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Mechanisms involved in the antiplatelet activity of magnesium in human platelets

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Summary. In this study, magnesium sulphate dose-dependently (0·6–3·0 mmol/l) inhibited platelet aggregation in human platelets stimulated by agonists. Furthermore, magnesium sulphate (3·0 mmol/l) markedly interfered with the binding of fluorescein isothiocanate-triflavin to the glycoprotein (GP)IIb/IIIa complex in platelets stimulated by collagen. Magnesium sulphate (1·5 and 3·0 mmol/l) also inhibited phosphoinositide breakdown and intracellular Ca^{+2} mobilization in human platelets stimulated by collagen. Magnesium sulphate (3·0 mmol/l) significantly inhibited thromboxane A_2 formation stimulated by collagen in platelets. Moreover, magnesium sulphate (1·5 and 3·0 mmol/l) obviously increased the fluorescence of platelet membranes tagged with diphenylhexatriene. In addition, magnesium sulphate (1·5 and 3·0 mmol/l) increased the formation of cyclic adenosine monophosphate (AMP) in platelets. Phosphorylation of a protein of *Mr* 47 000 (P47) was markedly inhibited by magnesium sulphate (1·5 mmol/l). In conclusion, the antiplatelet activity of magnesium sulphate may involve the following two pathways. (1) Magnesium sulphate may initially induce membrane fluidity changes with resulting interference of fibrinogen binding to the GPIIb/IIIa complex, followed by inhibition of phosphoinositide breakdown and thromboxane A_2 formation, thereby leading to inhibition of both intracellular Ca^{2+} mobilization and phosphorylation of P47. (2) Magnesium sulphate might also trigger the formation of cyclic AM, ultimately resulting in inhibition of the phosphorylation of P47 and intracellular Ca^{+2} mobilization.

Keywords: magnesium sulphate, platelet aggregation, GPIIb/IIIa complex, phospholipase C, protein kinase C.

Intravascular thrombosis is one of the generators of a wide variety of cardiovascular diseases. Initiation of an intraluminal thrombosis is believed to involve platelet adherence and aggregation. In normal circulation, platelets cannot aggregate by themselves. However, when a blood vessel is damaged, platelets adhere to the disrupted surface, and the adherent platelets release some biologically active constituents and aggregate (Sheu *et al*, 1994). Thus, platelet aggregation may play a crucial role in the atherothrombotic process. Indeed, antiplatelet agents (e.g. ticlopidine and aspirin) have been shown to reduce the incidence of stroke in high-risk patients (Hass *et al*, 1989).

Magnesium is an important intracellular cation and an obligatory cofactor of many enzymes in the human body. Magnesium deficiency and its association with platelet hyper-reactivity have been well recognized in a variety of diseases, including acute myocardial infarction (Rasmussen *et al*, 1988), pre-eclampsia (Lucas *et al*, 1995) and diabetes mellitus (Nadler *et al*, 1992). Magnesium has been shown to reduce platelet aggregation both *in vitro* and *ex vivo* (Hwang *et al*, 1992; Ravn *et al*, 1996a,b). Furthermore, *in vitro* studies have shown reduced platelet release of β-thromboglobulin and thromboxane B_2 with increasing magnesium concentrations (0·5–8·0 mmol/l) (Hwang *et al*, 1992; Ravn *et al*, 1996a,b). In addition, magnesium has also been shown to reduce thrombin-stimulated Ca^{+2} influx in platelets (Hwang *et al*, 1992). Shechter *et al* (2000) reported that oral magnesium therapy significantly improved the endothelial function of coronary artery diseases. Apart from the direct antiplatelet effect, an antithrombotic effect can also be derived from nitric oxide and prostacyclin, as magnesium has been shown to stimulate the release of these vasodilating and anti-aggregatory substances from the endothelium (Watson *et al*, 1986; Nadler *et al*, 1987).

On the other hand, studies of the mechanisms of magnesium in antiplatelet aggregation have rarely been compared with results from other antiplatelet drugs (such as aspirin and ticlopidine). We therefore systematically

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examined the influence of magnesium on washed human platelets and utilized the findings to characterize the mechanisms involved.

MATERIALS AND METHODS

Materials. Collagen (Type I, bovine achilles tendon), adenosine 5′-diphosphate (ADP), magnesium sulphate, arachidonic acid, EDTA, luciferin–luciferase, Dowex-1 (100–200 mesh; X8, chloride form), prostaglandin E1, trichloroacetic acid, EGTA, bovine serum albumin, acrylamide, sodium pyruvate, β-NADH, diphenylhexatriene (DPH), apyrase, heparin, thrombin and myoinositol were purchased from Sigma Chemicals (St Louis, MO, USA). Fura 2-AM and fluorescein isothiocyanate (FITC) were purchased from Molecular Probe (Eugene, OR, USA). *Trimeresurus flavoviridis* venom was purchased from Latoxan (Rosans, France). Myo-2-[^{3}H] inositol was purchased from Amersham (Buckinghamshire, HP, UK). Thromboxane B_2, cyclic adenosine monophosphate (AMP) and cyclic guanosine monophosphate (GMP) enzyme immunoassay (EIA) kits were purchased from Cayman (Ann Arbor, MI, USA).

Preparation of human platelet suspensions. Human platelet suspensions from human volunteers who had given informed consent, were prepared as previously described (Huang *et al*, 1991a). Briefly, blood was collected from healthy human volunteers, who had taken no medicine during the preceding 2 weeks, and was mixed with acid/citrate/glucose (9:1, v/v). After centrifugation at 120 ***g*** for 10 min at room temperature, the supernatant (platelet-rich plasma; PRP) was supplemented with prostaglandin E1 (PGE_1) (0·5 μmol/l) and heparin (6·4 IU/ml), then incubated for 10 min at 37°C and centrifuged at 500 ***g*** for 10 min. The washed platelets were finally suspended in Tyrode's solution, containing bovine serum albumin (BSA) (3·5 mg/ml), and adjusted to about $4·5 \times 10^8$ platelets/ml. The final concentration of Ca^{2+} in Tyrode's solution was 1 mmol/l.

