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LOCATION OF TUMOUR CELLS IN COLON TISSUE BY TEXAS RED LABELLED PENTOSAN POLYSULPHATE, AN INHIBITOR OF A CELL SURFACE PROTEASE

M. ANEES

Division of Biochemistry and Molecular Biology,
School of Biological Sciences, University of Manchester,
Manchester, M13 9PT, UK

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Pentosan polysulphate (PPS), a highly negatively charged polysaccharide, is a significant inhibitor of an isoenzymic form of a cell surface protease referred to as guanidinobenzoatase GB, associated with colonic carcinoma tissues in frozen sections and free GB in solution, in a concentration-dependent manner. However PPS failed to recognise and bind to the isoenzymic form of GB associated with normal colon epithelial cell surfaces. Texas red labelled PPS (TR-PPS) binds to the tumour cell surfaces of colonic carcinoma and colonic polyps and these cells fluoresce red, whilst the normal colon cell surfaces failed to bind the TR-PPS, and hence lacked red fluorescence. Polysulphonated suramin also selectively recognised and inhibited the colonic carcinoma GB isoenzyme. The kinetic data indicated that this inhibition was not caused by a mere polyanionic effect, since highly sulphated heparin failed to show a significant inhibition of colonic carcinoma GB, however trypan blue did show 50% inhibition. Kinetic studies have also shown that PPS is a non-competitive, reversible inhibitor of colonic carcinoma GB, with an apparent $K_m 6.8 \times 10^{-7}$ M. Gel analysis has shown that PPS binds to another site, distinct from the active centre, and after binding PPS changed the conformation of GB. These studies suggest that TR-PPS is a potent inhibitor of colonic carcinoma GB, and can be used as a novel fluorescent probe for the location of tumour cells in frozen sections of human colon tissues. PSS could also have potential as a vehicle for the transport of cytotoxic compounds to carcinoma cells of the colon.

KEY WORDS: Colon carcinoma, cell surface protease, guanidinobenzoatase, isoenzymes, pentosan polysulphate

INTRODUCTION

Guanidinobenzoatase (GB) is a tumour associated protease,[1] now known to be similar to,[2] but not identical to tissue plasminogen activator.[3] Previous studies have shown that GB has different isoenzymic forms, associated with normal and colonic carcinoma tissues.[4] GB

Correspondence: Dr. F.S. Steven, Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Manchester, Manchester, M13 9PT, UK.

Abbreviations: Guanidinobenzoatase (GB); Tissue plasminogen activator (t-PA); Plasminogen activator inhibitor (PAI-1); 9-Aminoacridine (9-AA); Pentosan polysulphate (PPS); Texas red pentosan polysulphate (TR-PPS); Methylumbelliferon (MU).

isoenzymes can be located on cell surfaces with an active site directed fluorescent probe, 9-aminoacridine (9-AA), a competitive inhibitor of GB.[5] These isoenzymic forms of GB can be differentiated by serum inhibitors,[6] competitive inhibitors,[7] cytoplasmic inhibitors,[8] and rhodamine labelled PAI-1.[9]

Pentosan polysulphate (PPS) is a semi-synthetic sulphated polysaccharide that has been previously investigated as an anti-coagulant and anti-metastatic agent.[10,11] Sulphated heparin-like compounds also act as anti-coagulants, and due to their potential ability to block growth factors they may prevent metastasis.[12,13] *In vitro*, PPS has been shown to inhibit cell growth of vascular smooth muscle cells,[14] angiogenesis,[15] and to suppress prostate tumour growth *in vivo*.[15] The particular interest shown in this compound was that it could be used as a nontoxic agent to inhibit the growth of prostate cancer at an early stage, when the tumour is not clinically evident.[15]

The purpose of the present study was to investigate the interaction of the PPS and other sulphated compounds with a cell surface enzyme GB isoenzymes, associated with colonic carcinoma and colonic polyps in frozen sections and free GB in solution employing conventional kinetic analysis.

From these studies it is concluded that the TR-PPS is a selective inhibitor for colonic carcinoma GB isoenzyme; therefore, TR-PPS is a successful probe for the location of tumour cells in frozen sections of colonic carcinoma and polyp tissues. PPS is a nontoxic compound and could possibly be used as a carrier of cytotoxic drugs or radioactive metals for the selective destruction of tumour cells within the colon.

