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Human platelet arylsulphatases: identification and capacity to destroy SRS-A

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Summary. Arylsulphatases IIA and IIB have been separately identified in human platelets by use of anion exchange chromatography and gel filtration. Arylsulphatase IIA had a molecular weight of 160,000 and a pH optimum of 4·5. Arylsulphatase IIB had a molecular weight of 60,000 and a pH optimum of 5·5. Both arylsulphatases IIA and IIB were inhibited by phosphate and sulphate ions characteristic of this enzyme class. Platelets, upon exposure to ionophore A-23187 or thrombin, discharged arylsulphatase coincident with β-glucuronidase release. Partially purified platelet arylsulphatase IIB inactivated rat SRS-A.

INTRODUCTION

The arylsulphatases (aryl-sulphate sulphohydrolases, EC 3.1.6.1) type IIA from human lung (Wasserman & Austen, 1976) and rat peritoneal mast cells (Orange & Moore, 1976), and type IIB from human lung (Wasserman & Austen, 1976) and human eosinophils (Wasserman, Goetzl & Austen, 1975) have been shown to inactivate a slow reacting substance of anaphylaxis (SRS-A). It has been suggested that the eosinophil or lung-derived arylsulphatases may play a regulatory role in immediate hypersensitivity reactions by destroying SRS-A. Arylsulphatase-like activity has also previously been reported in rabbit (Murata, Negata & Spicer, 1975), rat (Bentfeld & Bainton, 1975), and human (Polasek & Rotrekl, 1967) platelets. In addition, platelet-activating factor (PAF) (Benveniste, Henson & Cochrane, 1972) or thomboxane A2 (Dawson, Boot, Cockerill, Mallen & Osborne, 1976), generated as a consequence of immediate hypersensitivity reactions, is capable of aggregating and degranulating platelets. Thus, we reasoned that the platelet might also contribute to the destruction of SRS-A.

Therefore, we have examined and functionally characterized human platelet arylsulphatases. Herein we describe the identification of arylsulphatases of types IIA and IIB within human platelets as determined by diethylaminoethyl cellulose (DE-52) and Sephadex G-200 chromatography, pH optima and use of selective inhibitors. Arylsulphatase is released from platelets after interacting with thrombin or the ionophore A-23187. Further, platelet arylsulphatase IIB inactivates SRS-A. Platelet aggregation and degranulation accompanying immediate hypersensitivity reactions (Kravis & Henson, 1977) might, therefore, be a source

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Abbreviations: SRS-A, slow reacting substance of anaphylaxis; PAF, platelet activating factor; BSG–citrate, buffered glucose saline–citrate; P-NC, para-nitrocatechol; P-NCS, para-nitrocatechol sulphate; mU, milliunit; Tyr-hep, Tyrode's solution with heparin; BSG, buffered saline glucose.

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of arylsulphatase activity, which may contribute in the control of allergic reactions by destroying SRS-A.

MATERIALS AND METHODS

Materials

Sephadex G-200 fine, blue dextran, ovalbumin, chymotrypsinogen (Pharmacia Fine Chemicals, Piscataway, New Jersey); atropine sulphate, 4-nitrocatechol, *p*-nitrocatechol sulphate, phenolphthalein glucuronic acid (0·01 M, pH 7·0), pyruvic acid type II-Na salt, β-nicotinamide, adenine dinucleotide reduced form—disodium salt Grade III, β-glucuronidase type VI-A: *Escherichia coli*, lactic dehydrogenase type XII: human red blood cells, thrombin-bovine plasma grade I (Sigma Chemical Co., St Louis, Missouri); disodium ethylenediaminetetraacetate (Fisher Scientific Co., Fair Lawn, New Jersey); DEAE–cellulose (DE-52) (Whatman Chemicals, Div. W. & R. Balston, Maidstone, Kent, England); amberlite XAD-7 (Mallinkrodt Chemical Works, St Louis, Missouri); histamine diphosphate grade A (Calbiochem. Inc., San Diego, California); and mepyramine maleate (Merck, Sharp and Dohme, Inc., West Point, Pennsylvania) were obtained from the manufacturers. The ionophore A-23187 was kindly provided by the Eli Lilly Co. A-23187 was dissolved in dimethyl sulphoxide (10 mg/ml) and kept at −20°.