Platelet aggregation. The turbidimetric method used a Lumi-Aggregometer (Payton, Canada) as described previously (Born & Cross, 1963). Briefly, platelet suspensions (0·4 ml) were prewarmed to 37°C for 2 min and then magnesium sulphate was added for 3 min before the addition of platelet-aggregation inducers. The reaction was allowed to proceed for at least 6 min and the extent of aggregation was expressed as a percentage of the control (in the absence of magnesium sulphate). The degree of aggregation was expressed in light-transmission units. While measuring ATP release, 20 μl of a luciferin/luciferase mixture was added 1 min before the addition of agonists, and ATP release was compared with that of the control.

Analysis of the platelet surface glycoprotein (GP) IIb/IIIa complex by flow cytometry. Triflavin, a specific fibrinogen receptor (GPIIb/IIIa complex) antagonist, was prepared as previously described (Sheu *et al*, 1992). FITC-conjugated triflavin was also prepared as previously described (Sheu *et al*, 1996) and the final concentration was adjusted to 1 mg/ml. Human platelet suspensions were prepared as described above. Aliquots of platelet suspensions ($4·5 \times 10^8$/ml) were preincubated with magnesium sulphate (3·0 mmol/l) for 3 min, followed by the addition of 2 μl of FITC-triflavin. The suspensions were then incubated for another 5 min and the volume was adjusted to 1 ml/tube with Tyrode's solution. The suspensions were then assayed for fluorescein-labelled platelets with a flow cytometer (FACScan System; Becton Dickinson, San Jose, CA, USA). Data were collected from 50 000 platelets per experimental group. All experiments were repeated at least five times to ensure reproducibility.

Labelling of membrane phospholipids and measurement of the production of [^{3}H]-inositol phosphates. The method was carried out as previously described (Huang *et al*, 1991b). Briefly, citrated human PRP was centrifuged and the pellets were suspended in Tyrode's solution containing [^{3}H]-inositol (2·775 MBq/ml). Platelets were incubated for 2 h followed by centrifugation and finally resuspended in Ca^{2+}-free Tyrode's solution (1×10^9 platelets/ml). Magnesium sulphate was preincubated with 1 ml of loaded platelets at room temperature for 3 min and collagen (1 μg/ml) was then added to trigger aggregation. The reaction was stopped after 6 min and the samples were centrifuged at 1000 ***g*** for 4 min. The inositol phosphates of the supernatants were separated in a Dowex-1 anion exchange column. Only [^{3}H]-inositol monophosphate (IP) was measured as an index of total inositol phosphate formation.

Measurement of platelet [Ca^{2+}]i mobilization by Fura 2-AM fluorescence. Citrated whole blood was centrifuged at 120 ***g*** for 10 min. The supernatant was protected from light and incubated with Fura 2-AM (5 μmol/l) at 37°C for 1 h. Human platelet suspensions were then prepared as described above. Finally, the external Ca^{2+} concentration of the platelet suspensions was adjusted to 1 mmol/l. The rise in [Ca^{2+}]i was measured using a fluorescence spectrophotometer (CAF 110; Jasco, Tokyo, Japan) with excitation wavelengths of 340 and 380 nm, and an emission wavelength of 500 nm. The [Ca^{2+}]i was calculated from the fluorescence measured using 224 nmol/l as the Ca^{2+}–Fura 2 dissociation constant (Grynkiewicz *et al*, 1985).

Measurement of membrane fluidity by fluorescent probe. The intensity of fluorescence in human platelets was measured as described previously (Kitagawa *et al*, 1984). Briefly, platelets ($4·5 \times 10^8$/ml) were mixed with 9 volumes of sodium/potassium-Tris medium. A solution of DPH in dimethyl formamide was added to the suspension at a final concentration of 0·5 μmol/l. Platelets were preincubated with various concentrations of magnesium sulphate (1·5 and 3·0 mmol/l) for 3 min followed by the addition of DPH at 37°C for another 6 min. The relative fluorescence intensity of platelets was measured in a fluorescence spectrophotometer (Hitachi F4500, Tokyo, Japan) at 37°C.

Measurement of thromboxane B_2 formation. Washed human platelet suspensions ($4·5 \times 10^8$/ml) were preincubated for 3 min in the presence or absence of magnesium sulphate (1·5 and 3·0 mmol/l) before the addition of collagen (1 μg/ml). Six minutes after the addition of collagen, 2 mmol/l EDTA and 50 μmol/l indomethacin were added to the reaction suspensions. The vials were then centrifuged for 3 min at 15 000 ***g***. The thromboxane B_2 (TxB_2) levels of the supernatants were measured using an EIA kit according to the instructions of the manufacturer.

Determination of lactate dehydrogenase. Lactate dehydrogenase (LDH) was measured according to previously described methods (Wroblewski & Ladue, 1955). Platelets ($4{\cdot}5 \times 10^8$/ml) were preincubated with magnesium sulphate (3·0 mmol/l) for either 10 or 30 min, followed by centrifugation 10 000 ***g*** for 5 min. An aliquot of supernatant was incubated with phosphate buffer, containing 0·2 mg β-NADH, for 20 min at room temperature. Thereafter, 100 μl of pyruvate solution was added and the absorbance wavelength was read at 334 nm using an ultra violet visible recording spectrophotometer (UV-160; Shimazu, Kyoto, Japan). A maximal value of LDH was constructed from sonicated platelets.

Measurement of protein kinase C activity. Washed human platelets (2×10^9/ml) were incubated for 60 min at 37°C with phosphorus-32 (18·5 MBq/ml). Platelet suspensions were next washed twice with Tris-saline buffer. The [^{32}P]-labelled platelets were preincubated with magnesium sulphate (1·5 mmol/l) in an aggregometer for 3 min; collagen (1 μg/ml) was then added for 1 min to trigger protein kinase C activation. Activation was terminated by the addition of Laemmli sample buffer, and analysed by electrophoresis (12·5%; w/v) as described previously (Grabarek *et al*, 1992). The gels were dried, and the relative intensities of the radioactive bands were analysed using a Bio-imaging analyser system (FAL2000; Fuji, Tokyo, Japan) and were expressed as photostimulated luminescence/mm^2 (PSL).

Estimation of platelet cyclic AMP and cyclic GMP formations. The method of Karniguian *et al* (1982) was followed. Briefly, platelet suspensions were warmed to 37°C for 1 min, then PGE_1 (10 μmol/l), nitroglycerin (10 μmol/l) or magnesium sulphate (1·5 and 3·0 mμ) was added and incubated for 6 min. The incubation was stopped and the solution was immediately boiled for 5 min. After cooling to 4°C, the precipitated protein was collected as sediment after centrifugation. An aliquot (50 μl) of supernatant was used to determine the cyclic AMP and cyclic GMP contents by EIA, following acetylation of the samples as described by the kit manufacturer.

