reaction may contribute to increased fidelity will occur in a step after binding but preceding the second site of discrimination, i.e., hydrolysis of aminoacylated products. If the reaction of ATP and amino acid leads to a common intermediate for the interchange and aminoacylation reactions before dissociation of PP_i from the enzyme, the common intermediate will be partitioned between the two reactions. Consequently, the amount of enzyme available for the aminoacylation pathway will decrease. Since the alternate pathway (interchange) is quantitatively much more significant with noncognate amino acids as substrates, the overall rate of aminoacylation of tRNA by noncognate amino acids as substrates, relative to cognate amino acids, will decrease. For cognate amino acids, the rate of interchange relative to the rate of formation of aminoacyl adenylate and free PP_i (first partial reaction in aminoacylation) is much lower (or nonexistent) than for the noncognate amino acids so that the overall rate of aminoacylation is hardly affected. Thus, discrimination results because only noncognate amino acids divert the enzyme to a nonproductive pathway, thus decreasing their rate of aminoacylation. Evidence for the direct interchange reaction should be sought for other synthetases, particularly those in which the second site of discrimination (deacylation) is unimportant, e.g, cysteinyl-tRNA synthetase (Fersht & Dingwall, 1979a) and tyrosyl-tRNA synthetase (von der Haar & Cramer, 1976).

References

Alberty, R. A. (1969) *J. Biol. Chem. 244*, 3290.
Baldwin, A. N., & Berg, P. (1967) *J. Biol. Chem. 242*, 839.
Berg, P. (1958) *J. Biol. Chem. 233*, 501.
Blanquet, S., Fayat, G., Poiret, M., & Waller, J. P. (1975) *Eur. J. Biochem. 51*, 567.
Fersht, A. R., & Dingwall, C. (1979a) *Biochemistry 18*, 1245.
Fersht, A. R., & Dingwall, C. (1979b) *Biochemistry 18*, 1250.
Fersht, A. R., & Dingwall, C. (1979c) *Biochemistry 18*, 2627.
Fersht, A. R., Mulvey, R. S., & Koch, G. L. E. (1975) *Biochemistry 14*, 5.
Hopfield, J. J. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4135.
Jakubowski, H. (1978) *FEBS Lett. 95*, 235.
Janson, C. A., Degani, C., & Boyer, P. D. (1979) *J. Biol. Chem. 254*, 3743.
Josse, J. (1966) *J. Biol. Chem. 241*, 1938.
Kisselev, L. L., & Favorova, O. O. (1974) *Adv. Enzymol. 40*, 141.
Rossomando, E. F., Smith, L. T., & Cohn, M. (1979) *Biochemistry 18*, 5670.
Santi, D. V., Danenberg, P. V., & Satterly, P. (1971) *Biochemistry 10*, 4804.
von der Haar, F., & Cramer, F. (1976) *Biochemistry 15*, 4131.
Yamane, T., & Hopfield, J. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2246.

Arachidonic Acid Releasing Activity in Platelet Membranes: Effects of Sulfhydryl-Modifying Reagents†

Susan T. Silk,* Kenneth T. H. Wong, and Aaron J. Marcus

ABSTRACT: The effects of sulfhydryl-modifying agents on arachidonic acid releasing activity in isolated platelet membranes were studied. Release of arachidonic acid was inhibitable by 5,5′-dithiobis(2-nitrobenzoic acid) and *N*-ethylmaleimide but not by diazenedicarboxylic acid bis(dimethylamide) (diamide). A total of 51.4 nmol of sulfhydryls/mg of membrane protein reacted with 5,5′-dithiobis(2-nitrobenzoic acid) as four kinetically distinguishable classes, forming, in addition to mixed disulfides, inter- and/or intraprotein disulfides. While diamide cross-linking of 85% of the membrane sulfhydryls caused no inhibition, subsequent incubations with either *N*-ethylmaleimide or 5,5′-dithiobis(2-nitrobenzoic acid) inhibited the releasing activity to the same extent as in the membranes which had not been previously cross-linked. Therefore, modification of a few "essential" sulfhydryls was responsible for the rapid inhibition of arachidonic acid releasing activity. Membranes isolated from 5,5′-dithiobis(2-nitrobenzoic acid)-treated intact platelets exhibited less inhibition than membranes similarly treated following isolation. 5,5′-Dithiobis(2-nitrobenzoic acid) (4 mM) inhibited the former by 20% and the latter by 92%. Since no such difference in inhibition was observed with *N*-ethylmaleimide, it is possible that the membrane vesicles are either "inside out" or open to 5,5′-dithiobis(2-nitrobenzoic acid) which does not penetrate membranes [Smith, R. P. P., & Ellman, G. L. (1973) *J. Membr. Biol. 12*, 177–188]. The results are consistent with a model whereby essential sulfhydryls may be located on the inner surface of the platelet membrane. This is also supported by studies where the same 5,5′-dithiobis(2-nitrobenzoic acid) concentration inhibited oxygen consumption of thrombin- or collagen-stimulated platelets by 15–28%. These results suggest that the same enzyme(s) are involved in both arachidonic acid releasing activity in platelet membranes and in arachidonic acid mobilization in stimulated intact platelets.

