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ACTION OF IMMOBILIZED NEURAMINIDASE ON THE ACCUMULATION OF 5-HYDROXYTRYPTAMINE BY HUMAN PLATELETS

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Abstract—The accumulation of 5-HT was studied in human platelets following graded desialylation with neuraminidase immobilized on macrobeads. Platelets were removed from the soluble sialylglycoproteins of the plasma by gel filtration on Sepharose 2B; the uptake of 5-HT by gel filtered platelets over four hours was similar to that found using platelet rich plasma. Platelet aggregation induced by 10^{-5}M 5-HT and 10^{-6}M ADP was decreased following gel filtration and the use of Sepharose 2B insolubilized apyrase did not ameliorate this effect. Removal of up to 30 per cent of the platelet sialic acid, which results in changes in the glycoprotein pattern as demonstrated by two dimensional gel electrophoresis, appears to accelerate the rate of uptake of 5-HT. This is followed by a significantly decreased rate once 50% desialylation has been achieved. Native platelets and those from which 30 per cent of the sialic acid had been removed were refractory to resialylation with exogenous rat liver sialyltransferase though this enzyme preparation catalyses the resialylation of soluble asialoglycoprotein acceptor.

Sialic acids are ubiquitous components of animal cell surfaces [1, 2] and in the case of the platelet have been implicated as playing an important role in platelet aggregation and survival [3–5]. Two avenues for elucidating the role of sialic acids at the platelet periphery have been explored, namely the removal of sialosyl residues with neuraminidase [3–5] and the opposite approach in which extra sialic acid residues have been attached to the native platelet surface by using an exogenous sialyltransferase [6, 7]. Mester *et al.* [6] working with an impure enzyme system involving homogenized rat liver found that human platelet-rich plasma treated with this preparation and CMP-*N*-acetylneuraminic acid showed an increase in the amount of sialic acid bound to the platelet membrane. Platelets modified in this way [6] had a decreased aggregation response to ADP but an enhanced response to 5-hydroxytryptamine (5-HT).† It was concluded that the effect of incorporating sialic acid into platelets increased the number of receptor sites with which 5-HT reacted to cause aggregation. A subsequent paper by Szabados *et al.* [7] extended their work on platelets enriched in sialic acid to show that such cells had an accelerated uptake of 5-HT and led them to suggest that *N*-acetylneuraminic acid may also be a component of the transport receptor for this amine. In an attempt to characterize the molecular species undergoing exogenous sialylation in the platelet membrane we have attempted to repeat the procedures described by Mester *et al.* [6] and Szabados *et al.* [7].

This paper describes a novel neuraminidase treatment in which gel filtered platelets, freed of plasma sialoglycoproteins, are treated with the enzyme immobilized on Sepharose macrobeads. Such treated cells are found along with untreated cells to be refractory towards sialylation, using known quantities of characterized rat liver sialyltransferase. Interestingly, the effect on 5-HT uptake of platelets subjected to graded desialylation with immobilized neuraminidase appears to be biphasic, an initial acceleration being followed by a significant decreased rate of uptake once 50 per cent of the platelet sialic acid has been removed. This is the first study in which neuraminidase insolubilized on macrobeads has been used as a means of studying the effects of graded desialylation on 5-HT uptake by the human platelet. Data on the number of 5-HT receptors on the platelet surface is provided and the chemical nature of the transport receptor discussed.

MATERIALS AND METHODS

5-hydroxy[^{3}H]tryptamine creatine sulphate (two batches of 11 and 13.8 Ci/mmole used in this work), CMP-*N*-acetyl[4,5,6,7,8,9-^{14}C]neuraminic acid, ammonium salt (4.2 mCi/mmole), D-[1-^{14}C]mannitol (60 mCi/mmole) and [U-^{14}C]sucrose were purchased from The Radiochemical Centre, Amersham, England. Purified neuraminidase (EC 3.2.1.18) from *Vibrio cholerae* filtrate was obtained from Behringwerke A-G., Marburg/Lahn, Germany as an aqueous solution containing either 500 or 4635 units/ml, where 1 unit of activity is defined as the amount of enzyme that releases 1 μg of *N*-acetylneuraminic acid in 15 min at 37° from α_1 acid glycoprotein in an appropriate medium at pH 5.5. This preparation is stated by the manufacturer to be free

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† Abbreviations used: 5-HT, 5-hydroxytryptamine; PIPES, piperazine-*NN'*-bis-(2-ethanesulphonic acid).

of phospholipase C and neither proteinases nor aldolase activity could be demonstrated. Methysergide hydrogen maleinate (Sandoz Ltd., Basle, Switzerland) and chlorimipramine hydrochloride (Geigy Pharmaceuticals, Horsham, England) were kindly provided by Dr. K. J. Watling and Dr. A. V. P. Mackay, MRC Neurochemical Pharmacology Unit, Cambridge. All chemicals were of analytical quality unless otherwise specified.

Preparation of platelet rich plasma. Blood was drawn from healthy donors into one tenth volume of 3.8% w/v trisodium citrate and used within one hour of venopuncture. Following centrifugation at $200\,g_{av}$ for 20 min platelet-rich plasma was removed and centrifuged at $1500\,g_{av}$ for 5 sec to remove any contaminating red blood cells.

Preparation of gel-filtered platelets. An enriched preparation of platelets was prepared by sedimenting platelet rich plasma on a cushion of 34% w/v albumin (Fraction V from bovine plasma—Armour Pharmaceutical Co. Ltd., Eastbourne, England), adjusted to pH 6.5 with solid $NaHCO_3$, at $650\,g_{av}$ for 15 min following the procedure devised by Walsh [8]. The enriched suspension of platelets (5 ml) was separated from plasma by gel filtration on a column (8.5 × 2.6 cm) of Sepharose 2B (Pharmacia Fine Chemicals, Uppsala, Sweden) following closely the method of Tangen *et al.* [9]. A column was constructed from a plastic disposable syringe barrel equipped with an A26 adaptor (Pharmacia) fitted with a 40 μm mesh nylon net (Wright Scientific Ltd., Kenley, England). The eluting medium was 0.147 M NaCl containing 2.7 mM KCl, 2 mM $MgCl_2$, 5.6 mM glucose and 5 mM PIPES buffer (pH 7.3 and for experiments involving neuraminidase pH 6.4) and the column was eluted at a flow rate of 2 ml/min. Routinely platelets emerged from the column after the first 10 ml of eluate and two aliquots (15 ml each) were collected with a cell count ranging from 6–12×10^8 platelets/ml. Samples required with higher cell counts were prepared by a further centrifugation on albumin [8]. Platelet counts were performed by standard haemocytometry.

In addition to gel filtration on Sepharose 2B a number of samples were gel filtered on the same support enriched with purified apyrase immobilized on cyanogen bromide treated Sepharose 2B (effective concentration of apyrase 200 μg/ml; specific activity of enzyme 0.18 μmole inorganic phosphorus/min/mg). Apyrase is reported as containing platelet aggregating activity, caused by the presence of potato lectin which can be removed from the enzyme preparation by adsorption on insolubilized fetuin [10]. In this work all samples of apyrase (Sigma, Grade I) were purified by affinity chromatography on fetuin (Sigma, Type III) immobilized on Sepharose 4B (7.2 mg fetuin/ml of packed beads) following closely the procedure described by Wigham *et al.* [10].

