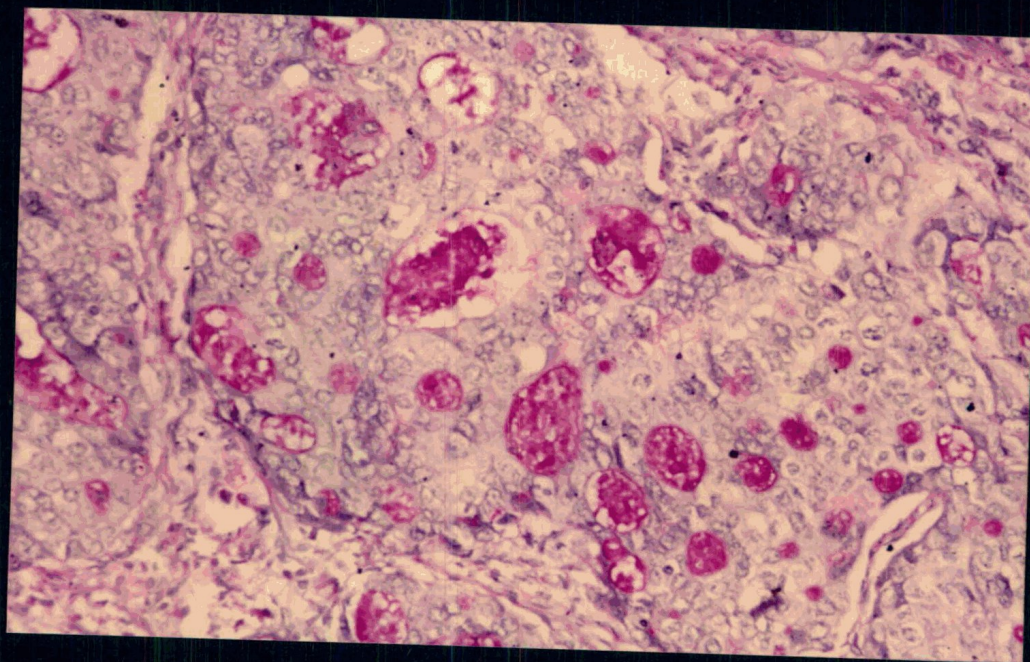


Fig 7. Many red staining carcinoma cells
PB-KOH-PAS X 200 CASE 1114-95

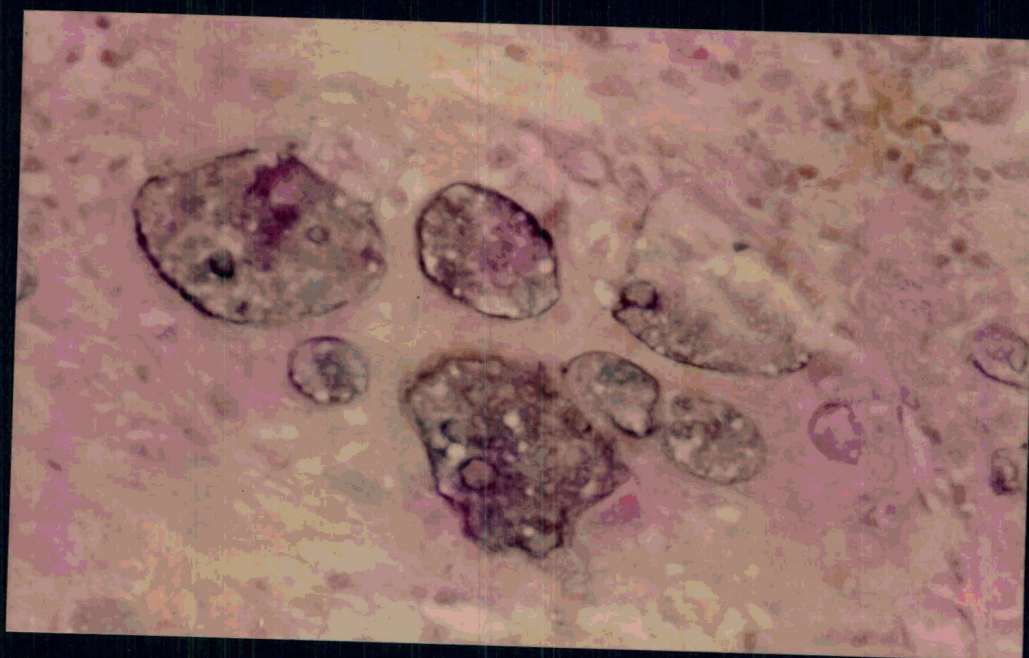


Fig 8. Brownish black mucin secreted by moderately
differentiated carcinoma HID-AB X 200 CASE 1114-5

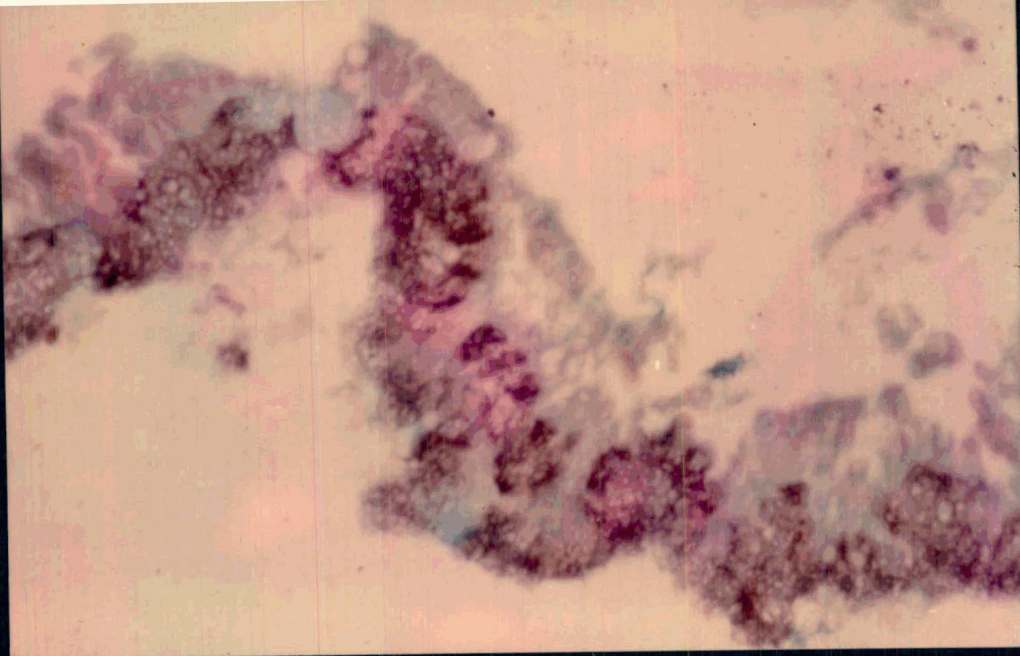


Fig 9. Brownish black mucin in well differentiated carcinoma HID-AB X 400 CASE 2698-95

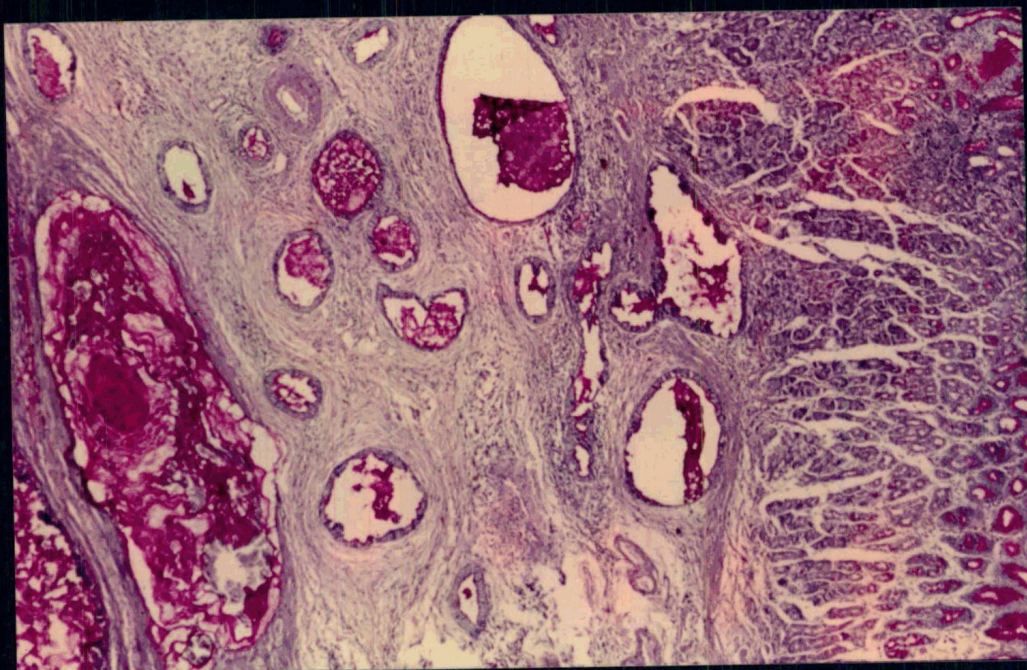


Fig 10 Red staining mucous in gastric mucosa [right] and tumor [left] PB-KOH-PAS X 40 CASE 2698-95