Estimation of nitrate in human platelet suspensions. Platelet suspensions (1×10^9/ml) were preincubated with collagen (2 μg/ml) and magnesium sulphate (1·5 and 3·0 mmol/l) for 6 min, followed by centrifugation (10 000 ***g***) for 5 min. The supernatants were deproteinized by incubation with 95% ethanol at 4°C for 30 min. The samples were then centrifuged for a further 7 min. It should be noted that the nitrate concentrations represent the total of both nitrite and nitrate concentrations in the platelet suspensions. This method reduced nitrate to NO via nitrite. The amount of nitrate in the platelet suspensions (10 μl) was measured by adding a reducing agent (0·8% VCl_3 in 1 mol/l HCl) to the purge vessel to convert nitrate to NO which was stripped from the platelet suspensions by a helium purge gas. The NO was then drawn into a Sievers nitric oxide analyser (Sievers 280 NOA; Sievers, Boulder, CO, USA). Nitrate concentrations were calculated by comparison with standard solutions of sodium nitrate.

Statistical analysis. Experimental results are expressed as the means ± SEM and are accompanied by the number of observations. Data were assessed by analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared using the Newman–Keuls method. A *P*-value less than 0·05 was considered statistically significant.

RESULTS

Effect of magnesium sulphate on platelet aggregation in human platelet suspensions

Magnesium sulphate (0·6–3·0 mmol/l) dose-dependently inhibited platelet aggregation stimulated by collagen (1 μg/ml), and thrombin (0·02 U/ml) in human platelets (Fig 1A and B). It also similarly inhibited ADP-induced (20 μmol/l) platelet aggregation in the presence of fibrinogen (300 μg/ml) (Fig 1B). Furthermore, magnesium

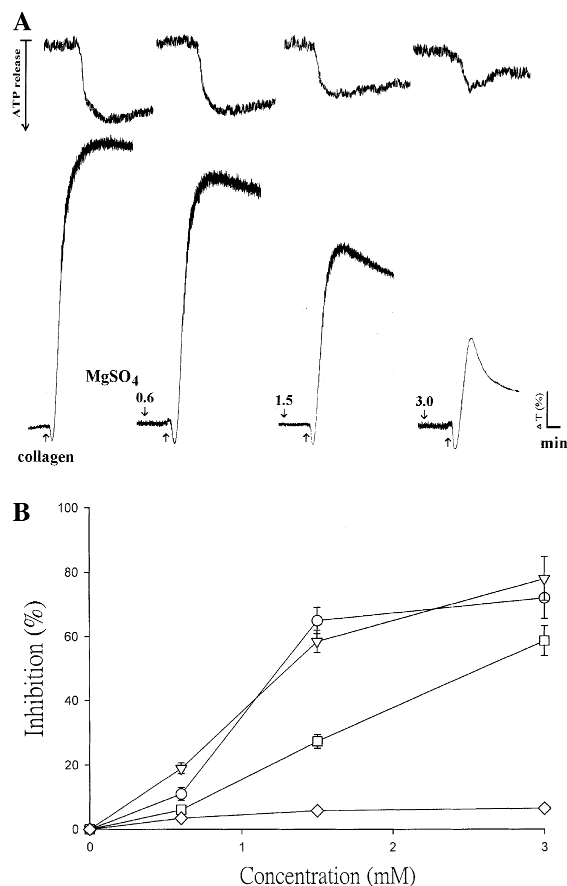

Fig 1. (A) Trace curves of magnesium sulphate on collagen (1 μg/ml)-induced aggregation and (B) concentration–inhibition curve of magnesium sulphate on collagen- (1 μg/ml, ○), ADP- (20 μmol/l, ▽), thrombin- (0·02 U/ml, □) and arachidonic acid- (100 μmol/l, ◇) induced platelet aggregation in washed human platelet suspensions. Platelets were preincubated with magnesium sulphate (0·6–3·0 mmol/l), agonists were then added to trigger aggregation (lower tracings) and ATP release (upper tracings) (A). Data are presented as a percentage of the control (means ± SEM, $n = 6$).

sulphate also inhibited the ATP release reaction when stimulated by agonists (i.e. collagen) (Fig 1A). The 50% inhibitory concentration (IC_{50}) values of magnesium sulphate for platelet aggregation induced by ADP, collagen and thrombin were estimated to be approximately 1·7, 1·8 and 2·6 mmol/l respectively. On the other hand, magnesium sulphate (0·6–3·0 mmol/l) did not significantly inhibit arachidonic acid (100 μmol/l) (Fig 1B) or the thromboxane A_2 analogue, U46619-induced (1 μmol/l) platelet aggregation (data not shown). Furthermore, magnesium chloride (1·5 and 3·0 mmol/l) also showed a similar dose-dependent inhibitory effect on agonist-induced platelet aggregation (data not shown). In the following experiments, we used collagen as an agonist to explore the inhibitory mechanisms of magnesium sulphate in platelet aggregation.

Effect of magnesium sulphate on collagen-induced GPIIb/IIIa complex exposure in human platelets

Triflavin is an Arg-Gly-Asp-containing disintegrin from *Trimeresurus flavoviridis* snake venom (Sheu *et al*, 1996). Triflavin inhibits platelet aggregation through direct interference with fibrinogen binding to the GPIIb/IIIa complex (Sheu *et al*, 1992). There is now a multitude of evidence suggesting that the binding of fibrinogen to the GPIIb/IIIa complex is the final common pathway for agonist-induced platelet aggregation. Therefore, we further evaluated whether or not magnesium sulphate binds directly to the platelet GPIIb/IIIa complex, leading to inhibition of platelet aggregation induced by agonists.

In this study, the relative intensity of the fluorescence of FITC-triflavin (2 μg/ml) bound directly to collagen-activated (1 μg/ml) platelets was 88·5 ± 9·6 ($n = 5$) and was markedly reduced in the presence of 5 mmol/l EDTA (negative control, 17·5 ± 3·2, $n = 5$) (data not shown). At a concentration of 3·0 mmol/l, magnesium sulphate markedly inhibited FITC-triflavin binding to the GPIIb/IIIa complex in platelet suspensions (19·6 ± 3·8, $n = 5$) (data not shown), indicating that the mechanism of magnesium sulphate's inhibitory effect on platelet aggregation may involve interference with fibrinogen binding to the GPIIb/IIIa complex.