Mobilization of arachidonic acid from the 2 position of platelet phospholipids is the first step in thromboxane and hydroxy acid synthesis (Marcus, 1978; Lands, 1979). Whereas there is a broader understanding of the thromboxane and hydroxy acid pathways, mechanisms involved in release of arachidonic acid are less well defined. Since thromboxane synthesis is only one of several biological responses of stimulated platelets (Schafer & Handin, 1979; Mustard & Packham, 1977), an understanding of arachidonate mobilization through the study of functional parameters has been difficult (Vanderhoek & Feinstein, 1978; Vallee et al., 1979).

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It appears likely that several lipolytic activities contribute to arachidonic acid release in activated platelets. A phospholipase A_2 activity has been postulated (Bills et al., 1977; Russell & Deykin, 1976; Rittenhouse-Simmons et al., 1977; Rittenhouse-Simmons & Deykin, 1977) and its Ca^{2+} dependence demonstrated in platelet membranes (Derksen & Cohen, 1975; Jesse & Cohen, 1976). The isolation from platelets of two such lipases has been reported (Jesse & Franson, 1979; McKean et al., 1979; Apitz-Castro et al., 1979). Another pathway has also been proposed (Bell et al., 1979) in which sequential action of a phosphatidylinositol-specific phospholipase C and a diglyceride lipase is responsible for arachidonic acid mobilization. The phospholipase C was localized in the platelet cytosol (Rittenhouse-Simmons, 1979; Mauco et al., 1979), while a diglyceride lipase activity, sensitive to sulfhydryl inhibitors, was located in platelet particulate fraction (Bell et al., 1979).

We have studied the Ca^{2+}-dependent arachidonic acid releasing activity (AARA)[1] in its endogenous milieu—the platelet membrane. In this experimental setting the enzymes responsible for arachidonate mobilization and their substrates are present in a relatively unperturbed relationship. The kinetics of AARA inhibition by several sulfhydryl-blocking reagents were investigated, and the results indicate that AARA in platelet membranes depends on the integrity of one or more "essential" sulfhydryls.

Experimental Procedures

Materials. EtMal, Nbs_2, bovine serum albumin (essentially fatty acid free), GSH, and Gly (crystalline, ammonia free) were from Sigma Chemical Co. Diamide and dithiothreitol (grade A) were from Calbiochem-Behring Corp. 97% MNNG was obtained from Aldrich Chemical Co. Nanograde organic solvents were from Mallinckrodt, Inc. [^{3}H]EtMal (sp act. 161 mCi/mmol) and Aquasol-2 scintillation counting solution were from New England Nuclear. Filtron-X scintillation counting solution was from National Diagnostics. Metricel GN-6 cellulose nitrate filters (0.45 μm) were from VWR Scientific Co. Heneicosaenoic acid (21:0) and fatty acid standards were from Supelco Chemical Co. Collagen was from Hormon-Chemie, München, West Germany. Human thrombin was kindly donated by Dr. John Fenton, II. Outdated human platelet concentrates were donated by and fresh human platelet concentrates purchased from the New York Blood Center.