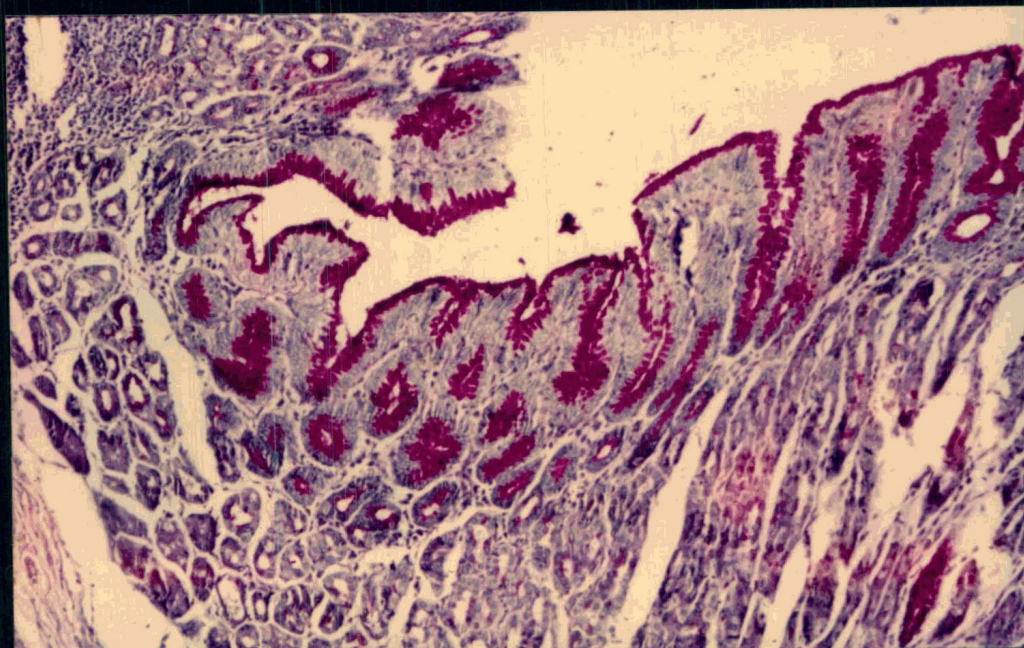


Fig 11 Extensive red staining of IM of gastric mucosa
PB-KOH-PAS X 100 CASE 2698-95

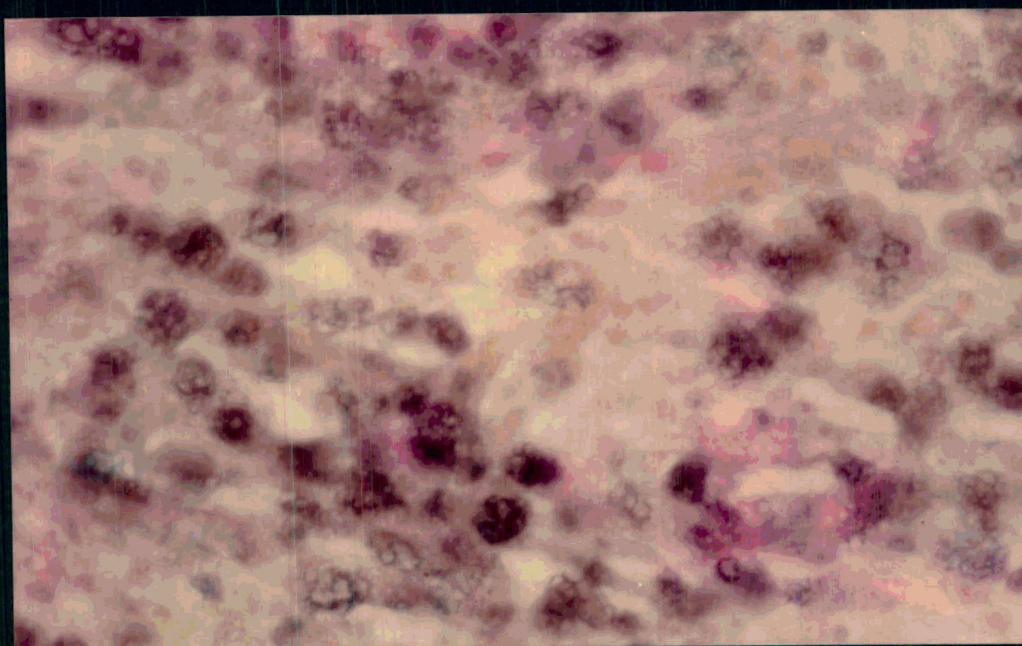


Fig 12 Brownish black staining of mucous in poorly
differentiated carcinoma HID-AB X 400 CASE 2698-95

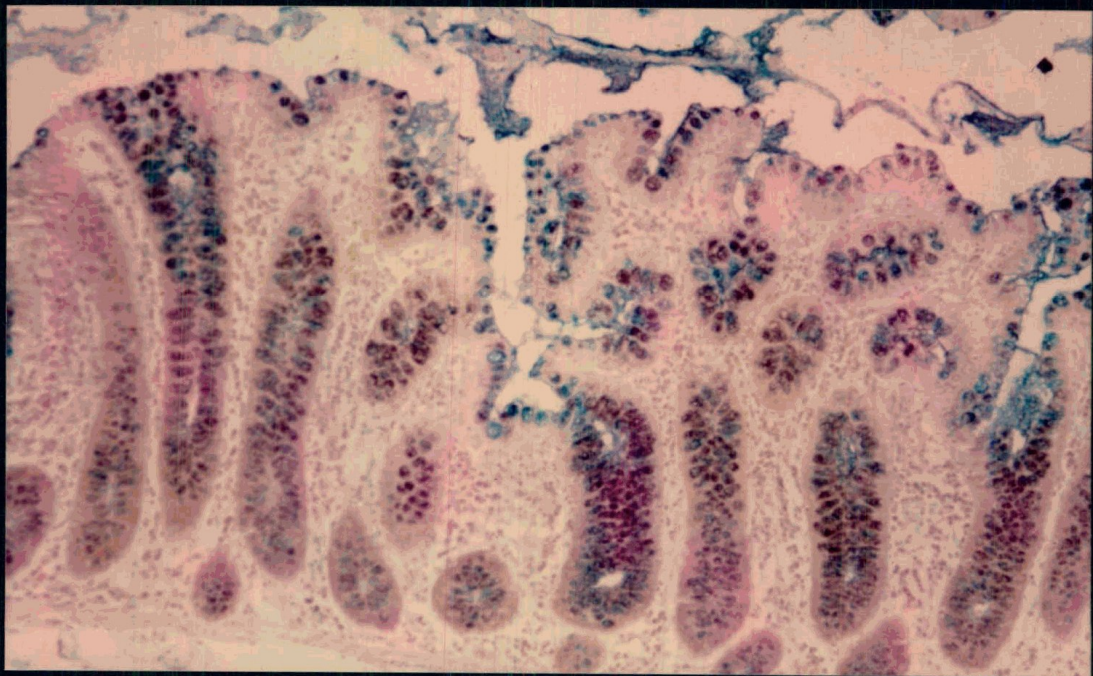


Fig 13 NORMAL CONTROL, SIGMOID
 Predominant brownish black sulphomucin secretion
 HID-AB X 100