Isolation of platelet arylsulphatases

Outdated platelet packs (greater than 72 h old) were obtained from the Blood Bank of the National Institutes of Health. Each unit of platelets was resuspended in 50 ml of buffered glucose saline–citrate (BSG–citrate) consisting of 117 mM NaCl; 13·6 mM Na citrate–dihydrate; 11 mM glucose; 7·6 mM Na_2HPO_4; 1·4 mM KH_2PO_4, pH 7·4 (Corash, Tan & Gralnick, 1977). Contaminating leucocytes were removed by centrifugation at 200 ***g*** for 10 min at room temperature. The actual platelet count in the platelet-rich supernatant was determined by phase microscopy. Platelets were washed three times in BSG–citrate by repeated centrifugation at 2000 ***g*** for 10 min at room temperature and the final preparation was free of contaminating leucocytes. The platelets were resuspended in 50 ml 0·1 M Tris, pH 8·0, per unit of platelets and homogenized with a Brinkman Polytron (Kinematica, Liyern, Sweden). The resultant suspension was centrifuged at 15,000 ***g*** for 15 min to remove cellular debris. The supernatant was removed, dialysed against 40 vol of 0·01 M Tris-HCl buffer, pH 8·0, at 4°, and applied to a 6 × 15 cm DE-52 cellulose column equilibrated in the same buffer. The column was washed with three bed volumes of the equilibrating buffer and eluted with a 1200 ml linear salt gradient to 0·4 M NaCl at 50 ml/h (Wasserman & Austen, 1976). Fractions of 4·0 ml were collected and 0·15 ml of each assessed for arylsulphatase activity. Effluent and eluate fractions containing arylsulphatase activity were concentrated to 2 ml each by ultrafiltration (Amicon Corp., Scientific Systems Division, Lexington, Massachusetts). Each fraction was applied to a 1·5 × 100 cm Sephadex G-200 column in 0·01 M Tris–HCl buffer, pH 8·0. The columns were eluted at 12 ml/h and 3·0 ml fractions collected. Aliquots of 0·15 ml were assessed for arylsuphatase activity.

Enzyme assays

Arylsulphatase was quantitated by measurement of the generation of *p*-nitrocatechol (*p*-NC) from the substrate *p*-nitrocatechol sulphate (*p*-NCS) (Metcalfe, Gadek, Raphael, Frank, Kaplan & Kaliner, 1977). Enzyme preparations previously dialysed against 0·5 M sodium acetate, pH 5·5, were added to 0·01 M *p*-NCS in 0·5 M sodium acetate, pH 5·5, in a final volume of 1·0 ml and incubated for 4–18 h at 37°. The reaction was terminated by the addition of 0·5 ml of 1 M NaOH and the *p*-NC present determined by colorimetric change at 515 nm (Gilford 250 Spectrophotometer, Gilford Instrument Co., Oberlin, Ohio). A standard curve was generated by the interaction of graded amounts of *p*-NC and base. One milliunit (mu) of enzyme activity is defined as that amount liberating 155 ng *p*-NC/h (1 nmol/h). The pH optimum of both arylsulphatases was determined in 0·5 M acetate buffer with 0·01 M *p*-NCS by altering the sodium acetate to acetic acid ratio.

β-glucuronidase activity was measured by the rate of hydrolysis of phenolphthalein glucuronide after a 4 h incubation at 37°, as determined at 540 nm in alkaline solution (Fishman, 1965). One unit of enzyme activity is that which liberates 1 μg phenolphthalein from a 1mM solution of phenolphthalein glucuronide over 1 h in 0·1 M acetate buffer, pH 4·5.

LDH was measured by consumption of reduced diphosphopyridine nucleotide during reduction of pyruvate to lactate (Bergmeyer, Bernt & Hess, 1965).