Platelet aggregometry. The aggregation of platelets in platelet rich plasma or suspensions in buffered medium (pH 7.3) adjusted with platelet poor plasma to similar cell count ($2.5–4.5 \times 10^8$ platelets/ml) was monitored in a Born–Michal Aggregometer (Mark IV) using the method described by Born [11].

Uptake of 5-hydroxytryptamine by platelets. 5-hydroxy[^{3}H]tryptamine creatine sulphate [400 Ci/mole] was prepared by adjusting labelled material with cold compound (Sigma). Uptake was then measured (final concentrations of 0.25–4.0 μM were examined) over periods of 10 to 60 sec using the centrifugation procedure of Drummond and Gordon [12].

For the study of the extended (up to 4 hr) uptake of 5-HT by platelets the procedure of Born and Gillson [13] was employed. Platelets were separated from the suspending plasma by centrifugation at $10{,}000\,g_{av}$ for 30 sec through silicone oil (MS 200; MS 702 (Hopkin and Williams) 1:9 v/v), the platelet pellet being frozen in liquid nitrogen to enable the complete removal of plasma to be accomplished.

Preparation of sialyltransferase (EC 2.4.99.1) enriched fraction. Wistar rats fasted for 20 hr were decapitated and the livers removed immediately onto ice and minced finely with scissors. Routinely, two livers were used for each preparation. An aliquot (50 ml) of ice cold homogenisation medium consisting of 0.5 M sucrose containing 1% w/v dextran (Sigma m.wt fraction 200,000–275,000), 5 mM $MgCl_2$ and made 37.5 mM with respect to Tris–maleate (pH 6.5) was added to the liver and after occasional stirring for 10 min the fluid was decanted from the liver tissue and replaced with a fresh aliquot (25 ml) of ice cold homogenisation fluid. The liver was then homogenised for 60 sec using a Polytron homogeniser (Type PT10-35, Kinematica GmbH, Luzern, Switzerland) set at the lowest speed. Following passage through a double layer of medical gauze the homogenate was centrifuged at $2000\,g_{av}$ for 20 min at 4°. After removing the supernatant fluid the friable upper third of the pellet was resuspended by gentle shaking with fresh homogenisation medium (1 ml) and layered onto a barrier consisting of 1.25 M sucrose containing 1% w/v dextran, 5 mM $MgCl_2$ and made 37.5 mM with respect to Tris–maleate (pH 6.5). Following centrifugation at $100{,}000\,g_{av}$ for 40 min at 4° the material collecting at the sucrose interface, shown by electron microscopy to be enriched in dictyosomes and corresponding to the Golgi apparatus-enriched fraction of Morré *et al.* [14], was removed into ten vol. of ice cold homogenisation medium with a wide bore Pasteur pipette and centrifuged at $2000\,g_{av}$ for 30 min at 4°. The resultant pellet was resuspended in a small quantity (300 μl) of ice cold homogenisation medium and used within one hour of preparation.

Sialyltransferase activity in crude liver homogenates and dictyosome enriched fractions was assayed using both glycoprotein and glycolipid acceptors. Activity against asialofetuin, prepared as described previously [15], was measured using CMP-*N*-acetyl [^{14}C]neuraminic acid by the method of Schachter *et al.* [16], except that the radioactive product was precipitated in 1% w/v phosphotungstic acid in 0.5 M HCl and processed as described by Bosmann [17]. Sialyltransferase activity towards glycolipid acceptors was measured by the method of Kaufman and Basu [18] using *N*-stearoyldihydrolactocerebroside [1-*O*-(β-D-lactosyl)*N*-octadecanoyl-DL-dihydrosphingosine] and *N*-palmitoyldihydrolacto-cerebroside [1-*O*-(β-D-lactosyl)*N*-hexadecanoyl-DL-dihydro-sphingosine] (Miles

Laboratories Ltd., Slough, England). Routinely the sialylated product was extracted from the reaction mixture into chloroform–methanol (2:1 v/v) and counted on glass paper squares [19]. Kaufman and Basu [18] have shown that it is possible to separate nucleotide-sugar and free sialic acid from the sialylated glycolipid by high voltage electrophoresis in 0.05 M borate buffer (pH 9.0). Using a Shandon model L24 water cooled apparatus operating at 3700 V for 50 min we demonstrated that chloroform–methanol extraction quantitatively removes sialylated lipid uncontaminated with sugar nucleotide and free sialic acid.

Treatment of platelets with exogenous sialyltransferase. Platelet rich plasma was treated with rat liver sialyltransferase according to the procedure described by Mester *et al.* [6]. As a source of sialyltransferase these authors used rat liver homogenate, of unspecified activity; the homogenate used here was prepared by the method of Schachter *et al.* [16] and when tested against asialofetuin as acceptor in the presence of detergent [16] had a specific activity of 12.1 nmoles *N*-acetylneuraminic acid transferred/hr/mg protein.

Samples of gel filtered platelets (1.3×10^8) before or after treatment with insolubilized neuraminidase (see below) were incubated at 37° for 90 min with CMP-*N*-acetyl-[^{14}C]neuraminic acid (35.7 nmoles) and sonicated dictyosome enriched material (90–105 μg protein; specific activity 147–170 nmoles *N*-acetylneuraminic acid transferred/hr/mg protein) in a total vol. of 110 μl of buffered saline (147 mM NaCl containing 2.7 mM KCl, 2 mM $MgCl_2$, 5.6 mM glucose and made 5 mM with respect to PIPES buffer (pH 6.4). A sample (55 μl) was taken and adjusted to 3.5×10^8 cells/ml for 5-HT uptake and another aliquot (35 μl) taken for radioactive determination.

Preparation of immobilized neuraminidase (EC 3.2.1.18). Cyanogen bromide activated Sepharose 6MB beads (0.5 g; Pharmacia, Sweden) were mixed end over end with neuraminidase (two batches were used in this work, one of 500 units/ml and the other of 4635 units/ml) and bovine serum albumin (2 mg; Sigma crystallised essentially globulin-free containing less than 0.005% fatty acids) dissolved in 0.5 M NaCl containing 0.2 M $NaHCO_3$ (pH 8.3) for 18 hr at 4°. Following extensive washing on 0.5 M NaCl containing 0.1 M $NaHCO_3$ (pH 8.3) the macrobeads were treated with 1 M ethanolamine (adjusted to pH 9.0 with concentrated HCl) for 2 hr at 4°. After washing in three cycles of 0.5 M NaCl containing 0.1 M acetate buffer (pH 4.0); water; 0.5 M NaCl containing 0.1 M $NaHCO_3$ (pH 8.3) the beads were suspended in 0.154 M NaCl containing 0.009 M $CaCl_2$ and made 0.05 M with respect to acetate buffer (pH 5.5). A sample of beads in which neuraminidase was excluded from the above coupling procedure was also prepared.