Solutions and Buffers. Buffer in the AARA assay was Gly, pH 9.5: 0.133 M Gly, 0.067 M KCl, and 0.532 g of bovine serum albumin/100 mL buffer, containing either 14.7 mM EDTA or 13.3 mM $CaCl_2$. Tris–citrate buffer, pH 6.3, contained 96 mM KCl, 72 mM Tris base, 1.48 mM citric acid, and 2.9 mM sodium citrate. Other buffers made isotonic with KCl were cacodylate, 0.05 M, Tris, 0.025 M, and phosphate, 0.05 M.

EtMal, diamide, and GSH solutions were prepared immediately prior to use. Nbs_2 stock solutions (10 or 20 mM) in pH 7.0 buffer were stored at −20 °C (4 weeks) and adjusted to proper pH and concentrations before use.

Membrane Preparation. (*a*) *Untreated Platelets.* Within 4 days following expiration, platelet concentrates were combined (9–12 units/transfer pack) and processed as described by Bressler et al. (1979). Platelets were washed with 300 mL of 0.9% NaCl and then with 300 mL of Tris–citrate buffer (pH 6.3) and suspended in 3 volumes of the same buffer. Cell disruption was carried out by nitrogen decompression (Broekman et al., 1974). Intact platelets were sedimented (5 min) at 625*g*. The homogenate was layered on an equal volume of 30% (w/v) sucrose containing 5 mM EDTA and ultracentrifuged in an SW 41 Ti rotor at 286400*g* for 90 min. The membrane fraction from the buffer–sucrose interphase was collected and stored at −60 °C.

(*b*) *Nbs_2- or EtMal-Treated Platelets.* Once-washed platelets, combined in 1.15% KCl to a volume of 2 mL/unit of platelet concentrate, were incubated at 25 °C with equal volume of reagent or buffer. Reactions were stopped with equivalent concentrations of GSH, and platelets were washed and processed as described for untreated platelets above. Treated and control platelets were homogenized simultaneously.

Membranes from untreated or treated platelets were thawed and washed in 3 volumes of 1.15% KCl, sedimented by ultracentrifugation in a fixed-angle rotor at 226400*g* for 90 min, suspended in 1.15% KCl to ~20 mg of protein/mL, and stored at −60 °C. This stock suspension was used within 3 months. All processing was carried out at 1–5 °C; 1.15% KCl was always used for membrane washing and suspension; proteins were determined (Miller, 1959) with human serum albumin as standard.

AARA Assay. Membranes were washed again prior to assay (5–15 mg of protein/13 mL). Incubations (3 h) at pH 9.5 and 37 °C were initiated by adding a 0.5-mL membrane suspension containing 1–2 mg of protein to 1.5 mL of assay buffer. The extent of arachidonic acid release was determined as described by Derksen & Cohen (1975), except that the reaction was stopped with 0.2 mL of 0.1 M EDTA in 2 M NaCl, and lipids were extracted twice with 4.4 mL of ethyl acetate. The free fatty acids in the extract were methylated for 1 h with diazomethane (Aldrich, MNNG–diazomethane apparatus) in 0.5 mL of diethyl ether and 50 μL of methanol (Fales et al., 1973). The methylated mixture in 100 μL of pentane was analyzed on a Hewlett-Packard 7620A gas–liquid chromatograph equipped with a flame ionization detector. Two stationary phases were used: 10% SP-222-PS and 10% SP-2340 (Supelco).

AARA was defined as the amount of arachidonic acid obtained in the presence of 10 mM Ca^{2+} minus that obtained in the presence of EDTA. Activity in the presence of an inhibitor was expressed as (AARA in the presence of inhibitor/AARA in untreated membranes) × 100.

Reaction of Membranes with [^{3}H]EtMal. Membrane suspensions (0.5 mL), preincubated for 1 min at 25 °C, were mixed with 0.5 mL of 3.0 mM [^{3}H]EtMal (sp act. 3.54×10^4–5.53×10^3 cpm/μmol). The reaction was stopped with 5 mL of 0.15 M nonradioactive EtMal in ice-cold buffer, and the suspension was immediately filtered through the Metricel filter on a Millipore manifold. Following rapid washing with 10 mL of buffer, suction was discontinued, and 10 mL of 0.15 M EtMal was equilibrated on the filter for 10 min. Air-dried filters were placed in scintillation vials and membrane proteins solubilized in 1 mL of 10% $NaDodSO_4$ (Cuatrecasas, 1971). Filters were then dissolved in 10 mL of Filtron-X scintillation fluid; 4 mL of Aquasol-2 was added, and samples were counted. Background (no membranes) averaged 28% of the counts. Counting efficiency was 12–15%.