MATERIALS AND METHODS

Colonic carcinoma and normal colon tissues were provided by Dr. I.C. Talbot of the ICRF Colorectal Unit, St. Marks Hospital, London. Frozen sections were cut in the Histology Department, University of Manchester. These tissues were also used for the extraction of GB.

PD-10 disposable columns were purchased from Pharmacia/LKB, Uppsalla, Sweden. 9-Aminoacridine (9-AA), Texas red acid chloride, cyanogen bromide, trypan blue, low molecular weight heparin and pentosan polysulphate (PPS) were purchased from Sigma Chemical Co. Ltd, St. Louis, USA. Suramin was a gift from Bayer AG, Germany.

Coupling of PPS with Texas Red Acid Chloride

Texas red acid chloride (0.5 mg/ml) was first converted into Texas red amine, by condensation of the reactive chloride with ammonia in solution, and the excess ammonia was removed by evaporation. Texas red amine was coupled to PPS by cyanogen bromide activation according to Axen,[16] with slight modification. In brief, 1 ml of cyanogen bromide (5 mg/ml) was added to 1 ml of PPS (5 mg/ml) at pH 10, and the reaction was allowed to proceed for 3–4 min. Texas red amine (5 μg) was added and the reaction mixture was left overnight with constant shaking. The reaction products were passed over a PD-10 column for the separation of free Texas red amine from the red labelled TR-PPS, which has a higher molecular weight.

TR-PPS Staining

The nuclei of the cells in frozen sections of colonic carcinoma and normal colon were stained with haematoxylin for 1 min and equilibrated with phosphate buffer saline (PBS) for 10 min. TR-PPS staining was carried out by covering each section on the slide with 10 μl of TR-PPS (9×10^{-5} M), for 30 min, followed by washing off the excess stain from the slides in fresh isotonic saline for 1 min. The presence of an active GB isoenzyme on the normal colonic cells was demonstrated by fluorescent staining with dansyl fluoride.

Artificial Transfer of Colonic Carcinoma GB to Normal Colon Cell Surface Receptor by Fibrin Overlay Technique

The fibrin overlay technique was developed for the location of plasminogen activators, by covering sections with a layer of fibrin fibrils.[17] Steven *et al.*[18] used this method for the dissociation of GB or GB-I complex from the protein receptors of GB on lung squamous cell carcinoma cell surfaces in frozen sections. GB was captured by the fibrin clot and the inhibitor was released into the liquid fraction. In this study in the first step normal colonic cells are stripped of their isoenzymic form GB by the fibrin overlay technique. At this stage the normal colon epithelial cells lack GB but have receptors to bind GB molecules. The presence of receptors for GB on the cell surfaces was demonstrated by incubating prelabelled dansyl colonic carcinoma GB with fibrin treated normal colon sections for 2 h, followed by fluorescence microscopy.[18] Thus the blue fluorescence showing the reformation of GB-receptor complexes (Scheme 1). These normal colon cells were then challenged with TR-PPS and observed under the fluorescent microscope.