The activity of the immobilized neuraminidase was measured by determining the release of free sialic acid when aliquots (0.1 ml packed beads) of the treated macrobeads were incubated for varying time periods at 37° with fetuin (1 mg; Sigma Type III containing 6.9% *N*-acetylneuraminic acid) in a total volume of 110 μl 0.154 M NaCl 0.009 M $CaCl_2$ made 0.05 M with respect to acetate buffer (pH 5.5).

Treatment of platelets with immobilized neuraminidase. Gel filtered platelets were prepared in 0.147 M NaCl containing 2.7 mM KCl, 2 mM $MgCl_2$, 5.6 mM glucose and made 5 mM with respect to PIPES buffer (pH 6.4) at a final concentration of 1.4×10^9 platelets/ml. Neuraminidase immobilized on macrobeads (0.2 ml packed beads) was incubated with aliquots of platelet suspensions (0.7 ml) made 2 mM with respect to $CaCl_2$ and shaken regularly for periods of up to 1 hr at 37°. At the end of the incubation period the beads were allowed to sediment at 1 *g* and samples of the bead-free platelet suspension examined for 5-HT uptake [12]; samples of cell free supernatant fluid were examined for the presence of free sialic acid [20]. Control experiments in which platelet suspensions were treated with neuraminidase-free albumin, immobilized on macrobeads, were also examined under identical conditions to those used with insolubilized enzyme.

Analytical procedures. Free sialic acids were determined by the 2-thiobarbituric acid method of Warren [20] using *N*-acetylneuraminic acid (Sigma type IV) as a standard. To determine total sialic acids in gel filtered platelets known quantities of cells were hydrolyzed in 0.05 M H_2SO_4 at 80° for 1 hr and the hydrolysates fractionated on Dowex 1-X8 resin (formate form) as described in detail elsewhere [21]. Protein was determined by the method of Lowry *et al.* [22], with bovine serum albumin prepared as a protein standard by Armour Pharmaceutical Co., Eastbourne, England. Apyrase (EC 3.6.1.5) and insolubilized apyrase were assayed against ADP, the trichloroacetic acid soluble inorganic phosphorous being measured by means of the method of Fiske and Subba Row [23]. To minimise the ADP blanks care was taken to develop the colour at 0° as described by Wallach and Kamat [24].

Thin layer chromatography was performed on thin layers (0.25 mm) of silica gel on 20 cm × 20 cm glass plates (Machery-Nagel, Düren, Germany). Gangliosides were extracted from gel-filtered platelets using the techniques described in detail for these cells by Marcus *et al.* [25] and the plates were developed twice with chloroform–methanol–2.5 M NH_4OH (60:40:9 by vol) as described by these authors [25]. Two dimensional gel electrophoresis on reduced platelet material was performed using 12 × 7 × 0.3 cm slab gels (Type GSC-8, Pharmacia Fine Chemical AB, Sweden) following the method of Clemetson *et al.* [26].

RESULTS

Properties of gel filtered platelets. The aggregation responses of platelets which have been concentrated on an albumin cushion followed by gel filtration on Sepharose 2B or Sepharose 2B containing immobilized apyrase are compared with platelet rich plasma in Fig. 1; these traces are typical of samples from the same subject and similar results were obtained from other donors. In these experiments the concentration of the gel filtered platelets was adjusted to 3.5×10^8 platelets/ml for direct comparison with platelet rich plasma. At a final concentration of 10^{-5} M ADP the aggregation responses of platelet rich plasma and gel filtered cells were identical,

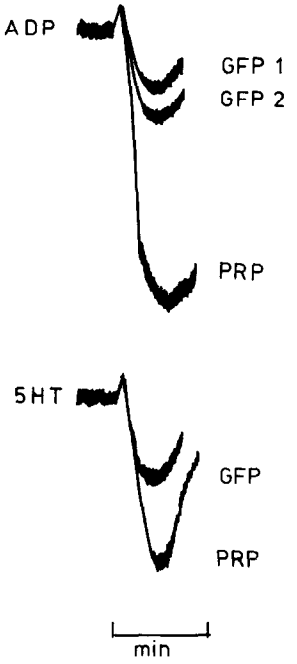

Fig. 1. Comparison of aggregation of platelet rich plasma (PRP) and gel filtered platelets induced by 10^{-6} M ADP (top) or 10^{-5} M 5-HT (bottom). Platelets concentrated on albumin and then gel filtered on Sepharose 2B or Sepharose 2B enriched with immobilized apyrase show a decreased extent of aggregation with ADP (trace GFP1) when suspended in platelet-poor plasma; further concentration on albumin produces no alteration to this trace. The effect of adding 200 μg/ml apyrase to PRP prior to albumin concentration and gel filtration is shown in trace GFP2. GFP refers to the trace obtained when platelets fractionated under conditions used in GFP1 and 2 above are aggregated with 5-HT when suspended in platelet poor plasma. In all the samples the cells were examined at 3.5×10^8 platelets/ml.

however at 10^{-6} M ADP platelets which had been centrifuged onto albumin and then gel filtered were decreased in the extent of aggregation (see Fig. 1, top curve). This diminished response was retained even when filtration on immobilized apyrase was performed. The response was increased slightly in extent when the initial albumin cushion centrifugation was performed in the presence of soluble apyrase (GFP2). With platelet aggregation induced by a final concentration of 10^{-5} M 5-HT there was a decrease in the extent but not in the velocity of aggregation of the gel filtered platelets.

Uptake of [^{3}H]-*5-hydroxytryptamine.* In the centrifugation procedure the uptake by gel filtered platelets of 0.5 and 2.5 μM 5-HT was linear over the 30 and 60 sec periods examined respectively. Linear regression analysis was used to obtain the parameter of the best-fitting straight line (see Fig. 2). Wilkinson analysis [27] of data obtained over the concentration range 0.5–4.0 μM 5-HT for the same donor gave K_m = 0.99 ± 0.11 μM and V_{max} = 0.44 ± 0.06 pmole/10^8 cells/sec; the K_m value is in excellent agreement with the value of 1.0 μM obtained by Gordon and Olverman [28] but the V_{max} is low. Wide variation in observed rate of 5-HT accumulation has been reported (see also Table 1) and may be a result of differences in the number of uptake sites on the donor's platelets rather than in their affinities for the amine.

To control for labelled intercellular amine trapped in the platelet pellet at zero time the extracellular volume was measured. Born and Bricknell [29] obtained values for the extracellular volume of platelet pellets of 0.35 μl/10^8 cells with inulin and 0.40 μl/10^8 cells using albumin, whereas Feinberg *et al.* [30] using human albumin reported a value of 0.29 μl/10^8 cells. A sample of untreated platelets gave in our hands values of 0.38 and 0.45 μl/10^8 cells as measured with mannitol and sucrose respectively.