Effect of magnesium sulphate on phosphoinositide breakdown in human platelet suspensions

Phosphoinositide breakdown occurs in platelets activated by many different agonists (Broekman *et al*, 1980). We found that collagen (1 μg/ml) induced the rapid formation of radioactive IP, IP_2 and IP_3 in human platelets loaded with [^{3}H]-inositol. We only measured [^{3}H]-IP formation as an index of total inositol phosphate formation. As shown in Fig 2, the addition of collagen (1 μg/ml) resulted in a rise of IP formation of about 2·3-fold compared with that in resting platelets [(5·8 ± 1·4 *vs* 13·5 ± 2·1) × 10^3 cpm]. In the presence of magnesium sulphate (1·5 and 3·0 mmol/l), the radioactivity of IP formation in collagen-stimulated human platelets markedly decreased. These results indicate that magnesium sulphate interferes with phosphoinositide breakdown in human platelets stimulated by collagen.

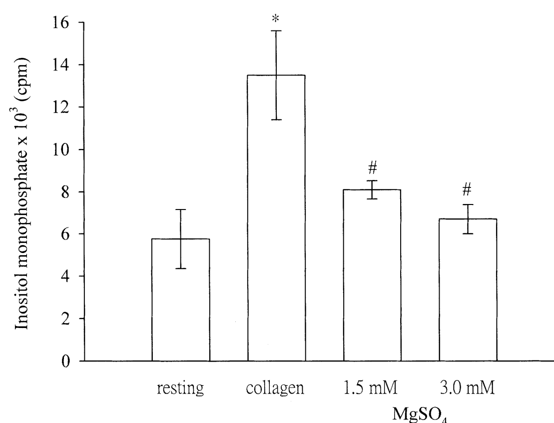

Fig 2. Effect of magnesium sulphate on collagen-induced inositol monophosphate formation in human platelet suspensions. Platelets were labelled with [^{3}H]-inositol and stimulated with or without collagen (1 μg/ml) in the presence of magnesium sulphate (1·5 and 3·0 mmol/l). Data are presented as the means ± SEM ($n = 4$). *$P < 0·05$ as compared with the resting group, #$P < 0·05$ as compared with the collagen group.

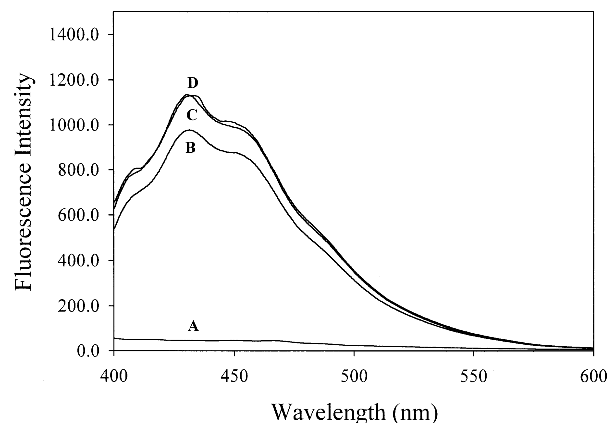

Fig 3. Fluorescence emission spectra of platelet membranes in the absence (A) or presence (B) of DPH (1 μmol/l). Curves C and D are the emission spectra of membranes labelled with DPH in the presence of magnesium sulphate (C) (1·5 mmol/l) and (D) (3·0 mmol/l) for 3 min. Profiles are representative examples of four similar experiments.

Effect of magnesium sulphate on [Ca^{2+}]$_i$ mobilization

Free cytoplasmic Ca^{2+} concentrations in human platelets were measured by the Fura 2-AM loading method. In this study, collagen (1 μg/ml) evoked an increase of [Ca^{2+}]i from 32·8 ± 5·0 to 225·8 ± 39·2 nmol/l (data not shown), which was markedly inhibited in the presence of magnesium sulphate (1·5 mmol/l, 71·3 ± 2·4%; 3·0 mmol/l, 86·7 ± 10·1%; $n = 4$) (data not shown). This suggests that magnesium sulphate exerts an inhibitory effect on [Ca^{2+}]$_i$ mobilization in human platelets stimulated by collagen.

Effect of magnesium sulphate on platelet membrane fluidity

Platelet membrane fluidity was measured in DPH-labelled human platelets. Measurements using the fluorescent probe

technique demonstrated that magnesium sulphate is capable of direct interaction with platelet membranes (Fig 3). Addition of magnesium sulphate (1·5 and 3·0 mmol/l) to platelet suspensions for 3 min resulted in a marked increase in DPH-related fluorescence intensity. This result implies that the inhibitory effect of magnesium sulphate on platelet aggregation may be due, at least in part, to the results of its effects on platelet membrane fluidity.

Effect of magnesium sulphate on TxB_2 formation

As shown in Table I, resting platelets produced relatively little TxB_2 compared with collagen-activated platelets. PGE1 (10 μmol/l) inhibited TxB_2 formation in collagen-activated platelets by 81% (data not shown). Furthermore, results obtained using various concentrations of magnesium sulphate indicated that magnesium sulphate (3·0 mmol/l) significantly inhibited TxB_2 formation in platelets stimulated by collagen (1 μg/ml) (Table I).

Table I. Effect of magnesium sulphate on thromboxane B_2 formation induced by collagen in washed human platelets.

	Dose	Thromboxane B_2 (ng/ml)
Resting		45·8 ± 13·4
Collagen (μg/ml)	1	153·2 ± 19·4*
+Magnesium sulphate (mmol/l)		
	1·5	110·2 ± 12·7
	3·0	85·5 ± 9·2†

*$P < 0·01$ as compared with the resting group.
†$P < 0·05$ as compared with the collagen group.
Platelet suspensions were preincubated with magnesium sulphate (1·5 and 3·0 mmol/l) for 3 min at 37°C and then collagen (1 μg/ml) was added to trigger thromboxane B_2 formation. Data are presented as the means ± SEM ($n = 5$).