Assay of GB by MUGB

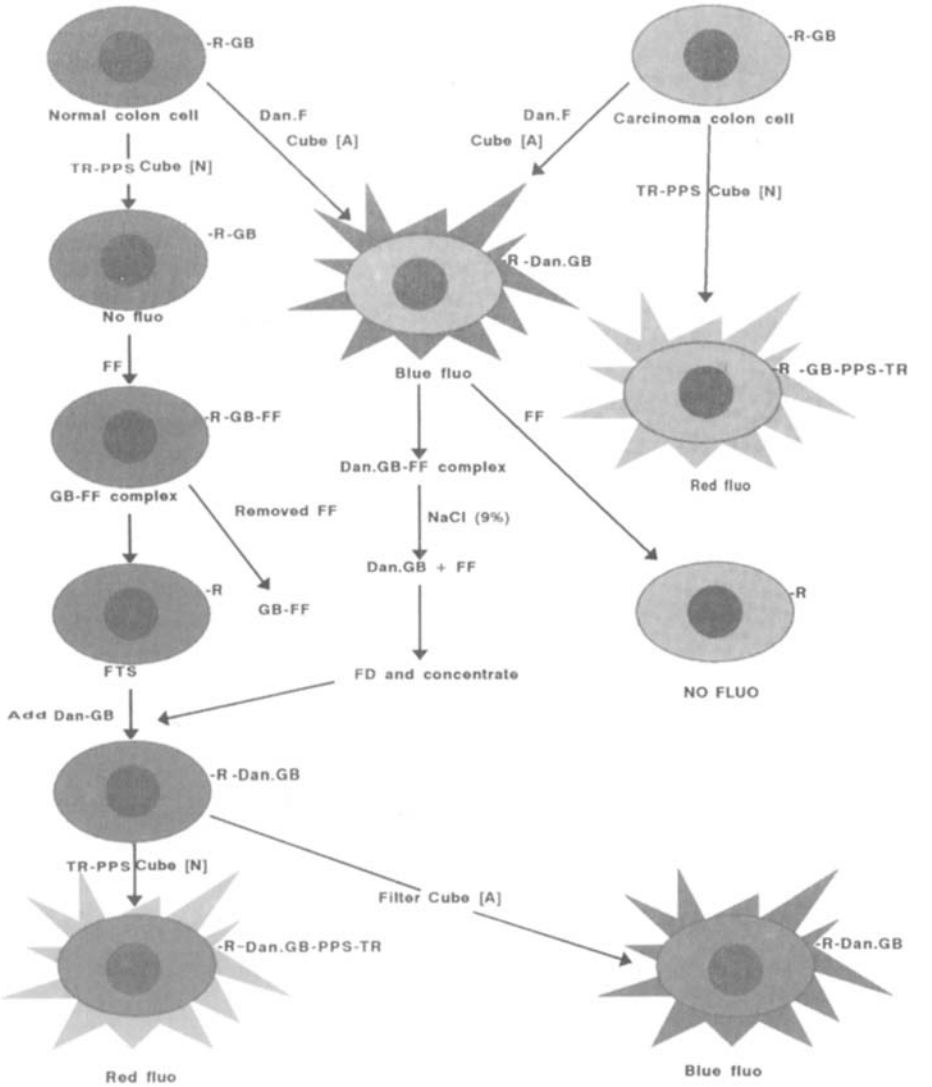
GB isoenzymes were purified from colon carcinoma and normal colon tissues as described earlier by using agmatine-sepharose affinity chromatography.[19] The GB activity was assayed with MUGB as substrate; the fluorescent product (MU) was measured by fluorescence spectrophotometry.[1] Cleavage of the substrate was monitored at an excitation wavelength of 323 nm and an emission wavelength of 446 nm.

Kinetic of Inhibition of GB

Inhibition experiments were carried out by pre-incubating purified GB (10 μg/ml) with the inhibitor for 10 min at 37°, prior to adding the substrate (2×10^{-4} M, final concentration). Lineweaver-Burk plots were used to define the type of inhibition by plotting 1/v against the reciprocal of variable amounts of substrate in the presence and absence of PPS.

Fluorescence Microscopy and Photography

Sections stained with TR-PPS were examined in the Leitz Diaplan fluorescence microscope, fitted with an automatic camera and Kodak ASA 400 colour film was used to record the data. Under these conditions cells with active GB were recognised by TR-PPS and exhibited red surface fluorescence.



SCHEME 1 Artificial transfer of colonic carcinoma GB on normal colon cell receptors.
Key. R: receptor, TR-PPS: Texas red pentosan polysulphate, Dan-F: dansyl fluoride, FF: fibrin fibrils, fluo: fluorescence, FD: freeze dried, FTS: fibrin treated sections, GB-FF: enzyme bound to fibrin. See Colour Plate III.

RESULTS AND DISCUSSIONS

Texas red labelled pentosan polysulphate (TR-PPS) is a significant inhibitor of an isoenzymic form of GB, associated with colonic carcinoma and colon polyps, and after binding makes these cell surfaces fluoresce red (Figures 1a, b, c). On the other hand TR-PPS failed to recognise and bind to the isoenzymic form of GB associated with the epithelial cell surfaces of normal colon and hence these cell lacked red fluorescence (Figure 2a).

Previous studies have shown that dansyl fluoride (Dan-F) is an active site directed fluorescent inhibitor of GB, which binds to both the colonic isoenzymic forms of the GB.[9] The GB on cell surfaces after binding Dan-F makes the cells fluoresce blue.[24] When the normal colon sections in Figure 2a were subsequently challenged with Dan-F, the cell surfaces bound Dan-F and fluoresced blue (Figure 2b). This suggests that active GB was present on the cell surfaces of normal colon but that these cells had previously failed to recognise and bind the TR-PPS.

