ELSEVIER

In vitro addition of glutathione to blood from zinc-deficient rats corrects platelet defects: Impaired aggregation and calcium uptake

Boyd L. O'Dell, Michelle Emery, Jinming Xia, and Jimmy D. Browning

Department of Biochemistry, University of Missouri, Columbia, MO 65211

Zinc deficiency in rats impairs platelet aggregation, and this defect is associated with the failure of platelets to take up external calcium. Recent data show that the red cell plasma membrane from zinc-deficient rats contains a lower than normal concentration of protein sulfhydryl groups, a defect that can be corrected readily in vivo by zinc repletion. The purpose of this study was to determine the effect of zinc deficiency on sulfhydryl concentration of platelet membrane proteins and to determine if impaired platelet function can be corrected in vitro by treatment of blood with glutathione (GSH), a physiological reducing agent. Immature male rats were fed low zinc diets based on egg white or EDTA-treated soy protein (<1 mg/kg Zn) and the same diets supplemented with 100 mg/kg Zn (control). For membrane analysis, rats were fed soy-based diets for 3 weeks; for reversal experiments they were fed egg white-based diets for 2 weeks. Compared with controls, rats fed the low zinc diets had approximately 15% lower concentration of membrane protein sulfhydryls and their platelets exhibited impaired aggregation coupled with decreased calcium uptake when stimulated with ADP. Treatment of whole blood from deficient rats with 0.2 mM GSH restored aggregation and calcium uptake of washed platelets to control levels. The presence of red cells was essential for the reversal process in as much as treatment of platelet-rich-plasma or washed platelets with GSH had no effect. Treatment of control blood with GSH did not affect platelet aggregation, but decreased calcium uptake. The lower calcium uptake by controls suggests that a feedback mechanism in deficient platelets was not corrected by GSH treatment. The results show that selective sulfhydryl groups in platelet membranes are lost during zinc deficiency and that platelet function can be restored to normal by treatment of blood with GSH. There seems to be an association of oxidation-susceptible thiol groups with a calcium channel and that these groups are protected by normal physiological levels of zinc. (J. Nutr. Biochem. 8:346–350, 1997) © *Elsevier Science Inc. 1997*

Keywords: zinc status; platelet aggregation; calcium uptake; glutathione; membrane sulfhydryls; rat

Introduction

Zinc deprivation in rats leads quickly to defective platelet function, including impaired aggregation of washed platelets as well as of platelet-rich-plasma.[1–3] Associated with the impaired aggregation is a decreased calcium uptake by platelets stimulated with aggregating agents such as ADP, thrombin, or fluoride.[3–5] The results suggest a defective calcium channel in plasma membranes. These and earlier observations have led to the speculation that the first limiting pathological defect of zinc deficiency relates to the loss of essential sulfhydryl groups in critical plasma membrane proteins.[6]

Recent results[7] show that the sulfhydryl concentration in the rat red cell plasma membrane is decreased by zinc deficiency and that the decrease is readily reversed by zinc repletion. The prompt in vivo reversal of apparent sulfhydryl oxidation raised the possibility of reversing this pathology of zinc deficiency in vitro by use of reagents that reduce protein disulfides to thiol groups. Platelets offer a good system to test function reversibility because their thiol-

This research was supported in part by NRICGP/USDA Grant No. 91-37200-6286.

Address reprint requests to Professor Boyd L. O'Dell at 112 Animal Science Research Center, University of Missouri, Columbia, MO 65211 USA.

Received October 25, 1996; accepted February 20, 1997.

655 Avenue of the Americas, New York, NY 10010

0955-2863/97/$17.00
PII S0955-2863(97)00020-X

disulfide status is causally related to aggregation.[8] Thiol reactive reagents, such as monobromobimame, *N*-ethylmaleimide, iodoacetamide, and *p*-chlormercuribenzoate inhibit aggregation,[8–11] and *N*-ethylmaleimide inhibits the calcium influx induced by collagen.[12] Phenylarsine oxide treatment of platelets inhibits both aggregation and calcium uptake, and subsequent treatment with dimercaptopropanol reverses the aggregation inhibition.[13]

The objective of this study was to determine the effect of zinc deprivation on the protein sulfhydryl concentration in platelet membranes and to determine the effect on platelet function of in vitro glutathione (GSH) treatment of blood from zinc deficient rats. Function was measured by aggregability and calcium uptake when washed platelets were stimulated with ADP. Platelet membranes from zinc deprived rats contained a lower concentration of protein sulfhydryls and function was restored to a normal level by treatment of whole blood with 0.2 mM GSH.