Table 1. Effects of desialylation of gel filtered platelets on the accumulation and surface binding of [^{3}H]-5-HT

Extent of desialylation of platelets treated with immobilized neuraminidase (%)	Rate of accumulation of [^{3}H]-5-HT* (pmoles/10^8 cells/sec)		Binding of [^{3}H]-5-HT at zero time* (1000 molecules/cell)	
	Untreated	Enzyme treated	Untreated	Enzyme treated
18.0	0.92 ± 0.07	1.10 ± 0.04	57 ± 16	18 ± 10
26.1	0.65 ± 0.05	0.79 ± 0.07	102 ± 12	66 ± 17
31.9	1.16 ± 0.07	1.22 ± 0.11	94 ± 16	62 ± 26
40.8	1.24 ± 0.06	1.08 ± 0.08	56 ± 14	32 ± 19
51.5	2.17 ± 0.09	1.38 ± 0.07	64 ± 21	25 ± 18
61.1	1.85 ± 0.06	1.36 ± 0.07	98 ± 15	49 ± 17
0	1.39 ± 0.07		78 ± 16	
	2.12 ± 0.11		73 ± 25	
	0.53 ± 0.03		42 ± 7	
	0.30 ± 0.04		39 ± 4	
	0.29 ± 0.02		48 ± 4	
	Mean ± S.E.M.		Mean ± S.E.M.	
	1.15 ± 0.21		68.3 ± 6.7	

Treatment of platelets with neuraminidase immobilized on macrobeads; binding and accumulation of [^{3}H]-5-HT is described in Materials and Methods.

* Each determination was performed in quadruplicate and the errors given are standard errors on the gradient and intercept of the regression line for [^{3}H]-5-HT over a 60 sec time course.

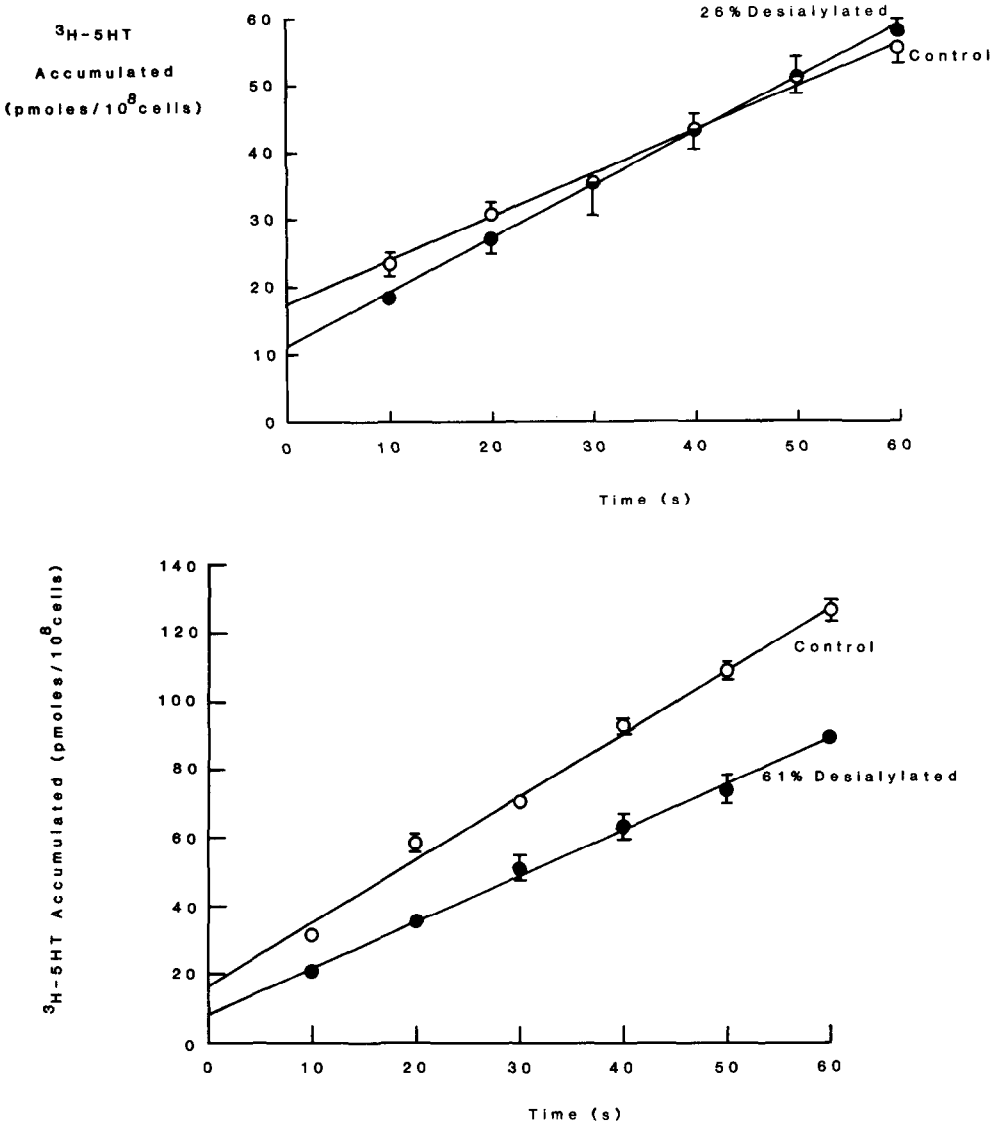

Fig. 2. The uptake of 2.5 μM 5-HT by gel filtered platelets and the effect of immobilized neuraminidase on the process. (A) ● = platelets from which 26% of the total available sialic acid has been removed, ○ = control. (B) ● = platelets from which 61% of the total available sialic acid has been removed, ○ = control. Aliquots (200 μl) of platelet suspension (4×10^8 cells/ml) were incubated with [^{3}H]-5-HT (2.5 μM initial concentration) at 37° and uptake measured as described in Materials and Methods. Each point represents mean ± S.E.M. of four determinations and the regression line is calculated by the method of least-squares.

The same measurements performed on platelets from which 27 per cent of the total sialic acid had been removed produced no significant changes with values of 0.27 and 0.50 μl/10^8 cells. In experiments performed at 0° in which extracellular space and non-specific binding of [^{3}H]-5-HT in the platelet pellet was measured (over range 0.001–10 μM [^{3}H]-5-HT) by competing for specific binding with cold amine [31] it was found that the '5-HT space' was greater than that measured with mannitol or sucrose and varied in magnitude depending on concentration of labelled amine used; this latter result is in agreement with Born and Bricknell [29]. There was close agreement between experiments performed on untreated platelets and platelets from which 40 per cent of the total sialic acid had been removed; at the initial concentration of 2.5 μM 5-HT at a value of 2.1 μl/10^8 platelets which corresponds to about 6000 molecules/cell, was obtained.