Further evidence for the selective binding of TR-PPS to the colonic carcinoma GB isoenzyme comes from the artificial transfer of the Dan-F labelled colonic carcinoma GB on to the receptor of normal colon cells after using the fibrin overlay technique.[18] GB from the normal colon cells was removed by exposure of sections to fibrin, and then, these sections were incubated with purified dansyl colonic carcinoma GB for 2 hours. This dansyl colonic carcinoma GB was demonstrated to bind to the morphologically normal colonic cells by the blue fluorescence (data similar to Figure 2b). The cells in the morphologically normal colon section, which originally lacked the ability to bind TR-PPS (Figure 2a) after the transfer of abnormal dansyl colonic carcinoma GB isoenzyme, now bind TR-PPS and fluoresced red (Figure 2c, Scheme 1). The data suggest that TR-PPS selectively binds to the isoenzymic form of GB from colonic carcinoma cells. The inhibition of colonic carcinoma GB with TR-PPS was demonstrated to be reversible with SDS[20,21] and the GB regained activity as judged by its ability to bind Dan-F and exhibit blue fluorescence (data similar to Figure 2b).

GB isolated from colonic carcinoma, continuously hydrolysed 4-methylumbelliferyl-*p*-guanidinobenzoate (MUGB), an active site titrant for trypsin-like enzymes.[22] Kinetic studies confirmed that TR-PPS is a potential inhibitor and caused 89% inhibition of colonic carcinoma GB (9×10^{-5} M), whilst the normal colon GB was only slightly inhibited (11%), when assayed with MUGB (Figure 3). The data in Figure 3 suggest that inhibition of colonic carcinoma GB was concentration-dependent. Polysulphonated suramin also recognised and inhibited the colonic carcinoma GB isoenzyme. This inhibition by suramin is of interest because recently it has been shown that suramin as a single agent is ineffective in treating metastatic colorectal cancer pretreated with fluoropyrimidine-based chemotherapy.[23] The inhibition was not caused by a mere polyanionic effect, since highly sulphated heparin failed to show a significant inhibition of colonic carcinoma GB isoenzyme, however trypan blue did show 50% inhibition (Figure 4).

Lineweaver-Burk plots confirmed that PSS was a non-competitive inhibitor of colonic carcinoma GB isoenzyme with an apparent $K_m 6.8 \times 10^{-7}$ M (Figure 5).

The interaction of purified colonic carcinoma GB with PPS was studied using agmatine-sepharose to immobilised GB (Scheme 2). When the bound enzyme was exposed to MUGB, cleavage took place with the release of the fluorescent product MU (Figure 6a). After exposure to PPS, enzyme activity was lost both on agmatine-sepharose and the