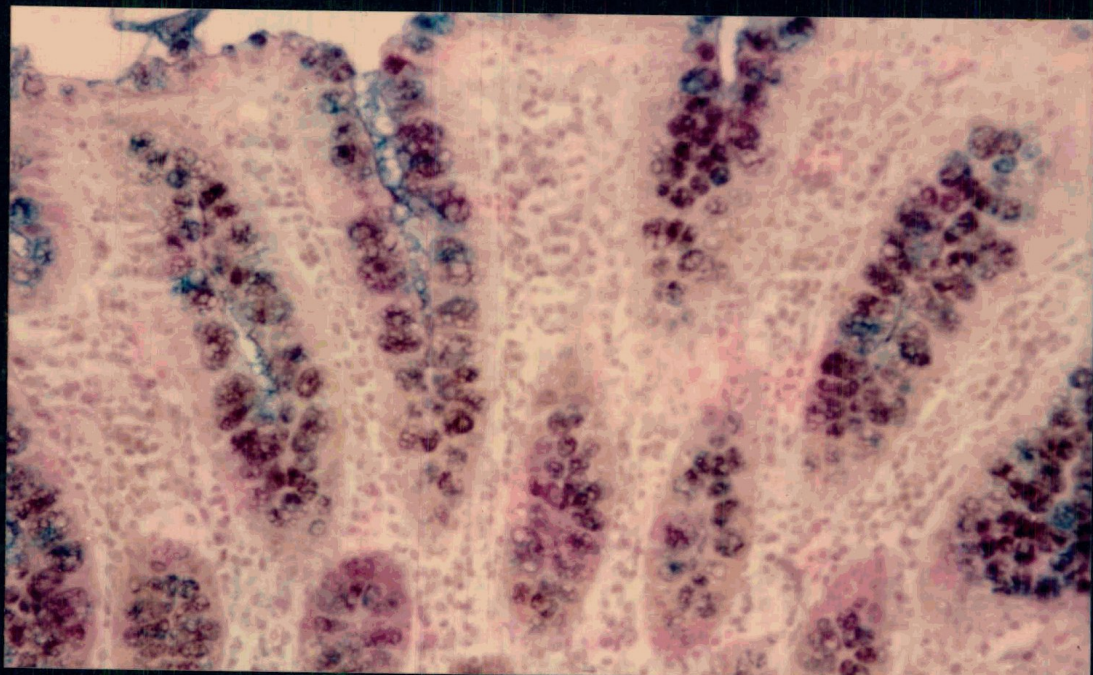


Fig 14 Close up view of Fig 13 HID-AB X 200

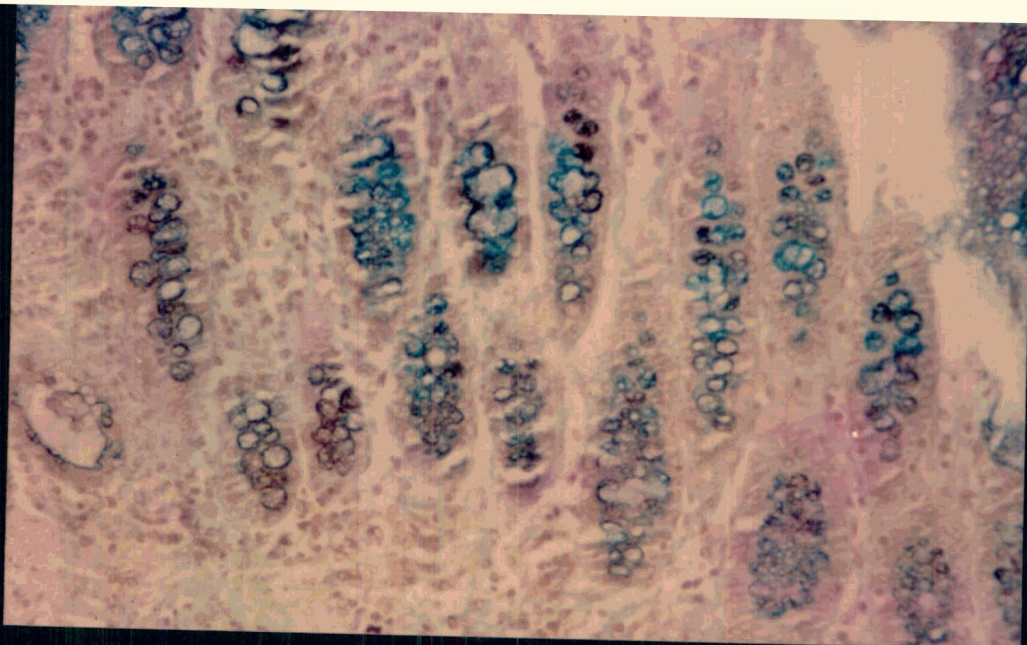


FIG 15 Predominant alcian blue positivity HID-AB X 200
CASE 894-95

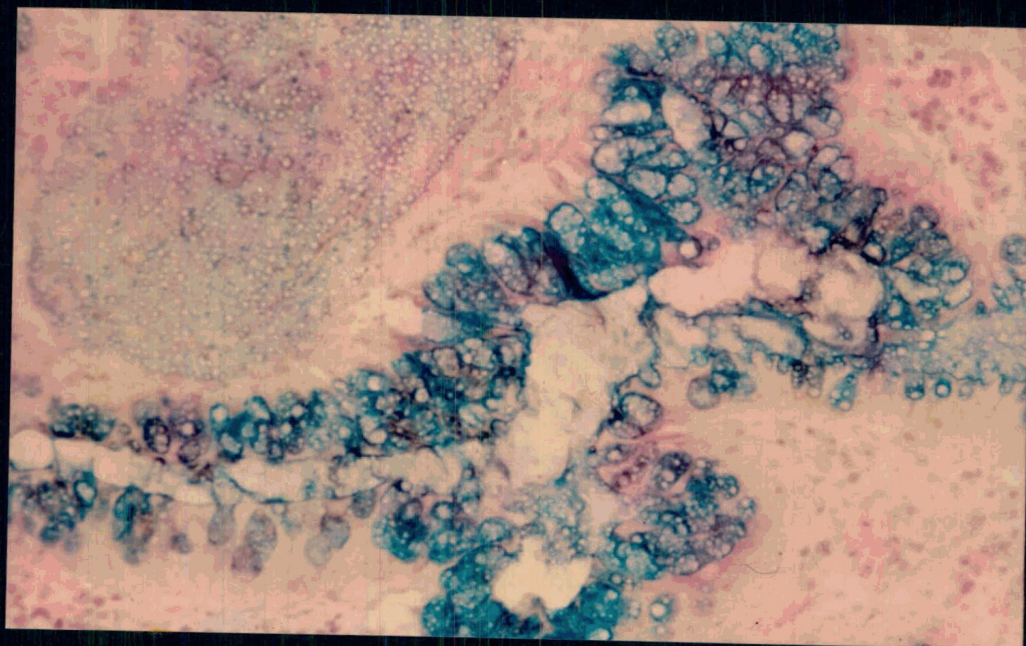


Fig 16 Diminished brownish black sulphomucin and predominant
blue sialomucin near sigmoid growth HID-AB X 400 CASE 894-95