Production and bioassay of SRS-A

SRS-A was generated from the rat peritoneal cavity by intraperitoneal challenge with 6 ml of a 1:25 dilution of

sheep anti-rat IgE in Tyrode's solution containing 10 units of heparin per ml (Tyr–hep). Rat peritoneal cavities were lavaged 20 min post-challenge with 10ml of Tyr–hep, and the collections were pooled and centrifuged at 400 *g* for 10 min at 4°. The yield of SRS-A averaged 200 units/rat. The supernatant was adjusted to 80% ethanol, left at 4° for 30 min, centrifuged at 3000 *g* for 10 min, and evaporated to dryness *in vacuo* at 50° in a Buchler flash evaporator (Buchler Instruments Div., Searle Analytic, Inc., Fort Lee, New Jersey). The SRS-A was resuspended in 5·0 ml of 0·1 M NaOH, incubated for 30 min at 37°, and applied over 0·5 h to a 100 ml amberlite XAD-7 column previously washed with 4 l acetone and 8 l distilled water. The column was washed with 400 ml of distilled water over 1 h followed by elution with 400 ml of 80% ethanol. The eluate was evaporated to dryness and stored at −70° (Orange, Murphy, Karnovsky & Austen, 1973). Samples were resuspended in distilled water before dilution into appropriate buffers for interaction with arylsulphatase. SRS-A was assayed on the atropinized, antihistamine-treated guinea-pig ileum as previously described (Brocklehurst, 1960). One unit of SRS-A activity was arbitrarily defined as the concentration required to produce a contraction of the isolated guinea-pig ileum equal in amplitude to that produced by 5·0 ng/ml of histamine base.

Interaction of arylsulphatase IIB with SRS-A

Reaction mixtures containing arylsulphatase IIB, SRS-A or arylsulphatase IIB and SRS-A in combination were incubated in 0·5 M sodium acetate, pH 5·5, at 37° for varying periods of time. At given intervals, aliquots were diluted in Tyrode's buffer, the pH adjusted to 7·8 with 0·01 M NaOH and the SRS-A activity determined by bioassay.

Platelet arylsulphatase release

Blood from normal volunteers who had ingested no aspirin for the preceding 72 h was drawn into 50 ml plastic syringes containing 1·0 ml 0·1 M EDTA, pH 7·4. Platelet-rich plasma was prepared by centrifugation at 200 *g* for 10 min at room temperature. The platelets were sedimented at 1000 *g* for 10 min, washed with BSG–citrate, counted under phase microscopy and resuspended in BSG at a concentration of 5×10^8 platelets/0·75 ml. The BSG consisted of 138·6 mM NaCl, 11 mM glucose, and 7·6 mM Na_2HPO_4, pH 7·4. At time 0, appropriate dilutions of the calcium ionophore A-23187 or thrombin in 0·25 ml BSG were added and the mixture incubated for 40 min at 37°. The reaction was terminated by pelleting the platelets at 2000 *g* for 10 min at 4° and the supernatant removed. The pellet was resuspended in 1·0 ml of BSG, freeze-thawed six times, centrifuged at 2000 *g* for 10 min at 4°, and the supernatant removed. Both the reaction supernatant and pellet extract were dialysed against 0·5 M sodium acetate, pH 5·5, and kept at −70° until assayed. The percentage of total enzyme release was determined by the following equation:

$$\% = 100 \times \frac{\text{Units of enzyme released into supernatant}}{\text{Units of enzyme in platelet lysate and supernatant}}$$

RESULTS

Arylsulphatase activity of isolated platelets

Platelet-rich plasma was obtained from five normal volunteers and the arylsulphatase activity of their platelets found to be $3{\cdot}8 \pm 0{\cdot}3$ mu/5×10^8 platelets. Human eosinophils contain $470 \pm 3{\cdot}0$ mu of arylsulphatase activity/5×10^6 cells (Metcalfe *et al.*, 1977). When these data are corrected for the average number of platelets ($2{\cdot}9 \times 10^8$/cm^3) or eosinophils ($1{\cdot}5 \times 10$/cm^3) present in normal human blood, platelets contain 2·3 mu of activity per cm^3, while eosinophils have 14·1 mu/cm^3. Thus, platelets, on the average, have approximately 16% of the *p*-NCS-hydrolysing activity of eosinophils per cm^3 whole blood.