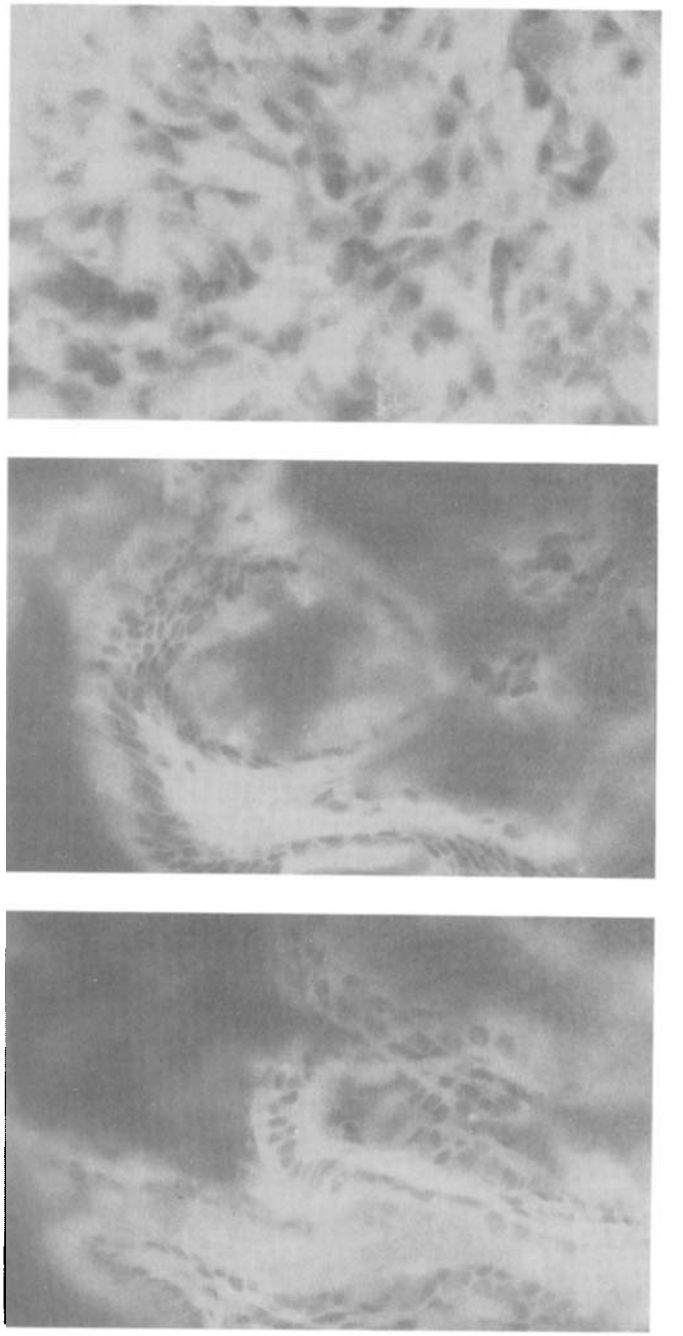


FIGURE 1 Frozen section of colonic carcinoma and colon polyps tissues directly stained with TR-PPS (9×10^{-5} M). (a) The cell surfaces of colonic carcinoma cells possessing active GB, bind TR-PPS and fluoresced red. (b,c) Colon polyps after binding TR-PPS fluoresced red. The nuclei of the cells appears dark, due to prior staining with haematoxylin. ×500. See Colour Plate IV.