Methods and materials

Materials

Fura 2-AM was purchased from Molecular Probes (Eugene, OR USA), and ADP, rat fibrinogen, bovine serum albumin, and glutathione were purchased from Sigma Chemical Co. (St. Louis, MO USA). Other reagents were of reagent grade.

Animals and diets

Immature (150 to 200 g) male rats of Wistar origin and produced in the departmental colony were housed in suspended stainless steel cages kept in a room at 22°C with a 12-hr light/dark cycle. Diet and deionized water were supplied ad libitum. There were two experiments. In the first experiment, designed to determine the effect of zinc deprivation on membrane sulfhydryl concentration, rats were fed for 21 days a low zinc diet based on EDTA-treated soy protein[14] or a control diet, the basal diet supplemented with 100 mg/kg zinc. A pair-fed group that consumed the control diet was included. In the second experiment designed to test in vitro reversal of platelet function by GSH treatment of whole blood, one-half of the rats consumed, for a period of 14 ± 3 days, a low-zinc diet based on egg white protein,[2] and one-half of the control diet, the basal supplemented with 100 mg Zn/kg diet. In this experiment, the aggregation evaluation was performed with one group of rats and the calcium uptake determination was performed with another. All rats were fasted overnight before blood was taken for measurement. The experimental protocols were approved by the University of Missouri-Columbia Animal Care and Use Committee.

Blood collection and treatment

The rats were anesthetized with ethyl ether and blood collected from the abdominal aorta. Blood was collected in a syringe containing anticoagulants dissolved in phosphate buffered saline (PBS; pH 7.4) to supply a final concentration of 1.0 mM citrate and 0.9 unit/mL of heparin. For GSH treatment, the anticoagulant solution contained GSH to supply a final concentration of 0.2 mM. Blood from one-half of each group of rats was collected in the basal anticoagulant (PBS) and one-half in anticoagulant containing GSH. The blood remained in the syringe at room temperature for approximately 15 min and was then centrifuged at 400 g for 10 min to prepare platelet-rich-plasma (PRP). The PRP was removed and treated with citrate, 10 mM final concentration, to facilitate preparation of washed platelets as described previously.[2] After centrifugation of the remaining red cell fraction, plasma was collected for zinc determination by atomic absorption spectrophometry.

Preparation of washed platelets

Platelets were sedimented from the citrated PRP by centrifugation at 600 g for 15 min. The pellet was gently resuspended in an equal volume of Ardlie and Han[15] buffer containing 5 mM HEPES (NaCl 137, $NaHCO_3$ 11.9, KCl 2.68, $MgCl_2$ 1.05, NaH_2PO_4 0.36, HEPES 5.0, and glucose 5.5 mM plus 3.5 g/L bovine serum albumin) adjusted to pH 6.5. The suspension was "rested" at room temperature for 5 min and pelleted again. The washed platelets were resuspended in 1.5 mL of Ardlie buffer adjusted to pH 7.4. Aggregation was performed after a 5 min rest.

Platelet aggregation

Aggregation was monitored by use of a dual channel aggregometer (Chrono-Log Corp., Haverstown, PA USA, Model 340) after the procedure described.[2] Briefly, 0.5 mL of a washed platelet suspension, containing 10 μg of rat fibrinogen and 1 mM Ca^{2+}, was treated first with a concentration of ADP (0.4 μM) that gave 50% of maximum aggregation in control platelets, then with 20 μM ADP to give maximal response. The response given by the lower concentration of ADP was calculated as a percent of the maximal response.