Separation and characterization of types IIA and IIB arylsulphatase from human platelets

Homogenates of purified platelet suspensions were subjected to DE-52 cellulose anion exchange chromatography. Arylsulphatase activity was detected in the effluent and was also found in the eluate at 12–16 mS (Fig. 1), consistent, respectively, with the presence of arylsulphatase IIB and IIA (Hook, Dodgson, Rose & Warwood, 1973). The arylsulphatase activities in effluent and eluant fractions were separately pooled, concentrated to 2·0 ml by ultrafiltration and filtered over a Sephadex G-200 column (Fig. 2). The arylsulphatase activity in the eluate fraction, eluting from DE-52 cellulose at 12–16 mS, chromatographed over Sephadex G-200 with an apparent molecular weight of 160,000, similar to arylsulphatase IIA from the rat peritoneal mast cell (Orange & Moore, 1976). The arylsulphatase activity in the effluent fraction had a molecular weight of approximately 60,000 on G-200 chromatography, consistent with Type IIB arylsul-

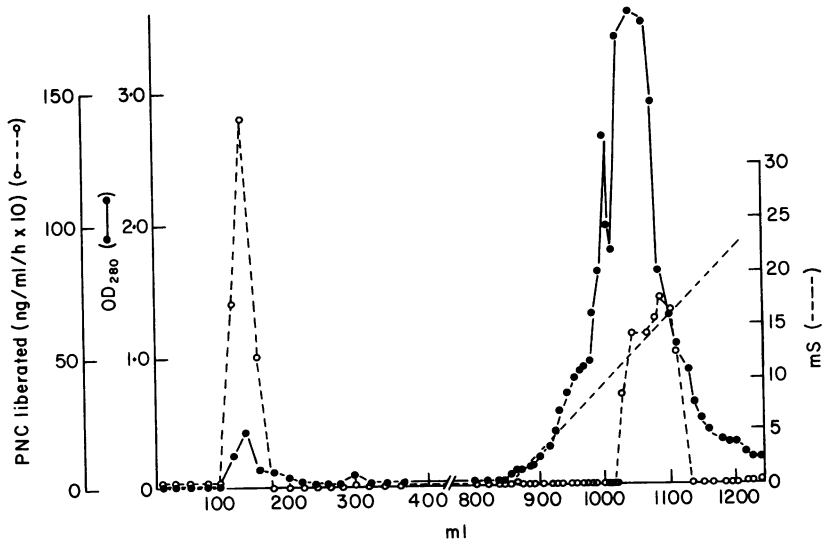

Figure 1. DE-52 cellulose anion exchange chromatography of arylsulphatase in human platelet extract. Enzyme activity from $2{\cdot}5 \times 10^{11}$ platelets assayed by cleavage of 0·01 M *p*-NCS at pH 5·5.

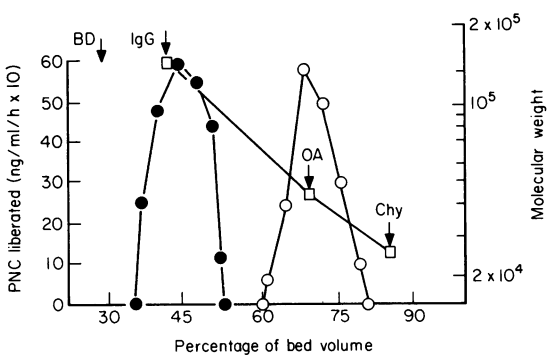

Figure 2. Molecular weight of human platelet arylsulphatase IIA (●) and arylsulphatase IIB (o) assessed by Sephadex G-200 gel filtration. Size markers included blue dextran (BD), human IgG (IgG), ovalbumin (OA) and chymotrypsinogen (Chy).