Determination of Nbs_2-Reacting Sulfhydryls (Ellman, 1959). (*a*) *Continuous Method.* Membranes suspended in

[1] Abbreviations used: AARA, arachidonic acid releasing activity; EtMal, *N*-ethylmaleimide; [^{3}H]EtMal, *N*-[*ethyl*-2-^{3}H]ethylmaleimide; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); Nbs, 5-thio-2-nitrobenzoic acid; diamide, diazenedicarboxylic acid bis(dimethylamide); GSH, reduced glutathione; MNNG, *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; $NaDodSO_4$, sodium dodecyl sulfate.

buffer to a protein concentration of 0.25–0.5 mg/mL were equilibrated at 25 °C for 1 h. Cuvettes (1 cm, containing 3 mL of either buffer or sample) were placed in the scatter transmission accessory of a Cary 118C double-beam spectrophotometer equipped with a high-intensity light source. The position of the phototube was fixed for the duration of the experiment (Butler, 1972). The base line was observed for 20 min to ensure stability; Nbs_2 was added rapidly with stirring to the reference and then to sample cuvettes ($t = 0$). Addition to sample, mixing, closing of compartment, and response time of the instrument required ~25 s. A_{412} was continuously recorded until equilibrium was attained (Figure 1). Membranes that settled out during the experiment were not used for kinetic studies. Finally, 10 μL of GSH of known concentration was added to the sample cuvette and the "effective" ϵ_{412} was calculated from ΔA_{412}.[2]

(*b*) *Direct Method.* Membranes were incubated in a known volume with Nbs_2 at 25 °C for 2 h (0.5 mg of protein/mL; ~2 mM Nbs_2, pH 7.2, cacodylate buffer, and 1 mM EDTA) and sedimented by ultracentrifugation in open poly(carbonate) tubes. A_{412} of the supernatants was read against appropriate blanks.

(*c*) *Indirect Method.* Following incubation with Nbs_2 as in the direct method, the membranes were washed twice, mixed disulfides were reduced with dithiothreitol, and Nbs was quantitated spectrophotometrically (Butterworth et al., 1967).

Nbs_2 and EtMal Inhibition of AARA. Once-washed membranes were added to buffer at 25 °C. The reagent in the same buffer was added with mixing and processed as described in the legends to Figures 2–5. Inhibition studies by micromolar Nbs_2 at pH 9.5 were done on twice-washed membranes.

Concentrations of Nbs in very dilute Nbs_2 solutions at pH 9.5 were determined from A_{412} readings. Membranes were incubated with Nbs-containing buffers, and AARA was assayed in the usual manner.

Oxygen Consumption Studies (Mürer, 1968). Blood collection, processing, and oxygen consumption studies were done as described by Bressler et al. (1979). An oxygen polarograph with a Clark-type electrode was used (Model 5331, Yellow Springs Instruments).

Effects of Diamide on AARA. Membranes were incubated with diamide at at final protein concentration of 1 mg/mL. Following the reaction, 1.15% KCl (7:5 v/v) was added, and membranes were sedimented by ultracentrifugation and assayed for AARA.

Determination of Residual Free Sulfhydryls following Incubation with EtMal or Diamide. Following incubation, membranes were washed twice and assayed for free sulfhydryls with Nbs_2 by the direct or the indirect method.

Results

Membranes isolated from either fresh or outdated platelet concentrates possessed Ca^{2+}-dependent AARA. The average release of arachidonic acid in the presence of 10 and 0.1 mM Ca^{2+} and 10 mM EDTA was 17.5, 4.4, and 1.8 nmol (mg of protein)$^{-1}$ h^{-1}, respectively. Oleic acid, the only other unsaturated fatty acid released to a significant degree, measured 3.8, 2.4, and 1.8 nmol (mg of protein)$^{-1}$ h^{-1}. Since the relative abundance of arachidonic to oleic acid in membrane phospholipids is 1.8:1 (Broekman et al., 1976), it follows that under our conditions preferential release of arachidonic acid took place. The AARA in unwashed membranes was stable at −60 °C for 8 months.