Platelet calcium uptake

Cytosolic calcium concentration was determined by fluorescence measurements made on suspensions of platelets loaded with fura 2 using the technique described previously.[4] Briefly, washed platelets in suspension medium were loaded by addition of 1 μM fura 2-AM and incubation at 37°C for 30 min. Calcium concentration was measured by the fluorescence ratio measured at 505 nm when excited at 340 and 380 nm in a dual wavelength fluorometer (Spex Industries, Edison, NJ USA). Internal calcium release was calculated as the difference between resting cytosolic calcium concentration when the platelets were suspended in medium containing 0.1 mM EGTA and the concentration after stimulation with 0.25 μM ADP. Calcium uptake was calculated as the difference between the cytosolic calcium concentration after internal release and that after stimulation with 0.25 μM ADP when the platelets were suspended in the same medium supplemented with 1 mM Ca^{2+}. This concentration (0.25 μM) of ADP produced calcium uptake on the linear portion of the response curve.[4]

Preparation of platelet membranes

Platelets were collected from PRP as described above and washed twice with 0.1 M phosphate buffer containing 0.5 mg/mL Na_2EDTA, pH 7.4. Washed platelets from approximately 5 mL of blood were suspended in 2 mL of the same buffer and sonicated by four 4-sec bursts using a Model VC375 Vibra Cell sonicator and microprobe (Sonics and Materials, Inc. Danbury, CT USA) at a setting of 4. The resulting mixed membranes were collected by centrifugation at 100,000 g for 30 min at 4°C. The supernate was replaced by 0.5 mL of the same buffer and sonicated again with two 10-sec bursts at a setting of 2. This procedure yielded approximately 0.5 mg of protein.

Determination of membrane sulfhydryl and protein concentrations

Sulfhydryl concentration in the solubilized membrane preparation was measured colorimetrically by the Ellman method as described

Table 1 Zinc status of rats used for determination of platelet aggregation and calcium uptake[1]

Diet	Weight Gain (g/d)	Plasma Zinc (μM)[2]
−ZN	1.4 ± 0.21*	8.5 ± 0.46*
+ZN	4.6 ± 0.28	15.7 ± 0.46

[1]Means ± SEM, $n = 14$. As indicated by the *, the −ZN group was significantly lower than controls (+ZN), $P < 0.001$.
[2]The plasma zinc concentrations are values measured on the anticoagulant diluted plasma samples corrected for dilution.

by Habeeb et al.[16] Briefly, to 1.5 mL of buffer, pH 8.0, containing 0.5 mg/mL of Na_2EDTA and 2% of sodium dodecylsulfate, were added approximately 120 μg of membrane protein. After solubilization of the membranes, 5,5′-dithio-bis-nitrobenzoic acid was added and the tube remained at room temperature for 15 min. Absorbance was measured at 412 nm and sulfhydryl concentration calculated from net absorbance and the reagent's molar absorptivity, 134,600 M^{-1} cm^{-1}. Protein was measured by the method of Lowry et al.[17] using bovine serum albumin as the standard.

Statistical treatment

The data in *Table 1* and *Figure 1* were analyzed by one-way analysis of variance (ANOVA). The other data were analyzed as a 2 × 2 factorial design using the General Linear Models (GLM) procedure of SAS (SAS Institute, Cary, NC USA). Statistical significance of the differences between treatments was assessed by the least squares means component of the GLM procedure.

Results

As shown by the data in *Table 1*, rats fed the low-zinc diet for 2 weeks based on egg white protein exhibited a depressed growth rate and low plasma zinc concentration. They were clearly zinc-deficient, but not as deficient as those fed a soy-based diet for three weeks. Aggregation of platelets from the untreated blood of these rats was impaired, and calcium uptake by platelets stimulated with ADP was depressed (*Figures 2* and *3*). Rats fed the low-zinc, soy protein-based diet for 3 weeks had plasma zinc concentrations of 3.5 μM compared with 14.1 μM for the ad libitum controls. Previous work has shown that platelet function is impaired in rats fed both diets.[2–4]

The sulfhydryl concentration in the platelet membrane proteins was significantly less in platelets from rats fed the low-zinc soy protein-based diet than in controls. As shown in *Figure 1*, zinc-deficient platelet membranes contained 45 nmol compared with 55 nmol for ad libitum-fed controls and 51 nmol per mg protein for pair-fed controls ($P < 0.05$).