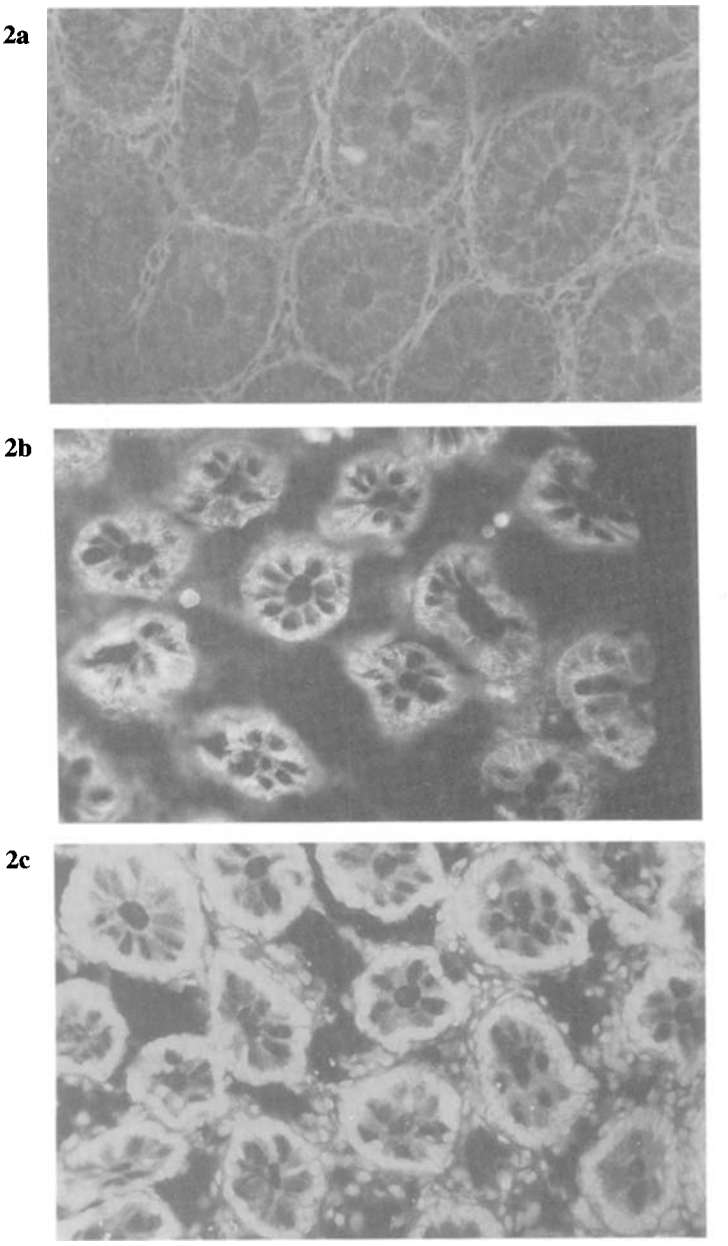


FIGURE 2 Frozen sections of normal colon tissue directly stained with TR-PPS, or after artificial transfer of colonic carcinoma GB. (a) The cell surfaces of the morphologically normal colon epithelial tissue failed to bind TR-PPS and did not fluoresce red. These cell possess a different isoenzymic form of GB, which did not recognise TR-PPS. ×750. (b) Frozen section of Figure 2a, after challenging with Dan-F. The normal colonic cell surfaces in this section, after binding Dan-F fluoresce blue. ×500. (c) Artificially transfer of the isoenzymic form of colonic carcinoma GB on to the receptors of normal colon cells. The normal epithelial cell surfaces (which originally lack red fluorescence), after the transfer of the isoenzymic form of colonic carcinoma GB, now bind with TR-PPS and fluoresced red. ×500. See Colour Plate V.

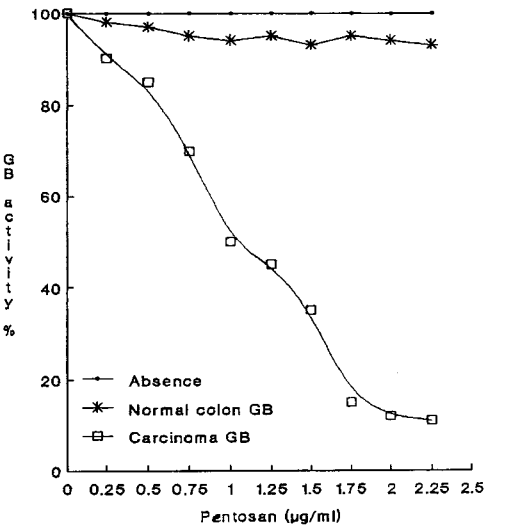


FIGURE 3 MUGB assay of normal and colonic carcinoma GB isoenzyme (10 μg/ml) incubated with the fluorogenic substrates MUGB, with and without PPS. The substrate, MUGB was continuously hydrolysed by both normal and colonic carcinoma GB in the absence of PPS, whilst only colonic carcinoma GB was significantly inhibited in the presence of PPS (9×10^{-5} M). The fluorescent product (MU) was measured by fluorescence spectrophotometry.

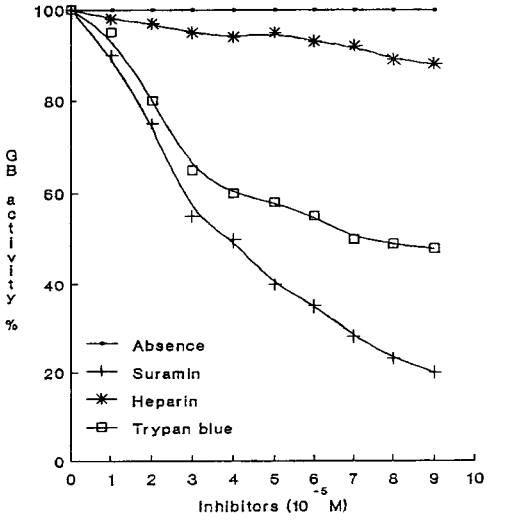


FIGURE 4 MUGB assay of colonic carcinoma GB isoenzyme (10 μg/ml) incubated with the substrate MUGB, with and without suramin. The MUGB was continuously hydrolysed by GB in the absence of suramin, whilst GB was markedly inhibited in the presence of suramin (9×10^{-5} M). Low molecular weight heparin failed to show significant inhibition, whilst trypan blue showed approximately 50% inhibition.