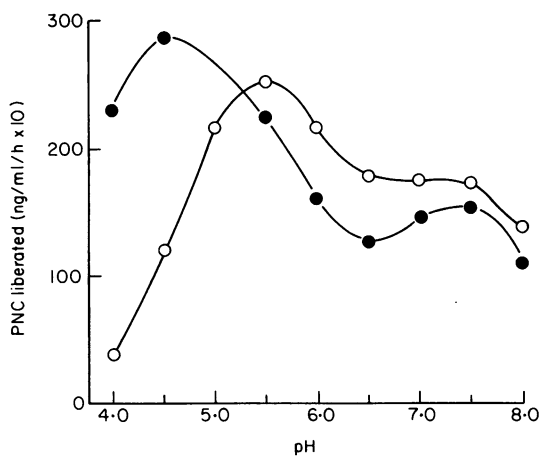

Figure 3. Effect of pH upon hydrolysis of *p*-NCS by purified human platelet arylsulphatase IIA (●) and arylsulphatase IIB (o).

phatase from human lung (Wasserman & Austen, 1976).

To determine the pH optimum for both arylsulphatase IIA and IIB, 15·0 mu/ml of each were interacted with 0·01 M *p*-NCS for 18 h at 37° at varying pH levels from 4·0 to 8·0 (Fig. 3). Arylsulphatase IIa had an optimal pH of 4·5, 78% optimal activity at pH 5·5, was depressed at pH 6·5 and demonstrated a slight increase in activity at pH 7·5. Arylsulphatase IIB had a pH optimum of 5·5, with a sharp decline in activity to 15% at pH 4·0 and a more gradual decrease under more alkaline conditions such that 70% of activity remained at pH 7·5. 15·0 mu/ml of both arylsulphatase IIA and IIB were reacted with substrate concentrations from 0·001 to 0·05 M *p*-NCS at their respective pH optimas for 18 h at 37°. Both enzyme activities were maximal at 0·01 M *p*-NCS in agreement with previous observations employing human lung arylsulphatase (Wasserman & Austen, 1976).

The effect of the introduction of sulphate or phosphate ions into the reaction mixture consisting of 15·0

mu/ml of arylsulphatase IIA or IIB and 0·01 M *p*-NCS was assessed after 18 h at 37°. Both arylsulphatase IIA and IIB were inhibited in a dose-related manner by phosphate and sulphate ions (Table 1), a finding that distinguishes Type II from Type I arylsulphatases (Roy, 1960).

Table 1. Inhibition of platelet type II arylsulphatases by sulphate and phosphate*

Ion	Concentration (μM)	Inhibition (%) IIA	IIB
Sulphate	100	33·0	29·0
	50	14·0	14·5
	10	0·0	0·0
Phosphate	10	21·4	88·4
	5	6·6	83·6
	1	0·0	49·0

* Reaction mixtures in 1·0 ml 0·5 M sodium acetate consisted of either arylsulphatase IIA (pH 4·5) or IIB (pH 5·5) plus 0·01 M *p*-NCS. Sodium sulphate or sodium phosphate was added and the reaction mixture incubated under standard conditions. Percentage inhibition was determined by comparison to mixtures seen in parallel in the absence of sulphate or phosphate.

Release of platelet arylsulphatase activity by thrombin

The capacity of platelets to release arylsulphatase was determined by incubating 5×10^8 platelets/ml with the calcium ionophore A-23187 (1 μg/ml) or 1 and 5 units of thrombin/ml. Both thrombin, as previously shown (Holmsen & Day, 1970), and A-23187 induced arylsulphatase release (Table 2). β-glucuronidase activity is believed to reside in the platelet α-granule (Holmsen, 1975). It was observed that both thrombin and A-23187 induced β-glucuronidase release from platelets. The concordance appreciated between the release of arylsulphatase and β-glucuronidase indicates that arylsulphatase may also reside in the α-granule of human platelets, as has been suggested by cytochemical studies performed on the rabbit (Murata *et al.*, 1975) and rat platelet (Bentfeld & Bainton, 1975).