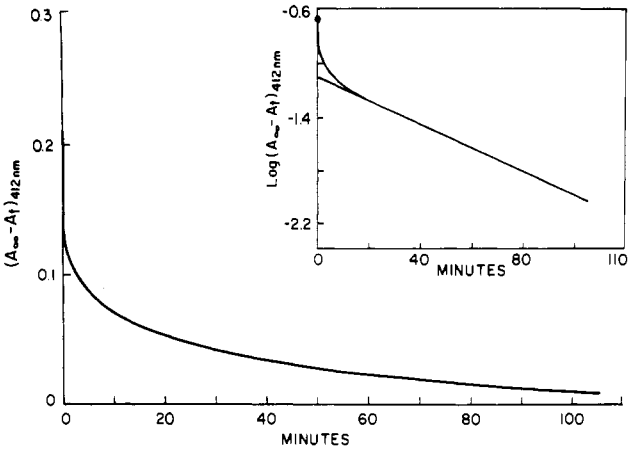

FIGURE 1: Reaction of Nbs_2 with platelet membrane sulfhydryls. Calculated differences between final absorbance (A_∞) and absorbance at time t (A_t) vs. time (0.63 mM Nbs_2; 0.28 mg of protein/mL; cacodylate buffer, pH 7.0, 1 mM EDTA). The total number of reacting sulfhydryls was determined from A_∞. (Insert) Semilogarithmic plot of the above data. Pseudo-first-order reaction rate constants and percent sulfhydryls for each class were obtained from slopes of successive tangents and their intercepts at $t = 0$ (Murphy, 1976).

Table I: Kinetic Parameters for Reaction of Nbs_2 with Platelet Membrane Sulfhydryls[a]

parameter	class I	class II	class III	class IV
k_1 (min^{-1})	0.0261 ± 0.0057	0.281 ± 0.065	1.59 ± 0.48	>>1.59
% SH	40 ± 4	22 ± 2	14 ± 2	24 ± 5
$t_{1/2}$ (min)	26.6	2.48	0.44	<<0.44

$$\frac{\text{nmol of SH}}{\text{mg of protein}} = 51.4 \pm 2.6 \text{ (six experiments)}^{b,c}$$

$$\frac{\text{nmol of SH}}{\text{mg of protein}} = 46.6 \pm 1.8 \ (\epsilon = 1.36 \times 10^4; \text{ Ellman, 1959})$$

[a] 0.2 mL of 10 mM Nbs_2 was added to a 3-mL membrane suspension (0.25–0.5 mg of protein/mL final concentration). Final Nbs_2 concentration 0.63 mM. Three different membrane preparations were used. The buffers were cacodylate, pH 7.0 (10 mM Ca^{2+}, four experiments; 1 mM EDTA, three experiments; no additions, one experiment). Values listed as mean ± SD. Further experimental details are given under Experimental Procedures and Figure 1. [b] Of the eight experiments, only six with known protein concentrations were used to determine the total number of sulfhydryls. [c] Calculated from equilibrium absorbance values and effective molar extinction coefficients ($\epsilon_{effective} = 1.05 \times 10^4$–$1.45 \times 10^4$ M^{-1} cm^{-1}).

Reaction of Nbs_2 with Membrane Sulfhydryls. Reactions of membrane sulfhydryls with excess Nbs_2 were subjected to pseudo-first-order kinetic analysis (Figure 1, Table I). Four classes of sulfhydryls were distinguishable. Because class IV sulfhydryls reacted within the response time of the spectrophotometer (25 s), their rate constant could not be determined. A total of 51.4 or 46.6 nmol of free SH/mg of protein was obtained, depending on the values of the extinction coefficient used in the calculations (Table I). The presence or absence of Ca^{2+} had no discernible effect on either the rates or the total number of reacting sulfhydryls. In addition, the direct method of analysis gave 45.5 ± 6.7 nmol of SH/mg (nine determinations), while the value obtained by the indirect method was

[2] The presence of light-scattering membranes in the chromophore-containing solutions may influence the measured absorbances in a complex manner (Butler & Norris, 1960). Absorbance amplificiation due to sample turbidity was partially eliminated by use of the scattered transmission accessory; however, a probable decrease in absorbance due to sample fluorescence was uncorrected for. Therefore, following equilibration, an $\epsilon_{effective,412}$ was obtained from the increase in absorbance upon addition of a known quantity of GSH.