Effect of magnesium sulphate on LDH released from platelet cytosol

In this study, magnesium sulphate (3·0 mmol/l) treatment of human platelets for 10 min did not significantly increase LDH activity compared with resting platelets (resting platelets, 25·8 ± 3·4 units *vs* magnesium sulphate-treated platelets, 28·1 ± 3·9 units, $n = 4$), even when the incubation time of magnesium sulphate with platelets was prolonged to 30 min (29·4 ± 3·3 units, $n = 4$) (data not shown). This indicates that although magnesium sulphate (1·5 and 3·0 mmol/l) significantly changes the fluidity of platelet membranes, by itself, it does not affect platelet permeabilization or induce platelet cytolysis under this range of concentrations.

Effect of magnesium sulphate on the formation of cyclic AMP, cyclic GMP and nitrate

The level of cyclic AMP in unstimulated platelets was low (31·5 ± 3·5 pmol/10^9 platelets). Addition of PGE_1 (10 μmol/l) increased the cyclic AMP level to 236·7 ± 34·2 pmol/10^9 platelets (Table II). When platelet suspensions were preincubated with magnesium sulphate (1·5 and 3·0 mmol/l), the cyclic AMP level increased to 77·1 ± 8·6 and 112·4 ± 12·8 pmol/10^9 platelets respectively (Table II). We also performed similar studies measuring the cyclic GMP response. The level of cyclic GMP in unstimulated platelets was very low, but when nitroglycerin (10 μmol/l) was added to the platelet suspensions, cyclic GMP levels increased from the resting level to 158·6 ± 13·7 pmol/10^9 platelet (Table II). However, addition of magnesium sulphate (1·5 and 3·0 μmol/l) resulted in no significant increase in platelet cyclic GMP levels (16·5 ± 1·5 and 16·0 ± 1·2 pmol/10^9 platelets), even at a higher concentration (5·0 μmol/l) (data not shown).

On the other hand, NO was quantified using a sensitive and specific ozone redox-chemiluminescence detector. As shown in Table II, collagen (2·0 μg/ml) caused about a 2·9-fold rise in nitrate formation, compared with that in resting platelets. In the presence of magnesium sulphate (1·5 and 3·0 mmol/l), nitrate production did not significantly increase after incubation with platelets for 6 min (Table II).

Table II. Effect of magnesium sulphate on cyclic AMP, cyclic GMP and nitrate formation in washed human platelets.

	Concentration	Cyclic AMP (pmol/10^9 platelets)	Cyclic GMP (pmol/10^9 platelets)	Nitrate (μmol/l)
Resting		31·5 ± 3·5	18·5 ± 2·1	4·8 ± 0·4
Prostaglandin E_1 (μmol/l)	10	236·7 ± 34·2*		
Nitroglycerin (μmol/l)	10		158·6 ± 13·7*	
Collagen (μg/ml)	2			13·8 ± 0·9*
Magnesium sulphate (mmol/l)	1·5	77·1 ± 8·6*	16·5 ± 1·5	5·9 ± 1·0
	3·0	112·4 ± 12·8*	16·0 ± 1·2	5·6 ± 0·6

*$P < 0·001$ as compared with the resting groups.
Platelet suspensions were preincubated with magnesium sulphate (1·5 and 3·0 μmol/l) at 37°C. Addition of prostaglandin E1, nitroglycerin and collagen in platelet suspensions served as a positive control of cyclic AMP, cyclic GMP and nitrate respectively. Data are presented as the means ± SEM ($n = 5$).

However, nitrate production did not increase even after prolongation of the incubation time to 30 min (data not shown). These results imply that the antiplatelet activity of magnesium sulphate may act at least partly through stimulation of the cyclic AMP, but not the NO/cyclic GMP pathway, in human platelets.

Effect of magnesium sulphate on collagen-stimulated phosphorylation of the 47 kDa protein

Stimulation of platelets with a number of different agonists induces activation of protein kinase C, which then phosphorylates proteins of *Mr* 40 000–47 000 in addition to other proteins (Siess & Lapetina, 1989). In this study, phosphorylation was used to examine the role of magnesium sulphate in activation of protein kinase C in human platelets. When collagen (1 μg/ml) was added to human platelets prelabelled with $^{32}PO_4$ for 2 min, a protein with an apparent *Mr* of 47 000 (P47) was predominately phosphorylated as compared with resting platelets (Fig 4A and B). On the other hand, magnesium sulphate (1·5 mmol/l) markedly inhibited the phosphorylation of P47 in human platelets stimulated by collagen (1 μg/ml) (Fig 4). In this study, the extent of radioactivity in P47 was expressed as a relative detection density [photostimulated luminescence (PSL)/mm^2] of the radioactive bands.

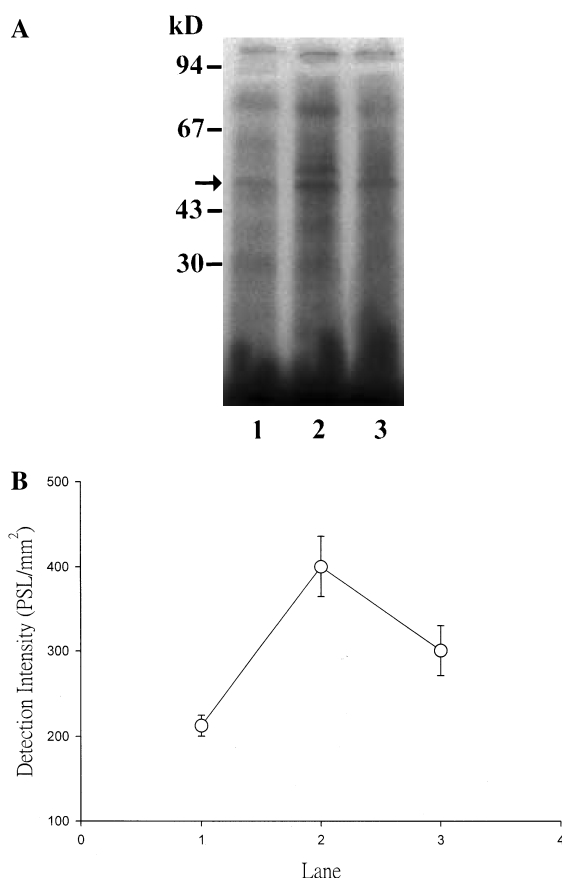

Fig 4. (A) Effect of magnesium sulphate on phosphorylation of a protein of *Mr* 47 000 (P47) in human platelets challenged with collagen. Platelets were preincubated with magnesium sulphate (1·5 mmol/l) before challenge with collagen (1 μg/ml). Lane 1, platelets with Tyrode's solution; lane 2, platelets with collagen (1 μg/ml); lane 3, platelets with magnesium sulphate (1·5 mmol/l) for 3 min followed by the addition of collagen (1 μg/ml). The arrow indicates a protein of *Mr* 47 000 (P47). Data are representative examples of four similar experiments. (B) The relative detection densities of the radioactive bands are expressed as PSL/mm^2. Data are presented as the means ± SEM ($n = 4$).