Table 2. Platelet arylsulphatase release

Stimulus	Percentage release* β-glucuronidase	Arylsulphatase
A-23187, 1μg/ml	8·8	11·4
Thrombin, 1 u/ml	10·8	13·6
5 u/ml	22·9	26·3

* Corrected for spontaneous β-glucuronidase release of 3·9% and arylsulphatase 3·4%. Control LDH release less than 4·0%.

Interaction of SRS-A with arylsulphatase IIB from human platelets

In order to determine if arylsulphatase from human platelets was capable of inactivating rat SRS-A, arylsulphatase IIB was incubated with partially purified rat SRS-A, employing a ratio of 1 unit of arylsulphatase: 400 units SRS-A for 3 or 5 h at 37°. The percentage inactivation of SRS-A following incubation for 3 h at 37° was 50%, and after 5 h was 75%. This compares favourably with inactivation of either rat or human SRS-A by arylsulphatases (Wasserman & Austen, 1976; Orange & Moore, 1976; Wasserman *et al.*, 1975).

DISCUSSION

Human platelets contain arylsulphatase at a concentration of 2·3 mu/cm³ of blood. Anion exchange chromatography followed by gel filtration of human platelet lysates separated the arylsulphatase activity into two forms. Arylsulphatase IIb does not adhere to DE-52 and has an apparent molecular weight of 60,000, characteristics which are similar to human eosinophil arylsulphatase IIB (Wasserman *et al.*, 1975). Arylsulphatase IIa elutes from DE-52 at 12–16 mS and has an apparent molecular weight in 0·01 M Tris–HCl buffer, pH 8·0, of 160,000, which is similar to arylsulphatase IIA from rat peritoneal mast cells (Orange & Moore, 1976). Arylsulphatase IIA had a pH optimum of 4·5 and arylsulphatase IIB a pH optimum of 5·5, findings consistent with pH optima of arylsulphatases IIA and IIB from human lung. Type II arylsulphatases are characteristically inhibited by phosphate and sulphate ions (Roy, 1960), and both of these ions demonstrate a dose-related inhibition of platelet arylsulphatases. Therefore, human platelets contain two forms of arylsulphatase much as has been described in the human lung (Wasserman & Austen, 1976) and rat basophil tumour (Wasserman & Austen, 1977).

Immediate hypersensitivit reactions in the rabbit involve the elaboration of mast cell or basophil-derived platelet-activating factor (PAF) (Kravis & Henson, 1977). The interaction between PAF and platelets leads to aggregation and degranulation and provides the cellular source in rabbits for biogenic amines (Henson, 1970) which cause the pathological events characteristic of allergic reactions. Factors derived from or generated by human immediate hypersensitivity reactions are also capable of causing similar platelet responses, although the role of the platelet in human allergic responses is unclear. Thrombin or A-23187 treatment of human platelets causes the secretion of both β-glucuronidase and arylsulphatase. The concomitant release of both enzymes suggests that they may both reside in the α-granule in the human platelet, an observation consistent with the histochemical localization of arylsulphatase in rabbit (Murata *et al.*, 1975) and rat platelets (Bentfeld & Bainton, 1975). Thus, human platelets encountering either PAF or thromboxane A_2 might be expected to release arylsulphatase.

The biological consequences of the immunological release of the chemical mediators of anaphylaxis have been adequately reviewed (Austen & Orange, 1975). One of the current concepts of the regulation of immediate hypersensitivity reactions involves the destruction of chemical mediators by cell-associated activities. The eosinophil, one of the cells selectively attracted to the site of allergic reaction by several mediators (Kay & Austen, 1971), has been demonstrated to contain a number of relevant enzymes. These include phospholipase D (PAF) (Kater, Goetzl & Austen, 1976), histaminase (histamine) (Zieger & Colten, 1977), and arylsulphatase IIB (SRS-A) (Wasserman *et al.*, 1975). Human platelet-derived arylsulphatase IIB is also an efficient inactivator of rat SRS-A. Therefore, the human platelet joins the human eosinophil in being capable of responding to and inactivating the mediators of anaphylaxis. The intravascular location of large numbers of platelets suggests that they may be uniquely capable of limiting the allergic reaction from extending into this compartment.

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