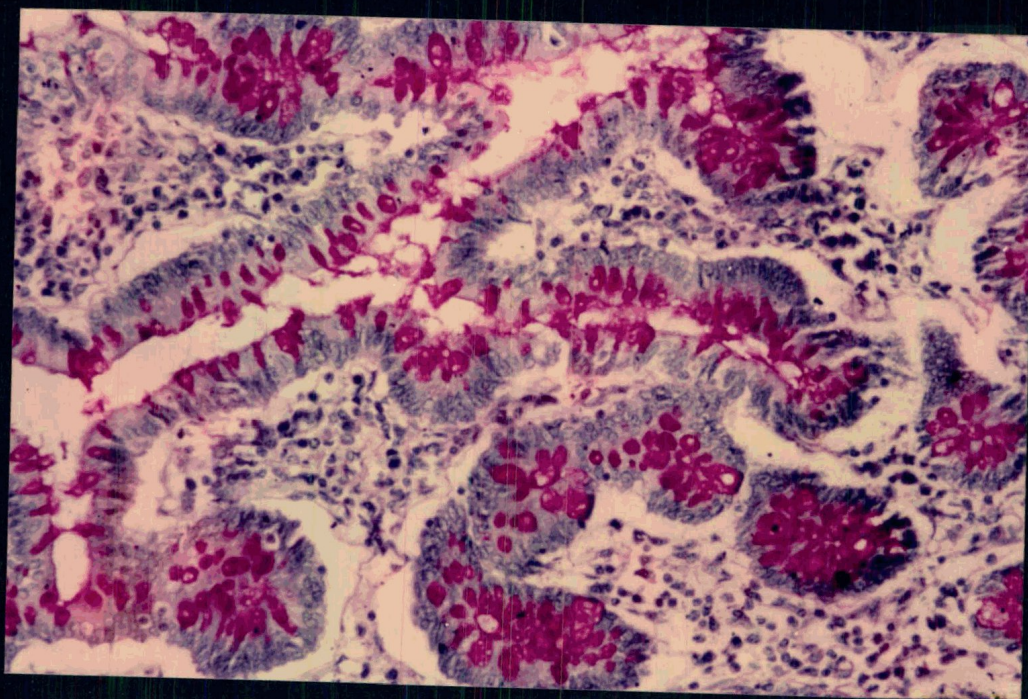
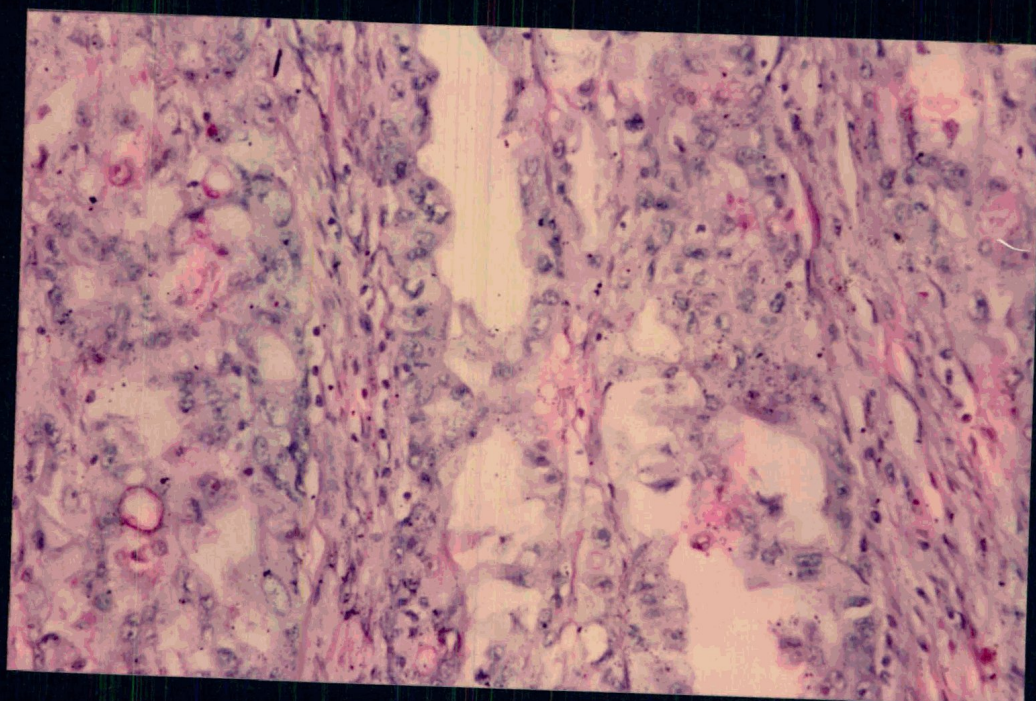


Fig 17 All red staining goblet cells in normal ascending
colon mucosa PB-KOH-PAS X 200 CASE 2068-95



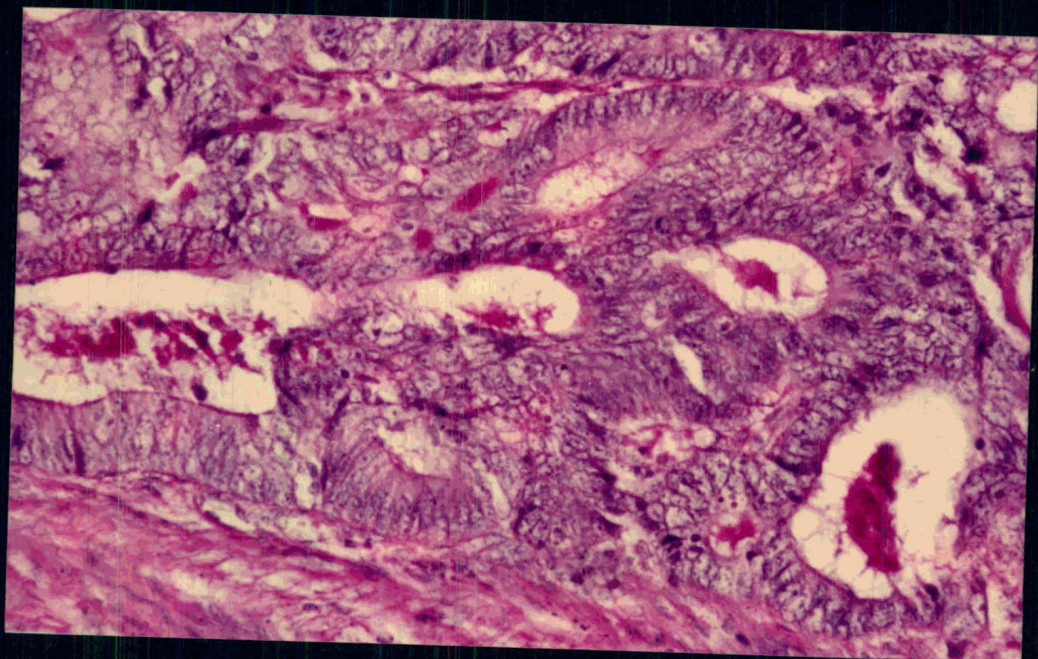
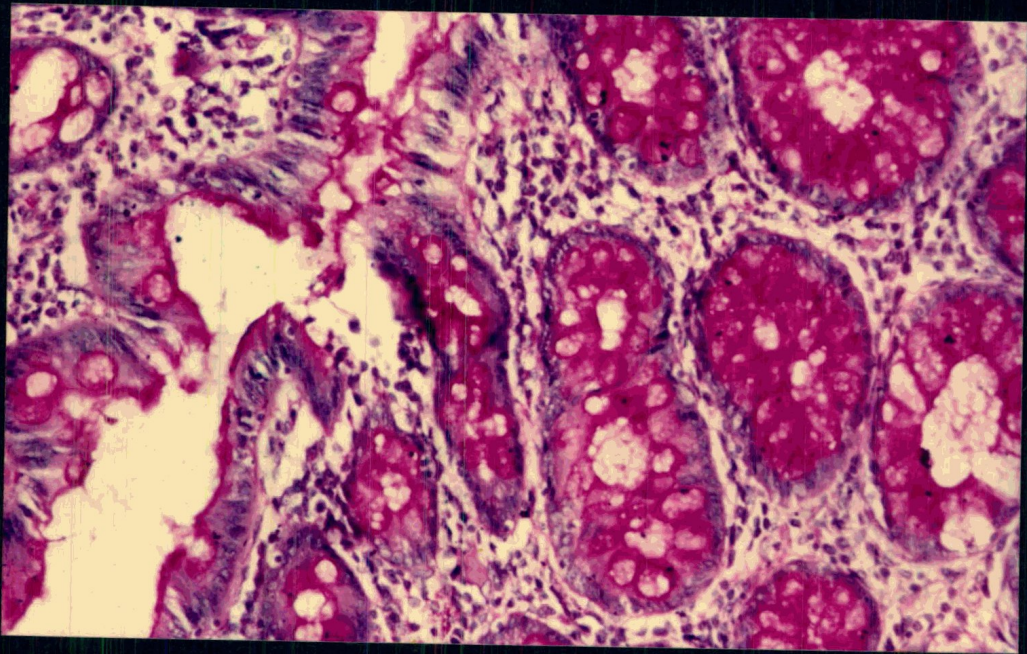


Fig 20 Minimal extracellular mucin stained, none in cells
PB-KOH-PAS CASE 1050-90