DISCUSSION

Magnesium sulphate is used widely to prevent seizures in pregnant women with hypertension (Lucas *et al*, 1995). The principal objective of this study was to describe the detailed mechanisms involved in the inhibition of agonist-induced human platelet aggregation by magnesium sulphate. This inhibitory effect of magnesium sulphate was demonstrable with the use of various agonists: collagen, thrombin and ADP. The inhibition was directly proportional to the pharmacological concentrations of magnesium sulphate used. Lucas *et al* (1995) suggested that the magnesium sulphate regimen for pre-eclampsia consisted of an initial loading dose of 4 g of magnesium sulphate (i.v.) followed by a 10 g intramuscular dose and a maintenance dose of 5 g given intramuscularly every for 4 h. In general, the therapeutic concentration is considered to be between 2 and 4 mmol/l (Pritchard, 1979; Sibai *et al*, 1984). However, this is based on clinical experience and not directly related to the suppression of eclamptic convulsions. In this study, magnesium sulphate was employed at concentrations of about 0·6–3·0 mmol/l which inhibited platelet aggregation induced by agonists. This result indicates that the pharmacological concentrations of magnesium sulphate employed to inhibit platelet aggregation *in vitro* are reasonably close to those of blood concentrations obtained during a magnesium sulphate regimen *in vivo*. In this study, both platelet aggregation and the ATP release reaction induced by these agonists (i.e. collagen) appeared to be affected in the presence of magnesium sulphate. Therefore, this partly infers that magnesium sulphate may affect Ca^{2+} release from intracellular Ca^{2+} storage sites (i.e. dense tubular systems or dense bodies), and this is in accordance with the concept that intracellular Ca^{2+} release is responsible for the ATP release reaction (Charo *et al*, 1976). On the other hand, magnesium sulphate did not significantly inhibit U46619-induced (1 μmol/l) or arachidonic acid-induced (100 μmol/l) platelet aggregation, indicating that magnesium sulphate-mediated effects do not depend on the blockage of the arachidonic acid pathway.

Although the action mechanisms of various platelet aggregation agonists, such as collagen, ADP and thrombin, differ (Fig 5), magnesium sulphate significantly inhibited platelet aggregation that was stimulated by all of them. This implies that magnesium sulphate may block a common step shared by these inducers. These results also indicate that the site of action of magnesium sulphate is not at the receptor level of individual agonists. Triflavin acts by binding to the

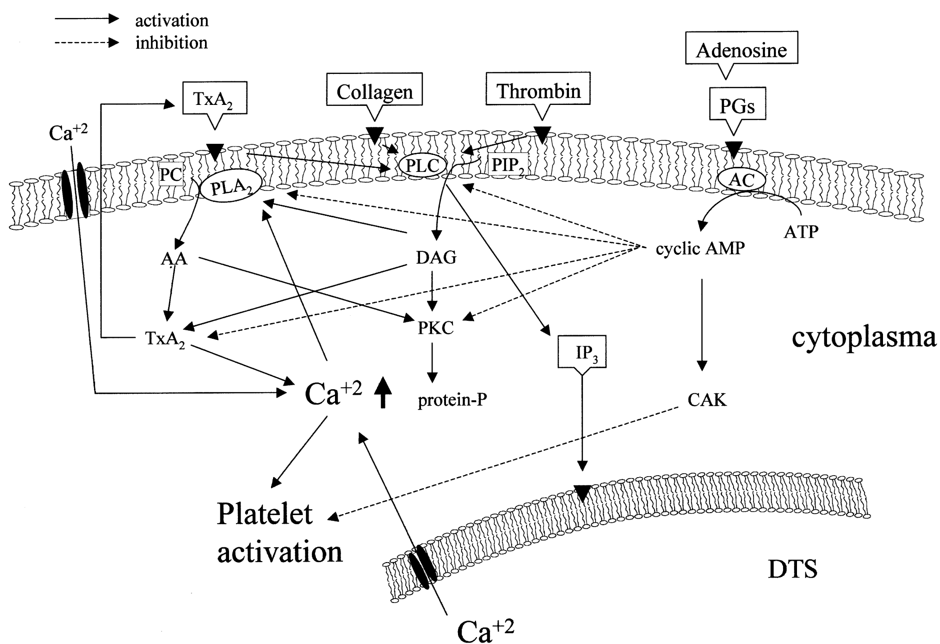

Fig 5. Signal transductions of platelet aggregation. Agonists can activate several phospholipases, including phospholipase C (PLC) and phospholipase A_2 (PLA2). The products of the action of phospholipase Con phosphatidylinositol 4,5-bisphosphate (PIP2) include 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). DAG stimulates protein kinase C (PKC), followed by phosphorylation of a 47 kDa protein. IP3 induces the release of Ca^{+2} from dense tubular systems (DTS). The major metabolite of arachidonic acid (AA) in platelets is thromboxane A_2 (TxA_2). Adenylate cyclase (AC) is activated by some prostaglandins (PGs) and adenosine, resulting in the hydrolysis of adenosine triphosphate (ATP) to cyclic 3′-5′-adenosine monophosphate (cyclic AMP). Cyclic 3′-5′-adenosine monophosphate can activate cyclic AMP-dependent protein kinase (CAK), resulting in the inhibition of platelet activation.