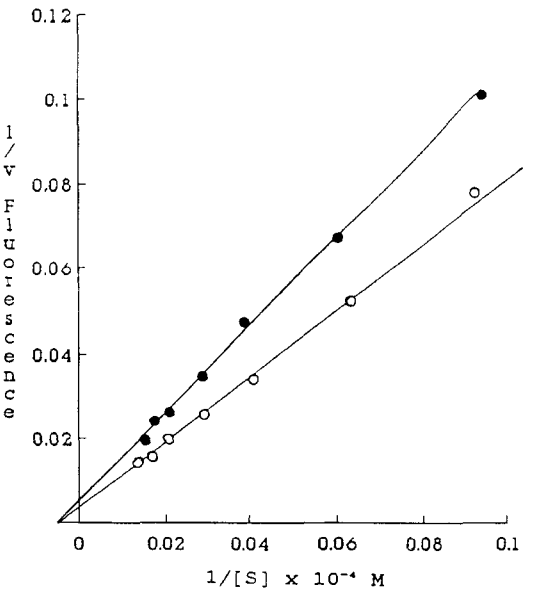


FIGURE 5 Lineweaver-Burk plot for the inhibition of colonic carcinoma GB isoenzyme. 1/v was plotted against variable concentrations of substrate 1/[S], in the presence (–●–) and absence (–○–) of PPS. The absence of a common intercept on the 1/v axis indicates that PPS is a non-competitive inhibitor of GB with calculated apparent $K_m 6.8 \times 10^{-7}$ M.

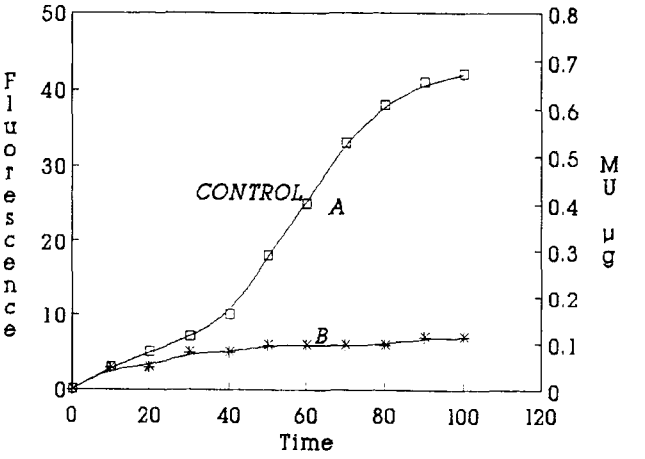
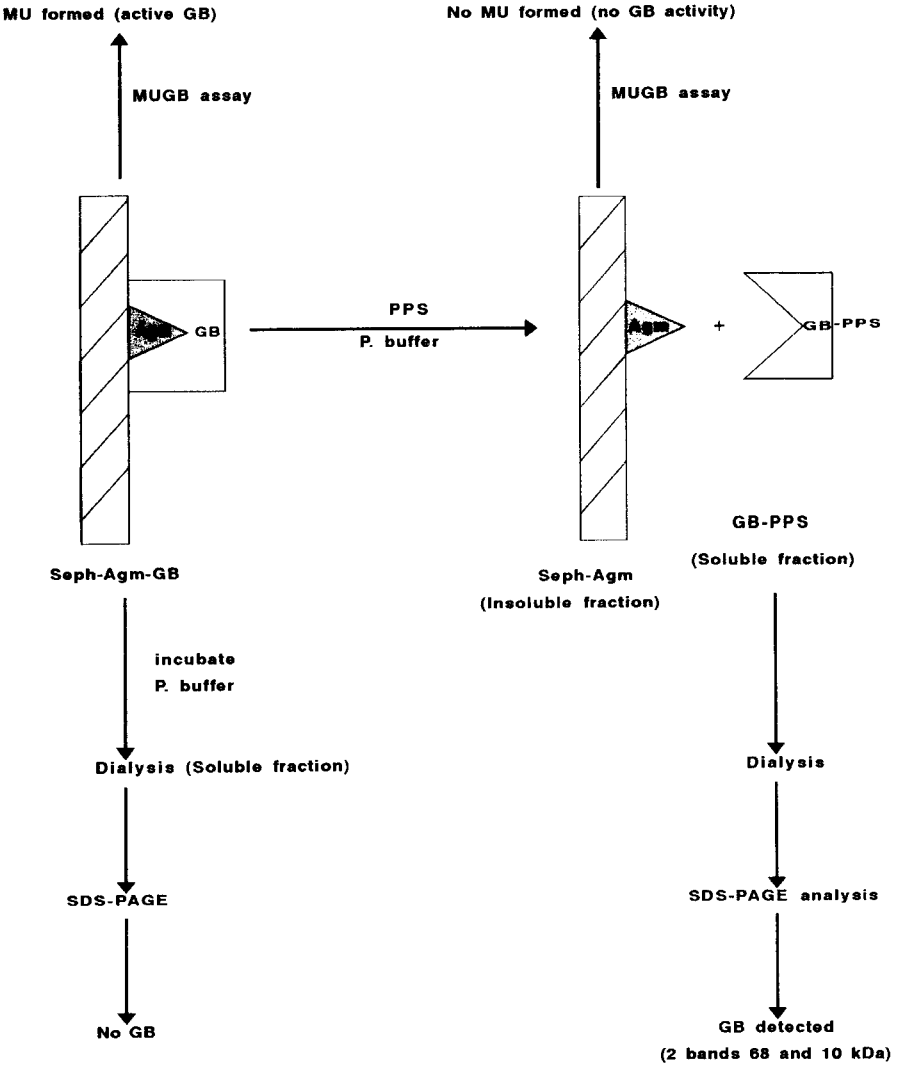