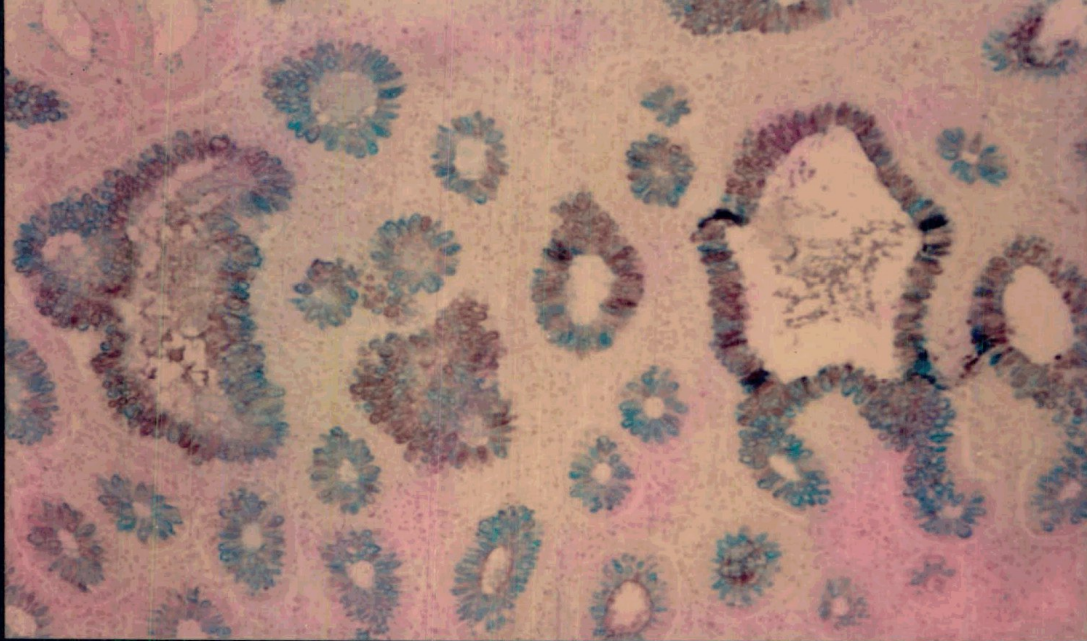


Fig 21 Pattern of sialomucin [blue] and sulphomucin [brownish black] in adenomatous polyp HID-AB X 100
CASE 265-91

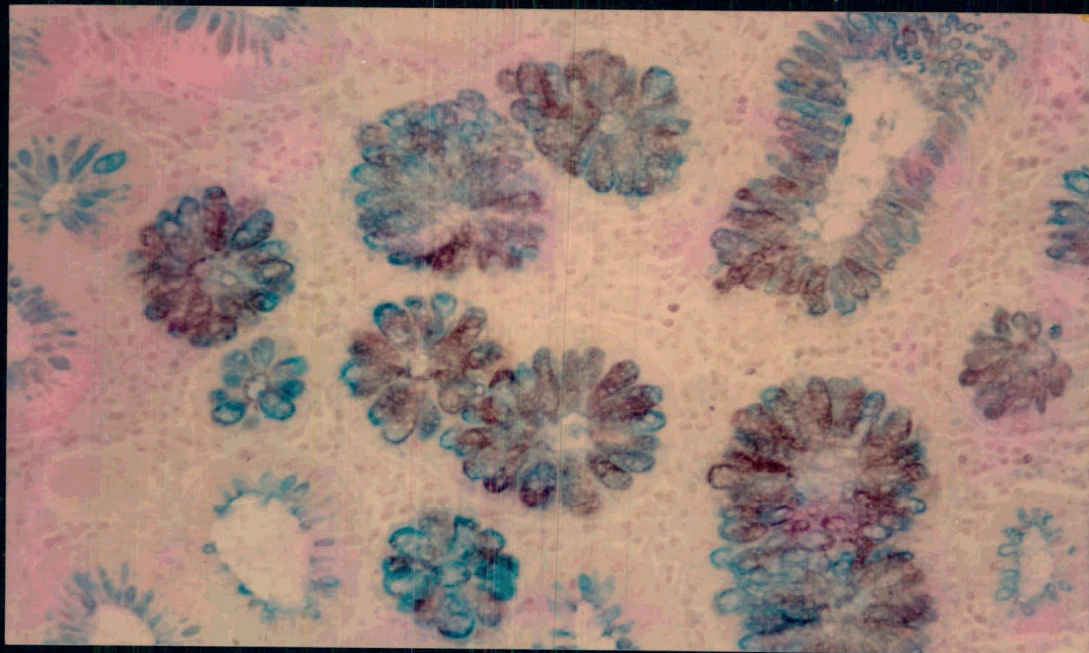


Fig 22 Higher power of same case HID-AB X 200
CASE 265-91

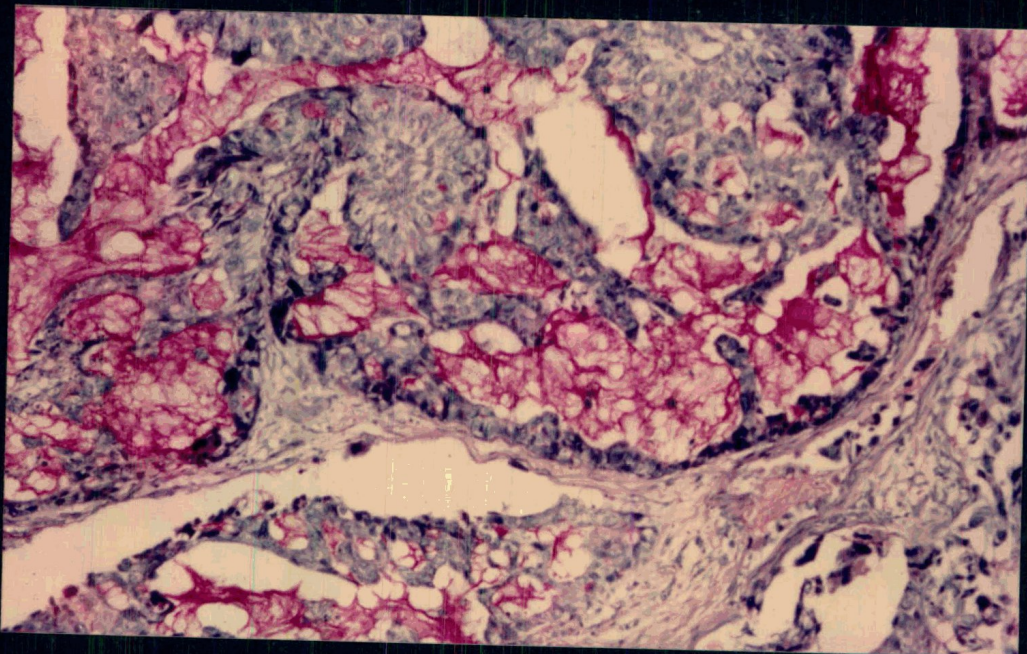


Fig 23 Red staining intra- and extracellular mucin in metastatic carcinoma in supraclavicular node [gastric primary] PB-KOH-PAS X 200 CASE F-477-95

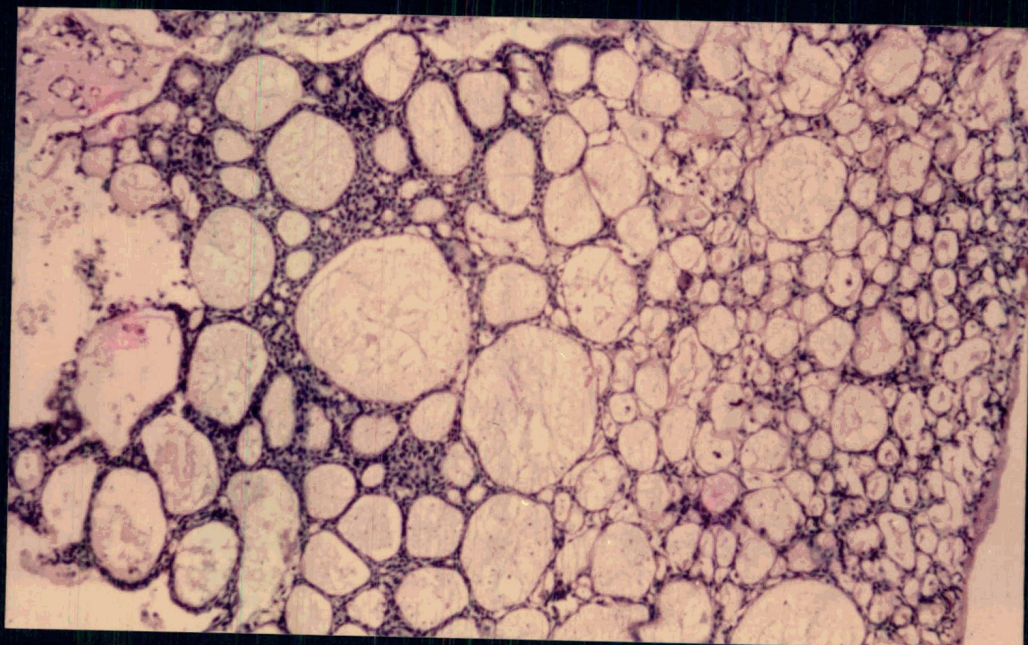


Fig 24 No staining of mucin in acini of adenoid cystic carcinoma of parotid PB-KOH-PAS X 100 CASE 2375-92