GPIIb/IIIa complex on the platelet surface membrane, resulting in interference with the interaction of fibrinogen with its specific receptor (Sheu *et al*, 1992, 1996, 1999). In this study, we found that magnesium sulphate markedly inhibited FITC-triflavin binding to the GPIIb/IIIa complex (data not shown). Furthermore, Gawaz *et al* (1994) reported that Mg^{+2} reduced fibrinogen binding to ADP- and collagen-stimulated platelets with an IC_{50} of about 3·0 mmol/l. It is well known that the fibrinogen–GPIIb/IIIa complex interaction is regulated by divalent cations, and that physiological concentrations of Ca^{+2} and Mg^{+2} support fibrinogen binding (Gulino *et al*, 1992). At pharmacological levels, however, Mg^{+2} may inhibit binding of fibrinogen to the GPIIb/IIIa complex by altering the receptor conformation. This might be caused by competition of Mg^{+2} with Ca^{+2} ions for Ca^{+2} binding sites in the GPIIb subunit (Gulino *et al*, 1992).

Conformational changes in the plasma membrane and/or changes in membrane fluidity represent a generally accepted mechanism for the antiplatelet effect of numerous drugs, including local anaesthetics, chlorpromazine and beta-blockers. Therefore, we wondered whether magnesium sulphate might also inhibit platelet aggregation by influencing membrane fluidity. To test this hypothesis, the fluorescent probe, DPH, was used to label biological membranes. In this study, magnesium sulphate (1·5 and 3·0 mmol/l) markedly decreased the DPH-relative fluorescence intensity of platelet membranes. These findings suggest that changes in platelet membrane fluidity may be the primary mechanism responsible for the antiplatelet effect of magnesium sulphate *in vitro*.

Furthermore, stimulation of platelets by agonists (i.e. collagen) results in phospholipase C-catalysed hydrolysis of the minor plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with concomitant formation of inositol 1,4,5-trisphosphate and diacylglycerol (Fig 5). There is strong evidence that inositol 1,4,5-trisphosphate induces the release of Ca^{2+} from intracellular stores (Berridge, 1983). Diacylglycerol activates protein kinase C, inducing protein phosphorylation and a release reaction (Fig 5). In this study, phosphoinositide breakdown of collagen-activated platelets was inhibited by magnesium sulphate, suggesting that signal transductions of antiplatelet aggregation by magnesium sulphate is related to inhibition of phosphoinositide breakdown. TxA_2 is an important mediator of the release reaction and aggregation of platelets (Hornby, 1982). Collagen-induced formation of TxB_2, a stable metabolite of TxA_2, was inhibited by magnesium sulphate (3·0 mmol/l) (Table I). It has been demonstrated that phosphoinositide breakdown can induce TxA_2 formation via free arachidonic acid release by diglyceride lipase or by endogenous phospholipase A2 from membrane phospholipids (Fig 5) (McKean *et al*, 1981). Thus, it seems likely that phosphoinositide breakdown and TxB_2 formation play a role in mediating the inhibitory effect of magnesium sulphate on human platelets.

The activation of human platelets is inhibited by two intracellular pathways, which are regulated by either cyclic AMP or cyclic GMP (Fig 5) (Walter *et al*, 1993). The importance of cyclic AMP in modulating platelet reactivity is well established (Karniguian *et al*, 1982). In addition to inhibiting most platelet responses, elevated levels of cyclic AMP decreased intracellular Ca^{2+} concentration by the uptake of Ca^{2+} into the dense tubular system and negatively affected the action of protein kinase C (Walter *et al*, 1993). In this study, we found that magnesium sulphate did not induce NO formation in human platelets. This result is in accordance with the cyclic GMP study, because the NO that is produced is biologically active, as most cellular actions of NO occur via stimulation of intracellular guanylate cyclase, leading to an increase in cyclic GMP (McDonald & Murad, 1996). Signalling by cyclic GMP somehow interferes with the agonist-stimulated phosphoinositide turnover that creates Ca^{2+}-mobilizing second messengers and protein kinase C (McDonald & Murad, 1996). Therefore, the inhibitory effect of magnesium sulphate on collagen-induced phosphorylation of P47 and platelet aggregation seems to be mediated, at least partly, by an increase in cyclic AMP levels in human platelets.

In conclusion, the observations of this study suggest that magnesium sulphate inhibits agonist-induced human platelet aggregation. This inhibitory effect may involve the following two mechanisms. (1) Magnesium sulphate may initially induce membrane fluidity changes on the platelet membrane, with a resulting interference of fibrinogen binding to the GPIIb/IIIa complex and activation of phospholipase C, followed by inhibition of phosphoinositide breakdown and thromboxane A_2 formation, thereby leading to inhibition of both intracellular Ca^{2+} mobilization and phosphorylation of P47. (2) Magnesium sulphate triggers the formation of cyclic AMP, which subsequently inhibits phosphoinositide breakdown and protein kinase C activity, finally resulting in inhibition of both the phosphorylation of P47 and intracellular Ca^{+2} mobilization.

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REFERENCES

Berridge, M.J. (1983) Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. *Biochemical Journal*, **212**, 249–258.

Born, G.V.R. & Cross, M.J. (1963) The aggregation of blood platelets. *Journal of Physiology*, **168**, 178–195.

Broekman, M.J., Ward, J.W. & Marcus, A.J. (1980) Phospholipid metabolism in phosphatidyl inositol, phosphatic acid and lysophospholipids. *Journal of Clinical Investigation*, **66**, 275–283.

Charo, I.F., Feinman, R.D. & Detwiler, T.C. (1976) Inhibition of platelet secretion by an antagonist of intracellular calcium. *Biochemical and Biophysical Research Communication*, **72**, 1462–1467.

Gawaz, M., Ott, I., Reininger, A.J. & Neumann, F.J. (1994) Effect of magnesium on platelet aggregation and adhesion. *Thrombosis and Haemostasis*, **72**, 912–918.

Grabarek, J., Raychowdhury, M., Ravid, K., Kent, K.C., Newman, P.J. & Ware, J.A. (1992) Identification and functional characterization of protein kinase C isozymes in platelets and HEL cells. *Journal of Biological Chemistry*, **267**, 10011–10017.

Grynkiewicz, G., Poenie, M. & Tsien, R.Y. (1985) A new generation of Ca^{2+} induces with greatly improved fluorescence properties. *Journal of Biological Chemistry*, **260**, 3440–3450.

Gulino, D., Boudignon, C., Zhang, L., Concord, E., Rabiet, M.J. & Marguerie, G. (1992) Ca^{+2}-binding properties of the platelet glycoprotein IIb ligand-interacting domain. *Journal of Biological Chemistry*, **267**, 11001–11007.