The effect of gel filtration on the uptake of 5-HT was studied over a 4 hr period by the method of Born and Gillson [13] and the results are displayed in Fig. 3. It can be seen that the gel filtered platelets and platelets concentrated on an albumin cushion

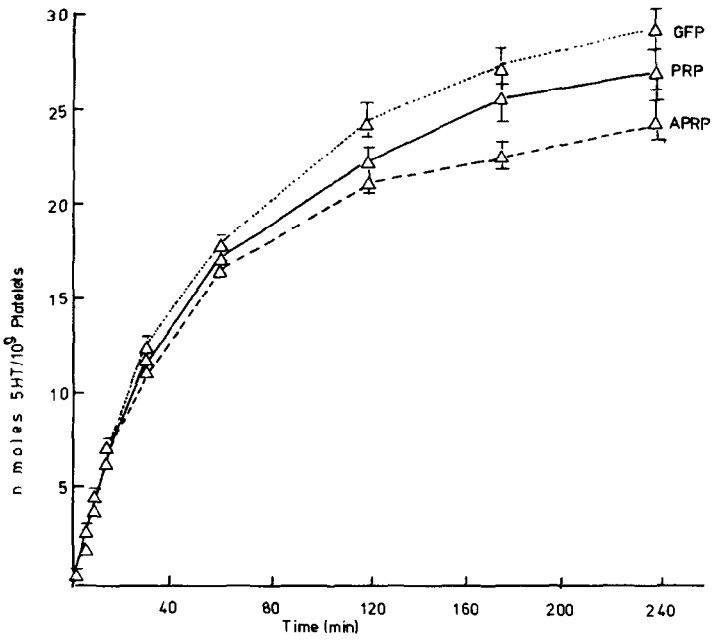

Fig. 3. Uptake of 5-HT over a 4-hr period by platelet-rich plasma and gel-filtered platelets. The concentration of 5-HT was maintained at 10 μM as described in Materials and Methods. PRP = platelet rich plasma, APRP = PRP which had been concentrated on an albumin cushion and GFP = APRP following gel filtration on Sepharose 2B. In all the experiments the cell count was adjusted to 3.5×10^8 platelets/ml. Each point represents the mean of duplicate experiments ± range.

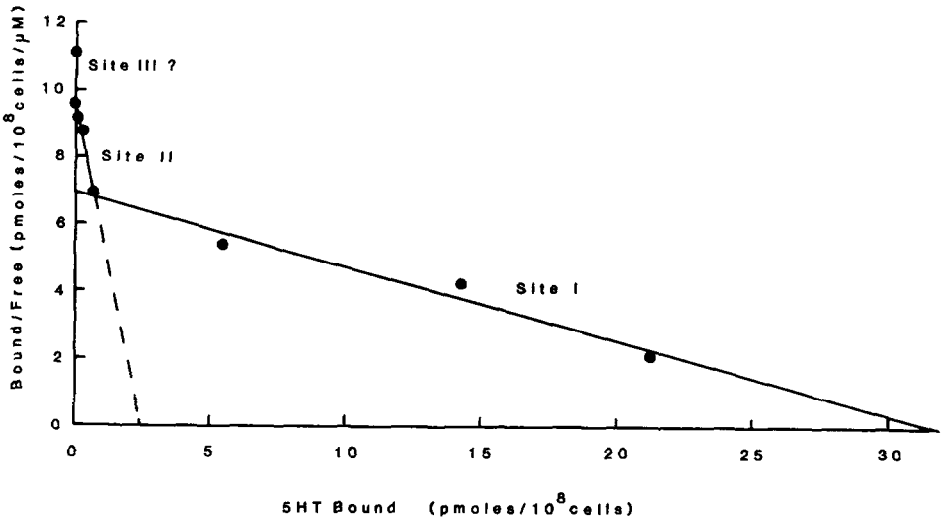

Fig. 4. Scatchard analysis of [^{3}H]-5-HT binding to gel filtered platelets at 0°. Aliquots (200 μl) of platelet suspension (4×10^8 cells/ml) were incubated at 0° and the amount of radiolabel measured as described in Materials and Methods. Specific binding of [^{3}H]-5-HT was determined by subtracting the values obtained when the incubations were performed in the presence of 10^{-4} M 5-HT (unlabelled); under which conditions binding of labelled 5-HT was proportional to the concentration of radiolabelled ligand. *Site I*: Apparent $K_d = 4.6$ μM, capacity 176,000 5-HT molecules/cell. *Site II*: Apparent $K_d = 0.26$ μM, capacity 15,000 5-HT molecules/cell. The possibility of a third high affinity site cannot be excluded.

Table 2. Substrate specificity of the sialyltransferase activity of the rat liver dictyosome-enriched fraction

Substrate	Specific activity* (nmoles sialyl residues transferred/hr/mg protein)	Activity ratio†	Per cent sialylation of substrate‡
Asialofetuin	137.5 ± 5.3	30	12.0
N-Stearoyl-dihydrolactocerebroside	3.7 ± 0.50	19	0.8
N-Palmitoyl-dihydrolactocerebroside	1.9 ± 0.07	10	0.4

* Sialyltransferase activity was assessed in the presence of detergent as described in Materials and Methods using dictyosome-enriched material (208 μg protein per assay). The assays were performed in triplicate and mean ± S.E.M. is quoted.

† Refers to the ratio of the specific activity of the dictyosome-enriched fraction relative to the starting homogenate.

‡ 239 and 100 nmoles *N*-acetylneuraminic acid represents 100% sialylation of the glycoprotein and glycolipid substrates respectively.

have very similar long term uptake and storage properties to platelets present in platelet rich plasma.

From the effects of chlorimipramine and methysergide on the rate of accumulation of 2.5 μM 5-HT over 60 sec the estimated IC_{50} for chlorimipramine is 6×10^{-9} M and for methysergide 10^{-4} M. This latter value is in good agreement with the value of 125 μM obtained by Born *et al.* [32] for the K_i of this drug though the IC_{50} with chlorimipramine is well below the value of 3.2×10^{-7} M reported by Todrick and Tait [33] obtained using platelet-rich plasma; in the presence of plasma the effective concentration is likely to be lowered, by binding to proteins.

Surface binding of 5-hydroxytryptamine. Scatchard analysis of [^{3}H]-5-HT binding to gel filtered platelets of 0° is shown in Fig. 4 and indicates that there are at least two distinct binding sites, arbitrarily called sites I and II. The capacity and affinities of Site I are $K_d = 4.6$ μM, capacity = 29.2 pmoles/10^8 cells. 176,000 sites/cell and for site II are $K_d = 0.26$ μM, capacity = 2.5 pmoles/10^8 cells, 15,000 sites/cell. The possibility of a third low capacity (1000 sites/cell; $K_d = 0.014$ μM) site cannot be excluded.

Platelet sialic acid content and neuraminidase treatment. The total sialic content of three samples of gel filtered platelets was 71 ± 8 nmoles/10^9 cells a figure in excellent agreement with the figure of 76 ± 7 nmoles/10^9 cells calculated from the values quoted in the literature by four separate groups of workers [5, 34, 35, 36] for saline washed platelets, assuming 1.8 mg protein/10^9 cells.