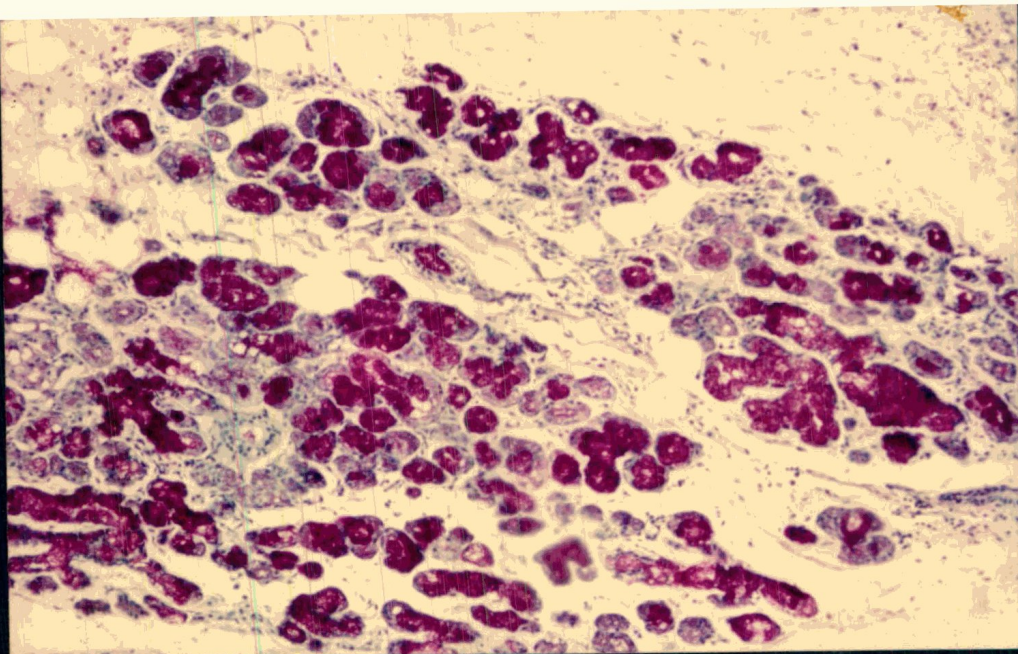


Fig 25 All salivary gland acini stained bright red
PB-KOH-PAS X 100 CASE 2375-92

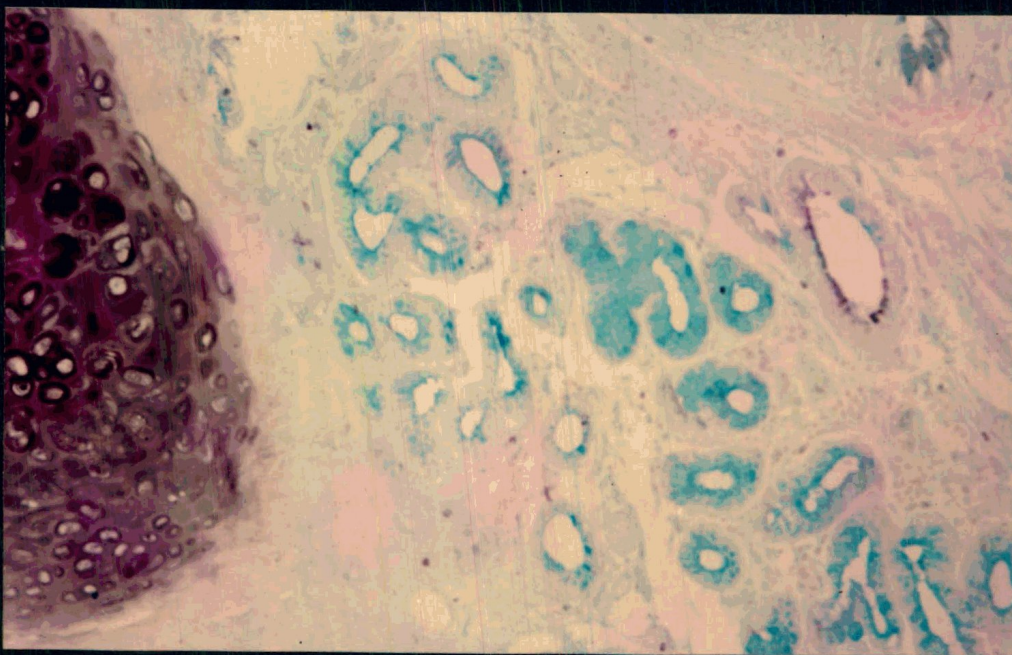


Fig 26 Brownish black staining of bronchial cartilage
HID-AB X 100 CASE 1230-95

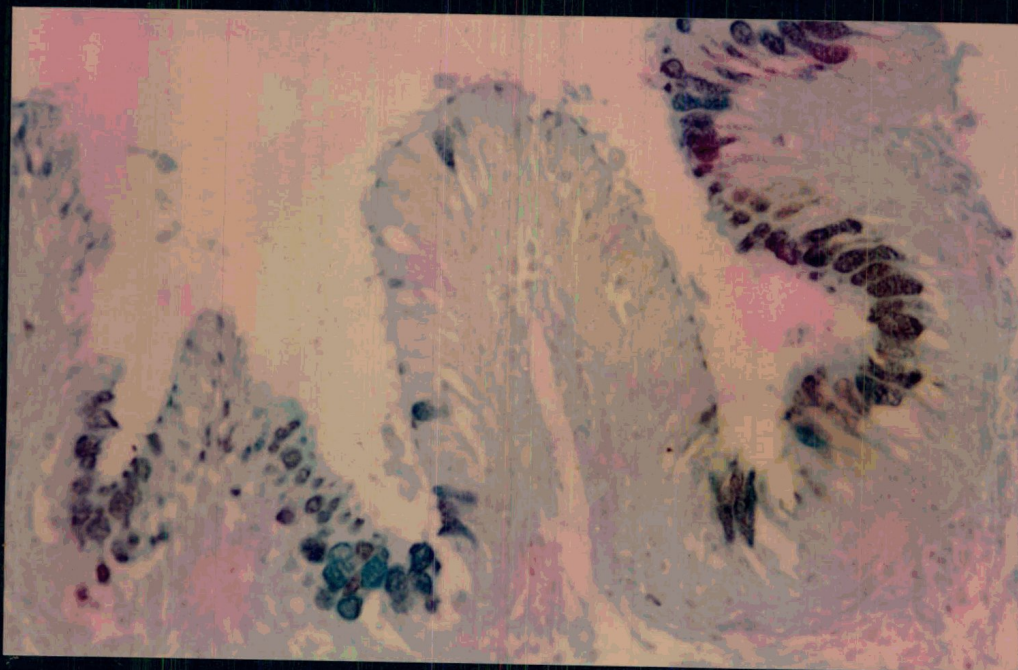


Fig 27 Brownish black selective staining of mucinous cells of normal bronchial mucosa HID-AB X 100 CASE 1230-95