Zinc deprivation in rats fed the egg white-based diet decreased the percent of maximal aggregation from 46 to 29% ($P < 0.01$), similar to data reported previously.[2] In vitro addition of 0.2 mM GSH to blood from zinc-deficient rats increased the aggregation response ($P < 0.05$) of washed platelets, restoring it to the control level, as shown in *Figure 2*. GSH treatment of control blood had no effect on platelet aggregation. The effect of glutathione was not observed when the blood was collected in citrate at a final

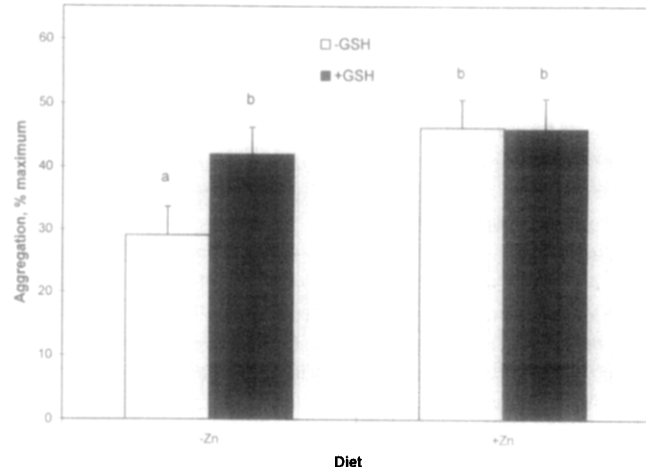

Figure 2 Effect on platelet aggregation of in vitro glutathione addition to blood from zinc deficient and control rats. $N = 11$; ANOVA, $P < 0.03$; zinc effect, $P < 0.01$. Designations are the same as *Figure 1*.

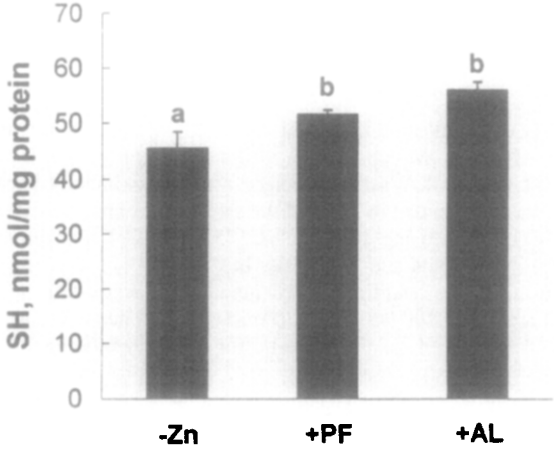

Figure 1 Zinc status and sulfhydryl concentration (nmol/mg protein) in platelet membrane proteins. Bars represent means and bar extensions represent SEM. $n = 7$; bars with different letters are statistically different, $P < 0.05$.

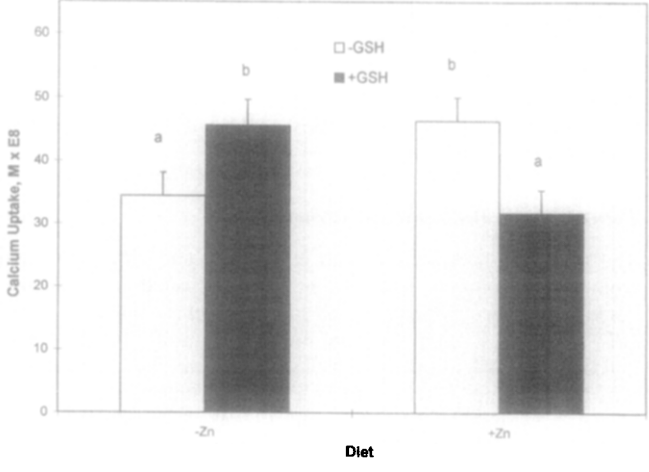

Figure 3 Effect on platelet calcium uptake of glutathione addition to blood from zinc deficient and control rats. $n = 7$; ANOVA, $P < 0.06$, zinc effect; $P < 0.04$. Designations are the same as *Figure 1*.

concentration of 10 mM (data not shown), suggesting that the concentration of free calcium must be maintained within a relatively narrow limit for the in vitro reaction to occur. It is also notable that addition of GSH to PRP from deficient rats at the same concentration for 15 min had no effect on aggregation (data not shown). Furthermore, the addition of 40 μM GSH directly to washed platelets in the aggregation cuvette for 10 min did not affect the response.

Zinc deficiency decreased significantly calcium uptake by agonist-stimulated platelets (*Figure 3*) as reported previously.[3–5] GSH treatment of whole blood from zinc-deficient rats restored calcium uptake to normal. However, GSH treatment decreased the calcium uptake of control platelets. Neither zinc deficiency nor GSH treatment had an effect on internal calcium release in deficient or control platelets (data not shown).