Hass, W.K., Easton, J.D., Adams, H.P., Pryse-Philips, W., Molony, B.A., Anderson, S. & Kamm, B. (1989) A randomized trial comparing ticlopidine hydrochloride with aspirin for the prevention of stroke in high-risk patients. *The New England Journal of Medicine*, **321**, 501–507.

Hornby, E.J. (1982) Evidence that prostaglandin endoperoxides can induce platelet aggregation in the absence of thromboxane A_2 production. *Biochemical Pharmacology*, **31**, 1158–1160.

Huang, T.F., Sheu, J.R. & Teng, C.M. (1991a) A potent antiplatelet peptide, triflavin, from Trimeresurus flavoviridis snake venom. *Biochemical Journal*, **277**, 351–357.

Huang, T.F., Sheu, J.R. & Teng, C.M. (1991b) Mechanism of action of a potent antiplatelet peptide, triflavin, from Trimeresurus flavoviridis snake venom. *Thrombosis and Haemostasis*, **66**, 489–493.

Hwang, D.L., Yen, C.F. & Nadler, J.L. (1992) Effect of intracellular magnesium on platelet activation and intracellular calcium mobilization. *American Journal of Hypertension*, **5**, 700–706.

Karniguian, A., Legrand, Y.J. & Caen, J.P. (1982) Prostaglandins: specific inhibition of platelet adhesion to collagen and relationship with c AMP level. *Prostaglandin*, **23**, 437–457.

Kitagawa, S., Shinohara, T. & Kametani, F. (1984) Effects of alcohols on ADP-induced aggregation and membrane fluidity of gel-filtered bovine blood platelets. *Journal of Membrane Biology*, **79**, 97–102.

Lucas, M.J., Leveno, K.J. & Cunningham, F.G. (1995) A comparison of magnesium sulfate with phenytoin for the prevention of eclampsia. *The New England Journal of Medicine*, **333**, 201–205.

McDonald, L.J. & Murad, F. (1996) Nitric oxide and cyclic GMP signaling. *Proceedings of the Society for Experimental Biology and Medicine*, **211**, 1–6.

McKean, M.L., Smith, J.B. & Silver, W.J. (1981) Formation of lysophosphatidylcholine in human platelets in response to thrombin. *Journal of Biological Chemistry*, **256**, 1522–1524.

Nadler, J.L., Goodson, S. & Rude, R.K. (1987) Evidence that prostacyclin mediates the vascular action of magnesium in humans. *Hypertension*, **9**, 379–383.

Nadler, J., Malayan, S. & Luong, H. (1992) Evidence that intracellular free magnesium deficiency plays a key role in increased platelet reactivity in type II diabetes mellitus. *Diabetes Care*, **15**, 835–841.

Pritchard, J.A. (1979) The use of magnesium sulfate in preeclampsia and eclampsia. *Journal of Reproductive Medicine*, **23**, 107–114.

Rasmussen, H.S., McNair, P., Goransson, L., Balslov, S., Larsen, O.G. & Aurup, P. (1988) Magnesium deficiency in patients with and without acute myocardial infarction uncovered by an intravenous loading test. *Archives of Internal Medicine*, **148**, 329–332.

Ravn, H.B., Vissinger, H., Kristensen, S.D. & Husted, S.E. (1996a) Magnesium inhibits platelet activity: an in vitro study. *Thrombosis and Haemostasis*, **76**, 88–93.

Ravn, H.B., Vissinger, H., Kristensen, S.D., Wennmalm, A., Thygesen, K. & Husted, S.E. (1996b) Magnesium inhibits platelet

activity: an infusion study in healthy volunteers. *Thrombosis and Haemostasis*, **75**, 939–944.

Shechter, M., Sharir, M., Paul Labrador, M.J., Forrester, J., Silver, B. & Merz, N.B. (2000) Oral magnesium therapy improves endothelial function in patients with coronary artery disease. *Circulation*, **102**, 2353–2358.

Sheu, J.R., Teng, C.M. & Huang, T.F. (1992) Triflavin, an RGD-containing antiplatelet peptide, binds to GP IIIa of ADP-stimulated platelets. *Biochemical and Biophysical Research Communication*, **189**, 1236–1242.

Sheu, J.R., Chao, S.H., Yen, M.H. & Huang, T.F. (1994) In vivo antithrombotic effect of triflavin, an Arg-Gly-Asp containing peptide on platelet plug formation in mesenteric microvessels of mice. *Thrombosis and Haemostasis*, **72**, 617–621.

Sheu, J.R., Lin, C.H., Peng, C.H. & Huang, T.F. (1996) Triflavin, an Arg-Gly-Asp-containing peptide, inhibits the adhesion of tumor cells to matrix protein via binding to multiple integrin receptors expressed on human hepatoma cells. *Proceedings of the Society for Experimental Biology and Medicine*, **213**, 71–79.

Sheu, J.R., Hung, W.C., Wu, C.H., Ma, M.C., Kan, Y.C., Lin, C.H., Lin, M.S., Luk, H.N. & Yen, M.H. (1999) Reduction in lipopolysaccharide-induced thrombocytopenia by triflavin in a rat model of septicemia. *Circulation*, **99**, 3056–3062.

Sibai, B.M., Graham, J.M. & McCubbin, J.H. (1984) A comparison of intravenous and intramuscular magnesium sulfate regimens in pre-eclampsia. *American Journal of Obstetrics and Gynecology*, **150**, 728–733.

Siess, W. & Lapetina, E.G. (1989) Platelet aggregation induced by alpha 2-adrenoceptor and protein kinase C activation. A novel synergism. *Biochemical Journal*, **263**, 377–385.

Walter, U., Eigenthaler, M., Geiger, J. & Reinhard, M. (1993) Role of cyclic nucleotide-dependent protein kinases and their common substrate VASP in the regulation of human platelets. *Advances in Experimental Medicine and Biology*, **344**, 237–249.

Watson, K.L., Moldow, L.F., Ogburn, P.L. & Jacob, H.S. (1986) Magnesium sulfate: rationale for its use in preeclampsia. *Proceedings of the National Academy of Sciences of the United States of America*, **83**, 1075–1078.

Wroblewski, F. & Ladue, J.S. (1955) Lactic dehydrogenase activity in blood. *Proceedings of the Society for Experimental Biology and Medicine*, **90**, 210–215.