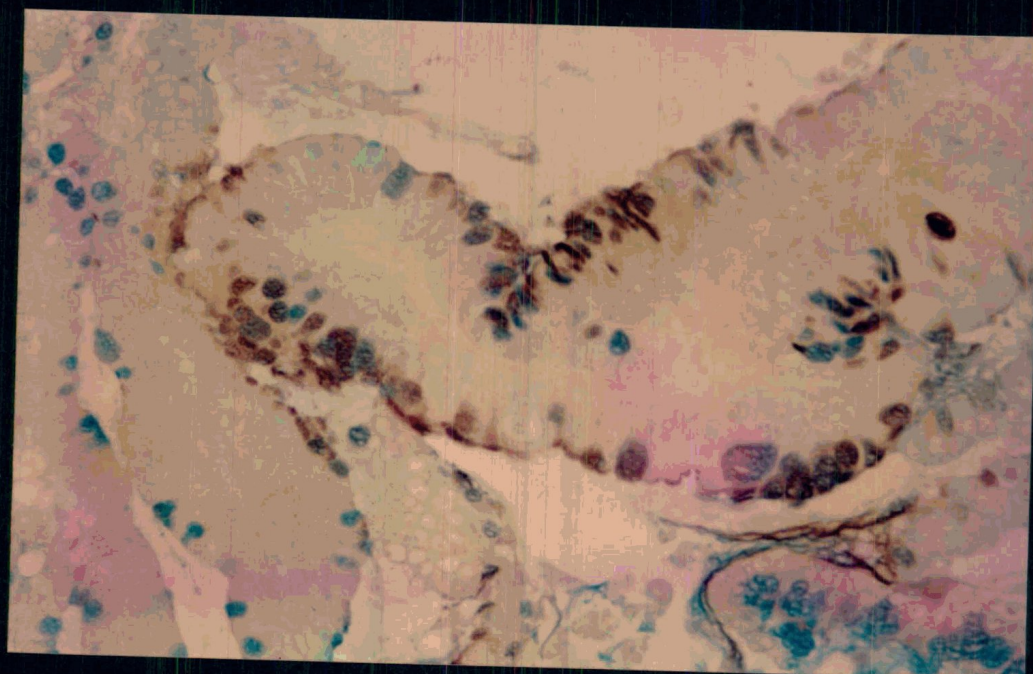


Fig 28 Brownish black [sulphomucin] and blue [sialomucin] staining goblet cells in neoplastic glands bladder carcinoma HID-AB X 200 CASE 583-95

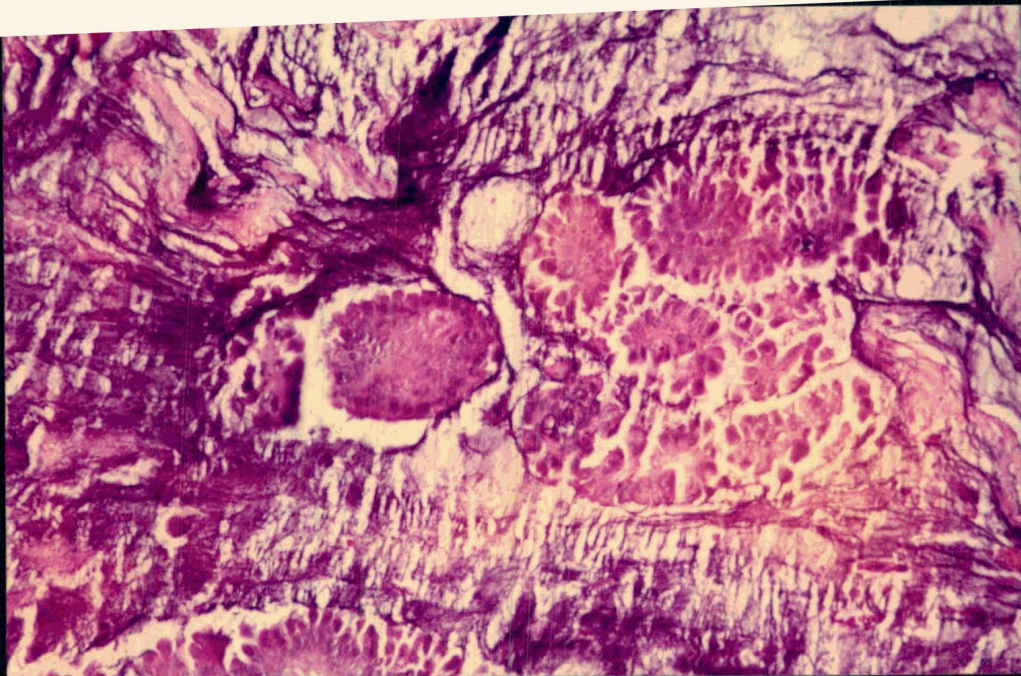


Fig 29 Purple-pink staining of extracellular mucin of
colloid carcinoma of breast AB-PAS X 200
CASE I-745-95

DISCUSSION

Mucin secretion by tissues or organs is a very ubiquitous phenomenon, particularly in the alimentary and respiratory systems. Mucins or mucosubstances are a very heterogenous group of mucopolysaccharides and can be specifically demonstrated in the various tissue by histochemical stains. In the gastrointestinal tract, neutral mucins are secreted by stomach, sialomucins by small intestines and sialo and sulphomucins by colon in normal circumstances. However, in various disorders, particularly neoplasms, mucosubstance totally alien to the part may be secreted. It would appear that biochemical variations in mucin secretion may represent an early precancerous change or in chronic inflammatory diseases indicate a reparative phenomenon. Study of mucin secretion in health and disease can indeed be very instructive in understanding pathogenesis of some disorders.

In this study, an attempt has been made to determine types of mucins secreted by tumors of gastrointestinal tract, mainly that of stomach and large intestine. Sections from the tumor and adjacent mucosa [normal or abnormal] have been subjected to three stains namely: AB-PAS [sialomucins] PB-KOH-PAS [O acetylated sialomucin] and HIS-AB [sial- and sulphomucins]. Mucin containing tumors of salivary glands, gall bladder, pancreas and lung have been similarly stained. Metastatic adenocarcinomas in lung and lymphnode were chosen to locate the primary site by differential mucin staining. Colloid carcinoma of breast, mucinous carcinoma of urinary bladder and a

myxoid neurofibroma were included. The choice of such a wide variety of tissues gave an opportunity to examine the various histochemical types of mucosubstances secreted by normal cells at these sites.

CARCINOMA OF STOMACH

Gastric carcinomas have been divided into intestinal and diffuse types, although a small proportion show features of both ⁴⁶. This classification has proved useful in the epidemiological studies. The intestinal type is more common in the older age group and has a better prognosis. Intestinal type of gastric cancers are considered to arise from areas of intestinal metaplasia [IM]. However, IM is a very common lesion seen in many conditions like chronic gastritis, peptic ulcer, adenoma etc and for practical purposes not all intestinal metaplasias can have a precancerous status. The role of IM and the development of over gastric cancer has been extensively studied by mucin histochemistry ^{14,15,16,17,18}. IM has been subdivided into complete [Type I] and incomplete [Type IIa and IIb] types. It has been shown that incomplete variant of IM predominantly secreting sulphomucin [Type IIb] is more commonly associated with intestinal type of gastric carcinoma. This variant of IM has been appropriately called "colonic" type because of histochemical resemblance to colonic goblet cells ⁴⁷.

In this series of 9 cases of gastric carcinoma, 2 did not show positivity with any of the three stains. In both cases, significant autolysis was present in the resected specimen

and this may explain lack of staining. The other 7 cases showed strong positive staining with AB-PAS [blue] and PB-KOH-PAS methods in the goblet cells of intestinal metaplasia [Figs: 2,5 and 3,6,11 respectively]. In most publications on the subject, it has been emphasized that intestinal metaplasia occurs in association with intestinal type and not the diffuse type of gastric carcinoma. However, in three cases of poorly differentiated carcinoma in this series, intestinal metaplasia was present and showed positivity with AB-PAS and PB-KOH-PAS [Fig 1 & 3]. The findings in this study support the view that sulphomucins [a normal colonic mucin] are consistently found in intestinal metaplasia and also the tumor proper. The HID staining was quite vivid in both moderately and poorly differentiated gastric carcinomas [Figs: 8,9 & 12]. This study has also demonstrated that O acetylated sialomucin normally present in left colon was detected in the gastric carcinoma and adjacent abnormal mucosa in most cases and often the staining [red] was quite extensive [Figs 6,7,10 & 11]

CARCINOMAS OF LARGE INTESTINE:

Transitional Mucosa : The use of high iron diamine-alcian blue [HID-AB] by Spicer ² in 1965, is a well established technique to simultaneously demonstrate both sulphomucin and sialomucin secretions in the colonic goblet cells. Many workers in the field have demonstrated important changes in the mucosubstances in the mucosa adjacent to a colonic carcinoma ^{27, 28 ,48} . The HID-AB stain has been able to delineate the transitional mucosa, which is often found adjacent to colonic carcinoma. On the routine H & E staining the glands in the transitional mucosa appear elongated or branched and

display enlarged goblet cells. However, the presence of transitional mucosa is best identified by demonstration of reversal of sulphomucin\sialomucin proportion. In the normal colonic mucosa, sulphomucin [stains brownish black with HID-AB] predominates [Figs: 13 & 14]. These photomicrographs clearly show the presence of sulphomucin in the lower 4\5th of the gland and the entire crypt and sialomucin [blue] restricted to surface epithelium and top portion of the glands. However, as one approaches the carcinomatous portion, the sulphomucins progressively diminish and sialomucins predominate [Figs: 15 & 16]. The frequent occurrence of this transitional mucosa adjacent to colo-rectal adenocarcinomas, adenomatous polyps, familial polyposis and its absence near non-carcinomatous neoplasms suggested that it is a premalignant event ^{27,28,49,50,51,52}

The reversal of sulphomucin\sialomucin proportion in the mucosa adjacent to colo-rectal growth was detected in 8 out of 9 cases in this series.