Discussion

The results of this study confirm earlier observations[1–5] that short-term deprivation of zinc in rats leads to impaired platelet function, including decreased aggregability and calcium uptake when stimulated. It is notable that in this series of studies similar observations were made regardless of the protein source, egg white versus soy,[2–4] or level of food intake by controls, pair-fed versus ad libitum.[2,3] In view of the consistent results obtained with the different diets and the lack of food intake effect, the results of two different experiments involving these variables were combined in this article. Further, it is assumed that the membrane thiols are affected similarly by zinc deficiency induced by the two different dietary treatments.

Associated with impaired platelet aggregation and calcium uptake in this study, there was a decreased concentration of thiols in platelet membrane proteins. The latter observation agrees with the observed decrease in thiol concentration of the erythrocyte plasma membrane in zinc-deficient rats.[7] The decreased concentration of sulfhydryl groups in platelet membranes observed here mimics defects observed in platelets treated with thiol reactive reagents.[8–12] These observations suggest strongly a causative relationship between platelet membrane thiol concentration and platelet function. Although the decrease in thiol concentration in platelet membranes from deficient rats is small, approximately 10% compared with pair-fed controls, it could well account for the depressed calcium uptake. The sulfhydryl concentration associated with calcium channels would constitute a small proportion of the total membrane sulfhydryls but might exert a dramatic effect on channel function. It is noteworthy that the membranes must be solubilized to detect a difference in thiol concentration. Determinations made on intact membranes gave lower values that were not different between deficient and control platelets. This suggests that the groups affected by zinc deficiency are "hidden" in the intact membrane.

The fact that both aggregation and calcium uptake of platelets from zinc-deficient rats are improved, in fact restored to normal, by GSH treatment of the whole blood, augments the concept that zinc deficiency results in a decreased thiol:disulfide ratio in membrane proteins critical to cell function. The mechanism by which GSH treatment effects reversal of platelet pathology is unclear, but it clearly involves the red cell component of the blood. Addition of GSH to PRP or washed platelet suspensions before stimulation of aggregation did not restore aggregability. Although it is possible that cells, other than erythrocytes, in whole blood were responsible for the stimulation, it seems unlikely in view of the fact that the PRP contained white blood cells and the well recognized fact that erythrocytes interact with platelets to enhance platelet reactivity.[18]

When stimulated by collagen, platelets in PRP containing metabolically active red cells produce a releasate that sensitizes other platelets to aggregate when they are stimulated by collagen at a later time.[19] Fixed or ATP-depleted red cells do not sensitize platelets in this manner. As observed in this study, a similar releasate seems to be involved in the reaction by which GSH increases the response of zinc-deficient platelets. The GSH concentration in red cells or plasma, or both, may be critical to the normal process of platelet aggregation. The GSH concentration in the plasma of zinc-deficient rats is not significantly lower than normal,[20] but it is lower in whole blood.[21] In any case, red cells must be present for GSH to sensitize platelets to stimulating agents. The red cell probably catalyzes formation of the releasate or requires GSH to effect its release.

In general there is an association between rat platelet aggregation and calcium uptake, but there seems to be a discrepancy in this study; GSH treatment of control blood decreased platelet calcium uptake but had no effect on aggregation. Although these phenomena may not be causally associated, it is highly likely that they are so related considering the observations made in other species[22] as well as the rat.[3] One explanation for the normal aggregation response associated with low calcium uptake in control platelets treated with GSH is that the active releasate exerts more than one effect. Another and more likely explanation, is that zinc-deficient platelets have two defects related to calcium uptake and only one is corrected by GSH treatment. The uptake of calcium by normal human platelets is inhibited by agents, such as phorbol esters and diacylglycerols, that stimulate protein kinase C (PKC).[23,24] Thus, greater activation of PKC in control than in zinc-deficient platelets would cause a decreased uptake of calcium. As we reported earlier, PKC activity is essential for rat platelet aggregation[3] and phorbol esters induce aggregation without the addition of external calcium.[25] Addition of external calcium (1 mM) accelerated aggregation of normal rat platelets, but had no effect on zinc-deficient platelets stimulated by phorbol myristate acetate (PMA). The mechanism by which addition of external calcium increases the response to PMA stimulation is unknown, but zinc status had no effect on the increase in intracellular calcium, as measured by fura-2, when external calcium was added to unstimulated platelets.[25] Calcium is essential for PKC activation and external calcium may bind to PKC in a compartment where it is not detectable by fura-2. It is postulated that in normal platelets there are two pools of uptake calcium, one detectable by fura that is a measure of the "calcium channel," and a nondetectable one that stimulates PKC. It seems that in zinc-deficient platelets, external calcium is not be able to enter the latter compartment when stimulated, and this defect is not corrected by GSH treat-

ment. Thus, the normal feedback inhibition of calcium uptake via PKC is impaired in zinc deficiency.