FIGURE 6 MUGB assay of GB-bound to agmatine-sepharose, pre-incubation and after incubation with PPS. In the control (A) GB-agmatine-sepharose was competitively dissociated from the active site of GB by the MUGB, and after 30 min, the enzyme activity was markedly increased. Whilst, in the test system (B), agmatine-sepharose after incubation with PPS, did not show any activity even after 100 min.



SCHEME 2 Conformational changes induced by PPS in GB bound to agmatine sepharose.
Key. Seph-Agm-GB: GB bound to sepharose agmatine, p: phosphate buffer, PPS: pentosan polysulphate, GB-PPS: enzyme inhibitor complex. See Colour Plate VI.

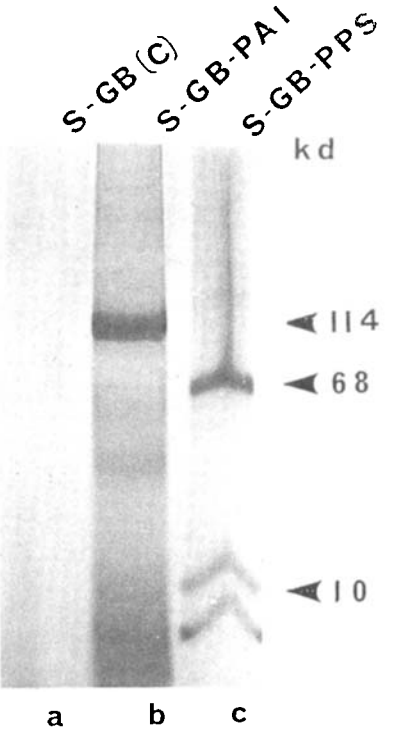


FIGURE 7 Gel electrophoresis analysis of the supernatant fluid from agmatine-sepharose bound GB (S-GB) in the presence and absence of PPS. In the absence of PPS no band of GB was detected (Track a), and in the presence of PPS (9×10^{-5} M), a single band of GB (68 kd) and two fragment of PPS (10 kd and 6 kd) were detected (Track c). In comparison the complex of GB-PAI-1 (114 kd), formed by the interaction of GB-bound to agmatine-sepharose with PAI-1, was stable and not dissociated on the SDS-polyacrylamide gel (Track b).

supernatant fluid (Figures 6b and 7a). However, this supernatant fluid contained GB which was dissociated from PPS in the presence of SDS (Figure 7c), in contrast to the interaction with PAI-1 and GB which resulted in loss of enzyme activity and an SDS stable complex of 114 kd (Figure 7b). It is likely that the PPS dissociated GB from agmatine-sepharose by causing a conformational change in the GB. The GB-PPS interaction was disrupted by SDS during PAGE analysis, with the release of GB molecular weight 68 kd and two fragments of PPS.

From the above results it is concluded that PPS is a non-competitive, reversible inhibitor of colonic carcinoma GB isoenzyme. PPS binds to another site distinct from the active site by non-covalent interactions, which are disrupted by 2% SDS on polyacrylamide gel electrophoresis. TR-PPS binds selectively to the colonic carcinoma GB isoenzyme (Figures 1 and 3), therefore, it could be used diagnostically for the location of tumour cells in frozen sections of colon tissue. Since, PPS is a nontoxic agent it could be used in the future as a carrier of cytotoxic drugs for the selective destruction of tumour cells within the colon.

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