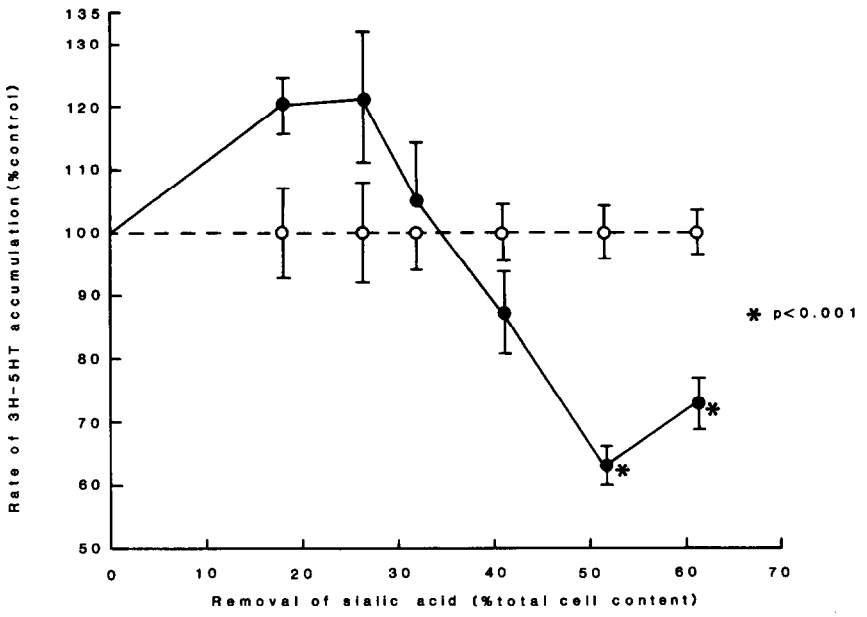

Fig. 5. Effect of graded desialylation on the uptake of 5-HT by gel filtered platelets. Data were normalised such that the rate of accumulation of 5-HT by untreated platelets was 100%. The gradient of the uptake curve at each degree of desialylation is expressed relative to the control cells. ● = platelets treated with immobilized neuraminidase, ○ = control. The error bars represent 1 S.E.M. and * = $P < 0.001$.

Table 3. Treatment of gel filtered platelets with exogenous sialyltransferase of the dictyosome-enriched fraction of rat liver

	Acceptor*	Specific activity of dictyosome-enriched fraction† (nmoles *N*-acetylneuraminic acid transferred/hr/mg protein)	Sialosyl residues transferred to acceptor‡ (nmoles *N*-acetylneuraminic acid)	Per cent sialylation before treatment with exogenous sialyltransferase	Per cent sialylation after treatment with exogenous sialyltransferase
Experiment 1	Untreated platelets	170	0.01 ± 0.006	100.0	100.1
	Platelets from which 30% total sialic acid removed following immobilized neuraminidase treatment	170	0.02 ± 0.006	70.0	70.2
	Asialofetuin	170	22.95 ± 1.36	0	6.4
Experiment 2	Untreated platelets	151	nil	100	100
Experiment 3	Untreated platelets	147	nil	100	100

* 1.26×10^8 platelets or 1 mg asialofetuin were treated for 90 min with sonicated dictyosome-enriched fraction (90, 105 and 95 μg protein for Expts 1, 2 and 3 respectively) as described in Materials and Methods.

† The specific activity of sonicated dictyosome enriched fraction was measured against asialofetuin in the absence of detergent. In Expt. 1 the transfer of sialosyl residues to asialofetuin was measured at the same time that the acceptor properties of the platelets were determined.

‡ Experiments performed in triplicate; mean ± S.E.M. quoted.

Two samples of neuraminidase (500 and 4635 units/ml respectively) immobilized on macrobeads were used and had measured activities of 40 and 140 units/ml packed beads, when assayed at pH 5.5. The bulk of the experiments were performed with the higher specific activity beads; under the conditions of treatment sialic acid was removed from gel filtered platelets at the rate of 0.6 nmole/min/10^9 cells. Over the four months period that these beads were used the enzyme activity was found experimentally to remain unchanged. No detectable sialic acid was released from platelets by incubating the cells with albumin immobilized on macrobeads.

Characterization of sialyltransferase preparation. The sialyltransferase activity of the rat liver dictyosome enriched fraction has a preferred specificity for the glycoprotein acceptor under the conditions assayed (Table 2); 14 per cent of this latter activity being recovered from the homogenate in the fraction. In the absence of detergent the relative specific activity of the dictyosome and homogenate fractions was decreased 95 and 98 per cent respectively. Sonication in an ice bath for a total of 2 min at 10 μm amplitude in bursts of 5 sec at 55 sec intervals increased the specific activity of the dictyosome enriched fraction and homogenate to levels comparable to those measured in the presence of detergent.