It is notable that PKC contains a cysteine-rich sequence in its regulatory domain that is essential for phorbol ester and zinc binding.[26,27] Conceivably, in zinc deficiency there is damage to the sulfhydryl rich area of membrane protein kinase, and this defect is not corrected by GSH treatment; hence there is not as much feedback inhibition to calcium uptake in zinc-deficient platelets as in controls. Another possibility is that control platelets are more sensitive to a low level of cytosolic calcium than are zinc deficient platelets.

The platelet pathology observed in zinc-deficient rats is clearly related to a defect in calcium uptake. Present results extend this association to decreased sulfhydryl concentration in platelet membranes. The fact that in vitro GSH treatment of whole blood from zinc-deficient rats restores their sensitivity to ADP stimulation adds credence to the concept that zinc deficiency affects plasma membrane function negatively by way of decreasing the thiol:disulfide ratio in critical plasma membrane proteins. The mechanism by which GSH treatment of blood effects the reversal of platelet pathology is unknown, but it seems to involve red cells and the production of another compound that acts on a calcium channel. Considering the fact that zinc-deficient platelet membranes contain a lower than normal concentration of thiol groups and that GSH restores platelet function, the intermediate may serve as a reducing agent to produce or protect sulfhydryl groups in a calcium channel. Although not determined experimentally, it is assumed that the GSH treatment of whole blood restores the platelet membrane thiols to normal concentration. Because zinc deficiency decreases calcium uptake not only by platelets but also by cortical[28] and hippocampal[29] synaptic membranes, zinc may serve generally to protect calcium and other channels dependent on functional thiols.

Acknowledgments

We thank Dr. Marilyn James-Kracke for the generous help in making available a Spex flurometer for measurement of cytosolic calcium. We also acknowledge the early contributions of Dr. Philip Gordon, who helped in development of the methodology for GSH treatment of zinc-deficient blood to reverse the platelet aggregation defect.

References

1 Gordon, P.R. and O'Dell, B.L. (1980). Rat platelet aggregation impaired by short-term zinc deficiency. *J. Nutr.* **110,** 2125–2129

2 Emery, M.P., Browning, J.D., and O'Dell, B.L. (1990). Impaired hemostasis and platelet aggregation in rats fed low zinc diets based on egg white protein. *J. Nutr.* **120,** 1062–1067

3 Xia, J. and O'Dell, B.L. (1995) Zinc deficiency in rats decreases thrombin-stimulated platelet aggregation by lowering protein kinase C activity secondary to impaired calcium uptake. *J. Nutr. Biochem.* **6,** 661–666

4 Emery, M.P. and O'Dell, B.L. (1991). Compromised zinc status in rats adversely affects calcium metabolism in platelets. *J. Nutr.* **121,** 1763–1768

5 Emery, M.P. and O'Dell, B.L. (1993). Low zinc status in rats impairs calcium uptake and aggregation of platelets stimulated by fluoride. *Proc. Soc. Exp. Biol. Med.* **203,** 480–484

6 O'Dell, B.L. (1981). Metabolic functions of zinc—a new look. In: *Trace Element Metabolism in Man and Animals*, (Howell, J.McC., Gawthorne, J.M., and White, C.L., eds.), p. 319–326, Australian Academy of Science, Canberra, Australia

7 Xia, J. and O'Dell, B.L. (1995). Relationship of plasma membrane sulfhydryl concentration to zinc deficiency pathology. *FASEB J.* **9,** A867

8 Zucker, M.B. and Mauss, E.A. (1986). Modification of platelet functions by monobromobimame, a fluorescent thiol group label. *Thromb. Haemostasis* **55,** 228–234

9 Aledort, L.M., Troup, S.B., and Weed, R.I. (1968). Inhibition of sulfhydryl-dependent platelet functions by penetrating and non-penetrating analogues of parachloromercuribenzene. *Blood* **31,** 471–479