Williams ⁵³ contends that the biological significance of transitional mucosa is controversial. The transitional mucosa has been noted in association with solitary rectal ulcer, diverticular disease, ischemic colitis and inflammatory or hyperplastic polyps ^{54,55,56,57, 58}

O acetylated Sialomucins:

Culling et al ²⁷ developed an ingenious histochemical technique, in which the normally masked O-acetylated sialic acids can be demonstrated. In this method [PB-KOH-PAS], periodate oxidation is followed by reduction with sodium borohydrate, which abolishes the normal PAS reactivity of the tissues. Subsequent saponification with KOH breaks the masking O-acetyl bonds, thus exposing the vic glycols which react with Schiff's reagent. The method, therefore, allows the staining of O acetylated sialomucin. Culling, Reid and Dunn ⁵⁹ showed the presence of O acetylated sialomucins in the colon and rectum, as well as terminal ileum, and its absence from the rest of the gastrointestinal tract. Culling et al ²⁹ published a data on differentiating lower gastrointestinal tract mucin from other mucins in primary or metastatic tumors. They came to the conclusion that metastatic mucin secreting carcinomas showing positive staining with PB-KOH are carcinomas arising from lower gastrointestinal tract. In another paper by Culling et al ³¹ it was realized that malignant change in an adenomatous polyp was accompanied by reduction in the staining for O acetylated sialomucin. In addition, it was shown that not all adenocarcinomas of large bowel contain O acetylated sialic acid.

In our series of 9 cases of colo-rectal carcinomas, PB-KOH stain was positive in the normal mucosa around the growth in 7 cases and the tumors revealed little or no positivity [Figs: 17,18,19 & 20].

COLONIC POLYPS

The polyps are included in this study in order to see any striking alterations in the mucin patterns. The adenomatous polyps and hyperplastic polyp revealed relative excess of sialomucin over sulphomucin content but this was not significant from the point of predicting dysplastic potential. None of the polyps had adjoining mucosa, which could have been studied. Agawa and Jass ³² studied sialic acid histochemistry in adenoma-carcinoma sequence and observed loss of O acetyl substitution in a series of 93 colo-rectal adenomas that included 14 malignant polyps. This qualitative change in occurred in tandem with increasing grades of dysplasia within the adenomas. Thus reduction of O acetyl sialic acid is considered as a marker for severe dysplasia and emerging carcinoma ^{37 38}. The PB-KOH-PAS method failed to stain 4 out of 5 adenomatous polyps but this remains unexplained because these polyps did not have dysplastic changes.

The villous adenoma revealed only sialomucin positivity [blue with HID-AB] and PB-KOH-PAS staining was negative. The significance of these findings is not clear. Villous adenomas reportedly have a high potential to progress to carcinoma. Many more cases will have to be studied by mucin histochemistry to evaluate the malignant potential of this condition.

MISCELLANEOUS LESIONS:

Since presence of O acetylated sialomucin is considered diagnostic of mucin secreting tumors of lower gastrointestinal tract, PB-KOH stain was carried out for some cases of metastatic adenocarcinomas. In one case of metastatic adenocarcinoma in lung [primary in colon] the mucin stained strongly with PB-KOH-PAS stain and this was also the case with metastatic adenocarcinoma in supraclavicular lymph node [Fig 23]. However, the primary in the latter case was in stomach. It is evident that O acetylated sialomucin is not only secreted by the tumors of lower gastrointestinal tract. In fact, most adenocarcinomas of stomach studied in this series revealed this type of sialomucin.

Mucin secreted by salivary gland tumors [Fig 24] did not stain for either sialomucin or sulphomucin. The normal salivary tissue contained O acetylated sialomucin [Fig 25]. Adenocarcinomas of pancreas and gall bladder revealed only AB-PAS positive sialomucins. Metastatic adenocarcinoma in lung [colonic primary] and that in supraclavicular lymph node , both stained strongly with PB-KOH [Fig 23]. In two cases of bronchiolo-alveolar carcinoma of lung , all stains were negative. However, the normal bronchial cartilage and bronchial columnar mucin containing cells were strongly positive for HID-AB [Figs: 26 & 27]. The mucin secreting adenocarcinomas of the urinary bladder revealed both sialo- and sulphomucins, which is characteristic of

colonic mucin [Fig 28]. The colloid carcinoma of the breast was more PAS positive than alcian blue and negative for other stains [Fig 29].

SUMMARY AND CONCLUSIONS:

1] Mucin histochemistry was carried out on 9 cases of gastric carcinoma, 9 cases of colorectal carcinoma, 5 cases of adenomatous polyps, 1 each villous adenoma , hyperplastic and juvenile polyp. The staining procedure included Alcian blue [sialomucins blue] , PB-KOH-PAS [O acetylated sialomucin red], and HID-AB sulphomucin brownish black and sialomucin blue].

2] This staining was carried out in 11 cases of mucin secreting neoplasms at sites other than gastrointestinal tract. These included one case each of acinic cell carcinoma and mucoepidermoid carcinoma of parotid gland, pancreatic and gall bladder carcinomas, metastatic adenocarcinoma in lung and lymphnode , two cases of bronchioloalveolar carcinomas, adenocarcinoma of urinary bladder, colloid carcinoma of breast and myxoid neurofibroma.

3] In all cases of resected specimens of carcinomas of stomach and large intestine (N=18) sections from mucosa adjacent to the growth and section well away from the growth were stained by these methods.

4] Intestinal metaplasia adjacent to gastric carcinoma consistently showed alcian blue and PB-KOH-PAS positivity and most also stained for high iron diamine with brownish

positive in metastatic tumor, certainly is a characteristic of both stomach and large bowel primary carcinoma.

8] Mucin adenocarcinoma of urinary bladder revealed colonic type of mucin pattern, namely: strong staining with high iron diamine. This finding corroborates with the published work.

9] All three stains, namely: AB-PAS, PB-KOH-PAS AND HID-AB, gave consistent reproducible results. It is suggested that mucin histochemistry should be employed in the study of mucins in health and disease at all sites, where mucosubstances are secreted. It is important to note that tissue fixation should be optimal, for autolysis gives fallacious results with these stains.

black colour. Thus, intestinal metaplasia, when associated with carcinoma contains sulphomucin, which is normally secreted by colon. Contrary to the belief at one time that O acetylated sialomucin is secreted only by lower gastrointestinal carcinomas, we found this mucin in most cases of gastric carcinoma. Again, we found association of sulphomucin secretion in intestinal metaplasia with both intestinal and diffuse type of gastric carcinoma, although others have demonstrated the association of intestinal metaplasia with intestinal type of gastric carcinoma..

5] The transitional mucosa or mucosa surrounding colo-rectal carcinomas consistently showed reversal of sulphomucin\sialomucin ratio. Whether this represents an early precancerous change, even before the occurrence of dysplasia, is not clear from this study.

6] Adenomatous polyps in this series did not show any alterations in the normal colonic type of mucin pattern, which could be considered to have precancerous status. However, the villous adenoma showed only sialomucin positivity, accompanied by absence of sulphomucin. Villous adenomas have much higher incidence of malignant transformation than adenomatous polyps. Clearly, much more work will have to be done in this regard.

7] Metastatic carcinoma in lung [colon primary] and one in supraclavicular node [gastric primary] stained brightly with PB-KOH-PAS sequence. The stain, when

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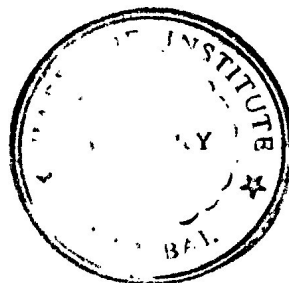
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