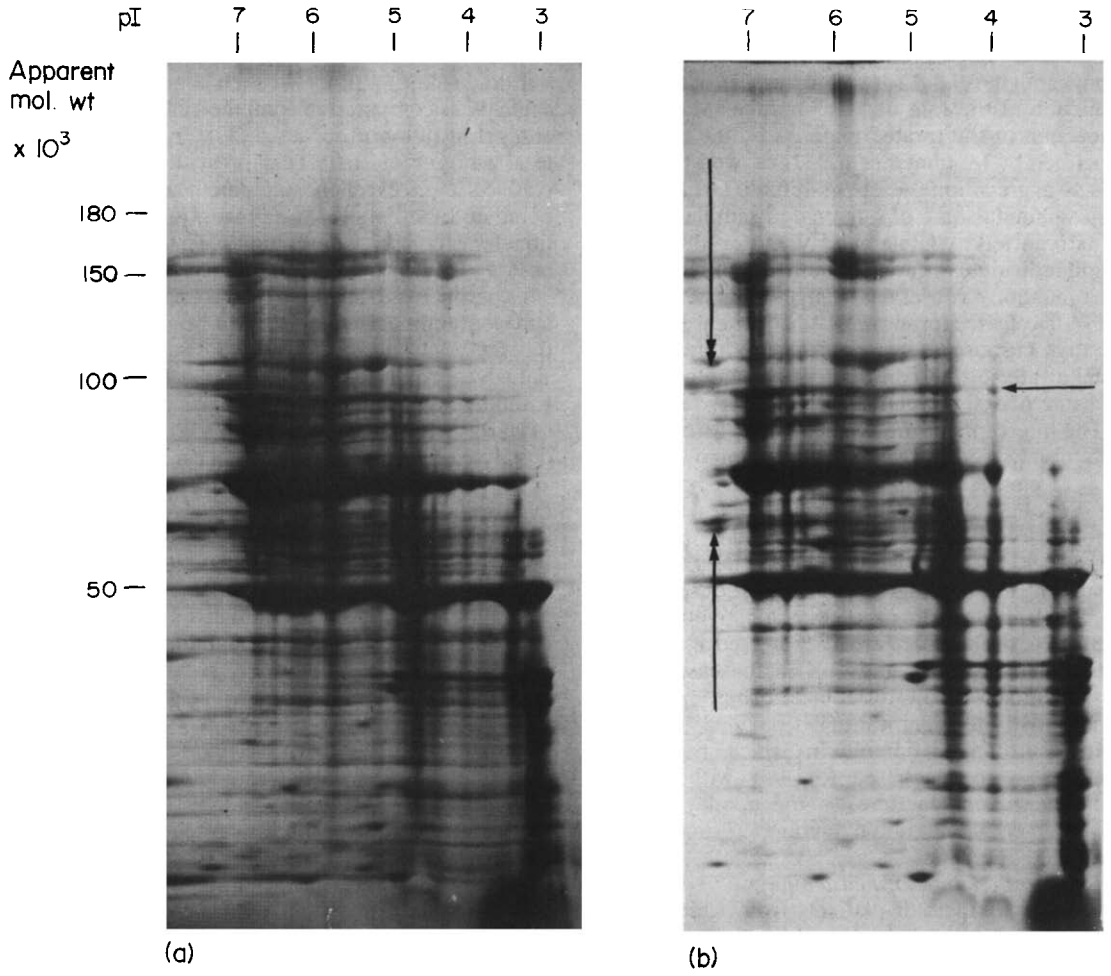

Fig. 6. Comparison of two-dimensional gel electrophoresis pattern of neuraminidase treated and untreated platelets. (A) Control sample. (B) Platelets from which 20% of the total sialic acid had been removed by treatment with immobilized neuraminidase. Equivalent amounts of platelets (8.4×10^8 cells) were processed in each case as described in Materials and Methods. The photographs are of gels stained with Coomassie brilliant blue. Platelets from which 20% of the available sialic acid had been removed possess an extra component of pI 4 and apparent molecular weight <100,000 (shown by horizontal arrow on B); this component also stains with periodic acid/Schiff reagent. In addition after the removal of 20% of the sialic acid Coomassie brilliant blue staining proteins of pI 7.0 in the range 100,000–50,000 apparent molecular weight are now detectable (double arrows in B—note absence of this material in A).

Attempted exogenous sialylation of platelets. No sialylation of platelets could be demonstrated when platelet rich plasma was treated with rat liver sialyltransferase preparation by the method of Mester *et al.* [6].

Treatment of gel filtered platelets with sonicated preparations of sialyltransferase, and CMP-*N*-acetylneuraminic acid was also without effect (see Table 3), even with cells from which 30 per cent of the total sialic acid had been removed. Under the conditions of the treatment transfer of sialosyl residues to soluble glycoprotein acceptor was demonstrated.

Effect of graded desialylation on 5-hydroxytryptamine uptake. The results of six separate experiments in which gel filtered platelets were desialylated to different extents are summarised in Table 1 and Fig. 5. In three experiments where less than 35 per cent of the total cell sialic acid content was removed the gradient of the uptake curve was greater than for the control cells by up to 23 per cent, though statistical analysis (Student's *t* test) showed no significant stimulation. At 50–60% desialylation there was a significant ($P < 0.001$ for the 46 degrees of freedom) reduction in the rate of 5-HT uptake. Microscopic inspection of the treated platelets by the technique described by Motamed *et al.* [37] showed that there was no aggregation (we are grateful to Dr. F. Michal for examining some of our treated samples).

As reported by Gielen and Viehöfer [38] we also found individual differences in the rate of [^{3}H]-5-HT accumulation (observed range 0.3–2.2 pmoles 5HT/10^8 cells/sec for untreated cells), hence for comparative purposes it was necessary to normalise the uptake rate so that control cells accumulated 5-HT at a rate of 100 per cent.

The intercepts of the uptake curves for untreated cells also varied but over a much narrower range (6.5–17 pmoles/10^8 cells at 2.5 μM [^{3}H]-5-HT) than for rate of uptake, the mean value of 11.4 ± 1.1 (11) pmoles/10^8 cells corresponds to $68{,}500 \pm 6500$ molecules per cell. In all cases following neuraminidase treatment the binding of 5-HT was reduced (range 34–68%), however, as they were relatively close to the origin, the standard errors expressed as a fraction were relatively large and only in the case of 61% desialylation was the difference in binding at zero time statistically significant ($P < 0.05$).

In control experiments in which gel filtered platelets were incubated for 1 hr with albumin immobilized on macrobeads, the uptake rate was 97.4 per cent and initial binding 97 per cent of untreated platelets.

Chemical analysis of neuraminidase treated platelets. Lipid extracts of platelets from which 20 per cent of the total sialic acid had been removed possessed an identical pattern of gangliosides when compared on thin layer chromatography with extracts obtained from untreated cells. Three gangliosides, haematoside, lacto-*N*-neotetraose and disialosyllactosyl ceramide known to be present in human platelets were identified by reference to known chromatographic properties [25]; the haematoside spot gave the strongest staining reaction. Similar glycoprotein patterns were found on staining two-dimensional gel electrophorograms with periodic acid-Schiff reagent; an extra component of pI 4.0 and apparent molecular weight < 100,000 (arrowed in Fig. 6) was found in the treated sample. Additional differences were found on staining with Coomassie brilliant blue (Fig. 6); there appears to be a higher concentration of proteins of pI 4 which form a vertical streak in the enzyme treated sample ranging from 120,000 to 15,000 apparent molecular weight. The treated platelets also possess an area of proteins of pI 7 and apparent molecular weight range 100,000–50,000 which appear to have shifted from a pI of 6 when compared to the control sample.

DISCUSSION

The function of sialosyl residues at the platelet surface has been examined by a number of laboratories using two approaches namely exogenous sialylation and neuraminidase treatment. In this work care has been taken to remove the platelets from their suspending plasma using gel filtration. A number of investigators have freed platelets of plasma by repeated centrifugation and resuspension in buffered saline, a process which is potentially damaging [9] as compared to combined albumin gradient and gel filtration procedures [39]. In our hands the total amount of sialic acid present in gel filtered platelets is in excellent agreement with the values obtained by others [5, 34–36] working with conventional washing procedures indicating that the method used by us has removed soluble sialoglycoproteins.

Experiments using exogenous sialyltransferases and neuraminidase are likely to be complicated by the fact that plasma is a rich source of soluble sialoglycoproteins; for example, the effects of neuraminidase treatment on platelet rich plasma are particularly difficult to interpret as the enzyme will desialyse concommitantly both plasma glycoproteins and the platelet surface. Apart from making the quantitation of the extent of the surface desialylation impossible to determine asialoplasma proteins are known to have modified properties [40]. The removal of sialic acid from fibrinogen, for example, reduces its ability to support ADP-induced aggregation [41] and thus the effect of neuraminidase on platelet surface properties could well be masked if such experiments were performed in the presence of partially sialylated plasma proteins.

Reports by Mester *et al.* [6] and Szabados *et al.* [7] that exogenous sialylation enhances aggregation in response to 5-HT [6] as well as accelerating the uptake of this agent [7] have caused much interest. Interest is heightened in this work because Davis *et al.* [4] found that neuraminidase treatment of platelet-rich plasma which is likely to remove sialic acid residues from the platelet surface caused an enhanced aggregation response to ADP and 5-HT. Unfortunately this latter study [4] presented no quantitative information on the degree of desialylation of the platelets. A study by Greenberg *et al.* [5], however, showed that removal of 15 to 66 per cent of total platelet sialic acid caused slightly enhanced responses of human and rabbit platelets to ADP; in the case of the rabbit platelet the removal of 65 per cent of the total sialic acid also slightly enhanced 5-HT-induced aggregation. It is difficult to reconcile these two conflicting bodies of infor-

mation and Greenberg *et al.* [5] have criticised the use by Mester *et al.* [6] of a crude enzyme to affect the increased sialylation which they point out may have altered platelet function.