10 Harbury, C.B. and Schrier, S.L. (1974). Modification of platelet sulfhydryl groups. *Thrombos. Diathes. Haemorrh.* **31,** 469–484

11 MacIntyre, D.E., Grainge, C.A., Drummond, A.H. and Gordon, J.L. (1977). Effect of thiol reagents on platelet transport processes and responses to stimuli. *Biochem. Pharmacol.* **26,** 319–323

12 Sato, T., Hashizume, T., and Fujii, T. (1987). *N*-Ethylmaleimide inhibits Ca^{2+} influx induced by collagen or arachidonate on rabbit platelets. *Biochim. Biophys. Acta* **928,** 266–271

13 Sugatani, J., Steinhelper, M.E., Saito, K., Olson, M.S., and Hanahan, D.J. (1987) Potential involvement of vicinal sulfhydryls in stimulus-induced rabbit platelet activation. *J. Biol. Chem.* **262,** 16995–17000

14 O'Dell, B.L., Browning, J.D., and Reeves, P.G. (1983). Plasma levels of prostaglandin metabolites in zinc-deficient female rats near term. *J. Nutr.* **113,** 760–765

15 Ardlie, N.G. and Han, P. (1974). Enzymatic basis for platelet aggregation and release: the significance of the "platelet atmosphere" and the relationship between platelet function and blood coagulation. *Br. J. Haemotol.* **26,** 331–355

16 Habeeb, A.F.S.A (1972). Reaction of protein sulfhydryls with Ellman's reagent. *Methods Enzymol* **34,** 457–464

17 Lowry, O.R., Rosebrough, N.J., and Randall, R.A. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275

18 Marcus, A.J. and Safier, L.B. (1993). Thromboregulation: multicellular modulation of platelet reactivity in hemostasis and thrombosis. *FASEB J.* **7,** 516–522

19 Valles, J., Santos, M.T., Aznar, J., Marcus, A.J., Martinez-Sales, V., Portoles, M., Broekman, M.J., and Safier, L.B. (1991). Erythrocytes metabolically enhance collagen-induced platelet responsiveness via increased thromboxane production, adenosine diphosphate release, and recruitment. *Blood* **78,** 154–162

20 Fernandez, M.A. and O'Dell, B.L. (1983). Effect of zinc deficiency on plasma glutathione in the rat. *Proc. Soc. Exp. Biol. Med.* **173,** 564–567

21 Mills, B.J., Lindeman, R.D., and Lang, C.A. (1981) Effect of zinc deficiency on blood glutathione levels. *J. Nutr.* **111,** 1098–1102

22 Siess, W. (1989) Molecular mechanisms of platelet activation. *Physiol. Rev.* **69,** 158–178

23 MacIntyre, D.E., McNicol, A., and Drummond, A.H. (1985) Tumour-promoting phorbol esters inhibit agonist-induced phosphatidate formation and Ca^{2+} flux in human platelets. *FEBS* **180,** 160–164

24 Valone, F.H. and Johnson, B. (1987) Modulation of platelet-activating-factor-induced calcium influx and intracellular calcium release in platelets by phorbol esters. *Biochem. J.* **247,** 669–674

25 Xia, J., Emery, M., and O'Dell, B.L. (1994). Zinc status and distribution of protein kinase C in rat platelets. *J. Nutr. Biochem.* **5,** 536–541

26 Ono, Y., Fujii, T., Igarashi, K., Kuno, T., Tanaka, C., Kikkawa, U., and Nishiszuka, Y. (1989). Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger sequence. *Proc. Natl. Acad. Sci. USA* **86,** 4868–4871

27 Ahmed, S., Kozma, R., Lee, J., Monfries, C., Harden, N., and Lim, L. (1991). The cysteine-rich domain of human proteins, neuronal chimaerin, protein kinase C and diacylglycerol kinase binds zinc. *Biochem. J.* **280,** 233–241

28 Browning, J.D. and O'Dell, B.L. (1994). Low zinc status in guinea pigs impairs calcium uptake by brain synaptosomes. *J. Nutr.* **124,** 436–443

29 Browning, J.D. and O'Dell, B.L. (1995). Low zinc status impairs calcium uptake by hippocampal synaptosomes stimulated by potassium but not by *n*-methyl-D-aspartate. *J. Nutr. Biochem.* **6,** 588–594