We have attempted without success to repeat the exogenous sialylation of platelets using either rat liver homogenate with platelet-rich plasma or a dictyosome enriched sialyltransferase preparation with gel filtered cells. It is difficult to reproduce exactly the conditions of Mester *et al.* [6] as the details of the rat liver homogenate are required as well as the activity of the preparation used in their sialylation experiments. In addition the conditions of Szabados *et al.* [7] are also difficult to reproduce; in this later paper the authors purified the homogenate by the method of Schachter *et al.* [16] but neither the degree of purification achieved nor the amount of transferase activity actually present in their experiments is cited. Detergent is known to be required for optimal sialyltransferase activity with glycoprotein acceptors [42] so it is perhaps surprising that Mester *et al.* [6] were able to transfer between 13.3 and 27.9 nmoles sialic acid per 10^9 platelets, an increase of up to 37 per cent of the sialic acid content reported for normal human platelets, in half an hour with a crude rat liver homogenate. In order to achieve comparable levels of sialyltransferase activity to those obtained in the presence of detergent we used sonicated dictyosome-enriched fractions. Whilst such fractions catalyse the sialylation of appropriate soluble glycoprotein acceptors no transfer of sialic acid took place when native platelets were used as potential acceptors. Further, when platelets were examined from which 30 per cent of the total platelet sialic acid had been removed by immobilized neuraminidase, to generate a significant number of potential sialoacceptor sites, no transfer of sialosyl residues was observed. These experiments which have been performed with known quantities of characterized sialyltransferase activity highlight the problems experienced in interpreting data obtained using crude enzyme systems [6] and must throw doubt on the extent to which *N*-acetylneuraminic acid may be regarded as a component of both the receptor sites with which 5-HT reacts to cause aggregation and the receptor responsible for initiating its active transport through the plasma membrane [6, 7].

In view of our inability to investigate by exogenous sialylation the role of *N*-acetylneuraminic acid in the active transport of 5-HT we have turned our attention to the use of neuraminidase. Up to now work in this latter area has been executed with soluble enzyme whilst in these studies we have developed the use of a novel procedure in which the enzyme is immobilized on macro (200–300 μm) beads of Sepharose 6B. Neuraminidase has been shown to penetrate cells [43] and by insolubilizing this glycosidase one can ensure that its actions are confined to the cell periphery. Additionally, macrobeads have the advantage over other bead supports in that they rapidly sediment at 1 *g* allowing the preparation of a bead-free cell suspension on which the effects of quantified, graded desialylation on various cellular processes may be measured readily.

Human platelets treated with neuraminidase have a reduced electrophoretic mobility [44] indicating that sialic acids make a significant contribution to the ionogenic properties of this cell. Interestingly only about 60 per cent of the total sialic acid of the intact platelet is available for release by this enzyme [34, 44], a degree of desialylation which we have been able to achieve in our experiments with insolubilized neuraminidase. Desialylation of platelets does not result in any ultrastructural changes [3], neither does the process induce spontaneous aggregation in saline medium [3] nor the release of platelet granule contents [5]. In 1973 Glynn [45] demonstrated that neuraminidase treatment reduced the uptake by platelets of 5-HT by 45 per cent. The amount of sialic acid removed was not measured, nor were the effects of graded desialylation investigated. Gielen and Viehöfer [38], however, reported that neuraminidase treatment stimulated the uptake of 5-HT over a 2-hr time course. Again the degree of desialylation was not measured and there was no indication as to whether the stimulation (up to about 20%) was statistically significant. By extending the neuraminidase treatment time Gielen and Viehöfer [38] stated that a decreased uptake of 5-HT was generally observed, though no quantitative data were presented. In our experiments, desialylation by up to 30 per cent of the total cellular sialic acid, using immobilized enzyme, may have slightly stimulated the rate of 5-HT uptake, though this was not statistically significant. Further removal of sialic acid residues produced a highly significant ($P < 0.001$) decrease of up to 40 per cent in the rate of accumulation at 50–60% desialylation. It would appear that the different effects observed by Glynn [46] and Gielen and Viehöfer [38] may be explained on the basis of a graded desialylation of the platelet surface.

Gielen and Viehöfer [38] make the point that it is not yet known whether the 5-HT receptor on the platelet surface is associated with sialoglycoproteins or with gangliosides deeper within the cell periphery. We find that within the limits of the sensitivity of the method the ganglioside pattern extractable from platelets lacking 20 per cent of their total sialic acid and untreated cells are identical. However at this stage of desialylation, when an increased rate of accumulation of 5-HT is measurable an extra periodic acid-Schiff positive component of pI 4 and apparent molecular weight <100,000 could be found by two dimensional gel electrophoresis in the treated sample. This staining procedure is not very sensitive and on staining the gel for protein other differences were found which would indicate that extensive modification of platelet membrane protein is taking place. The removal of charged sialosyl residues is known to cause changes in the orientation of surface components of cells [46] so it is possible that the modification of membrane glycoproteins in the initial stages of platelet desialylation (up to removal of 30% total sialic acid) may stimulate uptake of 5-HT as a result of surface reorientation phenomena.

The fact that there is no significant reduction in the rate of uptake of 5-HT until 50 per cent of the total cell sialic acid has been removed suggests that the translocation receptor may be some distance from the cell periphery and may be initially protected from the action of the immobilized enzyme. It has been proposed that the translocation receptor for 5-

HT is a ganglioside and gangliosides I and III extracted from human platelets have been shown to bind this amine *in vitro* at 4° [25] and are susceptible to the action of neuraminidase. Marcus *et al.* [25] have shown that ganglioside I (haematoside), the major ganglioside of the human platelet, binds irreversibly to 5-HT in the molar ratio of 218:1 at 4°. Using this information it can be calculated that the haematoside content of one platelet *in vitro* at 4° could bind 11,000 molecules of 5-HT, a figure which is close to the value of 15,000 sites per cell (Site II) which we obtained from Scatchard analysis of measurements made at the same temperature. At 4°, platelets do not actively transport 5-HT and the binding properties of the translocation receptor at this temperature may differ from its properties at 37°, thus the irreversible nature of 5-HT binding to the haematoside at 4° might not rule it out as a candidate for the translocation receptor. The molecular basis for a binding ratio of 218:1, however, is entirely unclear and it is questionable as to whether a similar ratio would be observed *in vivo*.

Woolley and Gommi [47] have demonstrated that the sensitivity of various tissues to 5-HT which is destroyed by neuramihidase may be partially restored by the addition of gangliosides [47]; however, Carroll and Sereda [48] have argued that the assumption that a material which restores activity is necessarily the naturally occurring receptor must be questioned. These latter authors [48] working with uterine muscle membranes point out that the sialic acid in this organelle is chiefly in the form of glycoproteins and that neuraminidase acts predominantly on non lipid-soluble sialic acids. The sialic acids of the glycoprotein, as opposed to glycolipids, are considered to be more peripherally located in cells [49] and therefore the ability to achieve a controlled and graded desialylation of cells with neuraminidase immobilized on macrobeads presents an additional technique for investigating this type of problem.

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