Table II: Nbs_2 Titration of Membrane Sulfhydryls[a]

Nbs_2 (μM)	time to reach equilibrium (min)	equilibrium values: half-cystines[b] (nmol/mg)	equilibrium values: S-Nbs (nmol/mg)	SH reacted in 10 min (nmol/mg)
5	1.7	7.9	2.1	10.0
25	74	14.3	15.4	22.9
50	94	23.8	21.8	34.8

[a] pH 9.5; 0.74–1.48 mg of membrane protein/mL. A_{412} was recorded continuously. [b] Extent of cross-linking was calculated from experimentally obtained equilibrium Nbs and known initial Nbs_2 concentrations (Flashner et al., 1972; Telegdi & Straub, 1973).

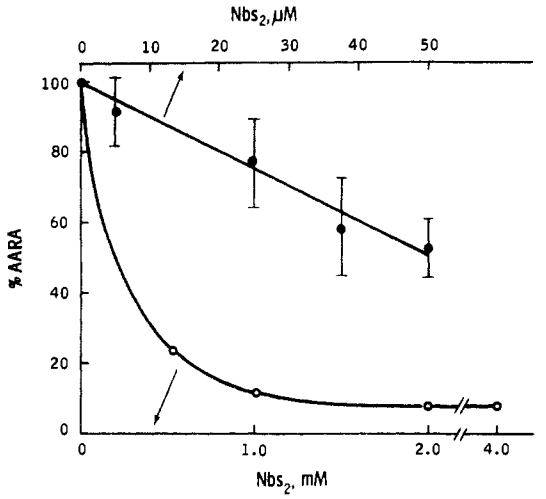

FIGURE 2: Effect of Nbs_2 on AARA. (○) Millimolar Nbs_2 concentrations (0.5 mg of protein/mL; phosphate buffer, pH 7.0; 20 min). Reaction was stopped by rapid cooling to 0 °C. Five milliliters of cold KCl was added to a 7.5-mL membrane suspension; the membranes were pelleted, resuspended in KCl, and assayed for AARA. (●) Micromolar Nbs_2 concentrations (0.9–1.4 mg of protein/mL; glycine buffer, pH 9.5, no albumin and no or 6.7 mM Ca^{2+}; 10 min). Two different membrane preparations were used (four experiments). Reaction with Nbs_2 was stopped by addition of 1 mL of Gly buffer (pH 9.5, albumin, Ca^{2+}) and membranes were assayed for AARA. Values are mean ± SD.

32.4 ± 2.3 nmol/mg (five determinations). Substantial cross-linking of sulfhydryls was observed during Nbs_2 titrations of membranes at pH 9.5 (Table II), which could account for the discrepancy in the total number of sulfhydryls obtained by the different techniques. The extent of cross-linking at pH 7.4 was the same as at pH 9.5 (not shown). The presence or absence of calcium did not affect the results.

AARA of Nbs_2-Treated Membranes. Inhibition of AARA by both millimolar and micromolar Nbs_2 concentrations at pH 7.0 and 9.5, respectively, is shown in Figure 2. Total inhibition was obtained when membranes were incubated at pH 8.0 with 4 mM Nbs_2 (20 min, data not shown). Figure 3 shows the kinetics of AARA inhibition by 0.63 mM Nbs_2. These rates were not affected by the presence of either 10 mM Ca^{2+} or 1 mM EDTA.

AARA in Membranes from Nbs_2-Treated Platelets and Oxygen Consumption Studies in Intact, Stimulated Nbs_2-Treated Platelets. AARA of membranes isolated from Nbs_2-treated platelets was compared with the activity of simultaneously isolated control membranes (Table III). Nbs_2 (4 mM) caused only 20% inhibition, while 8.6 mM Nbs_2 inhibited AARA by 75%. No significant difference in inhibition was observed in membranes from fresh or outdated platelet concentrates.

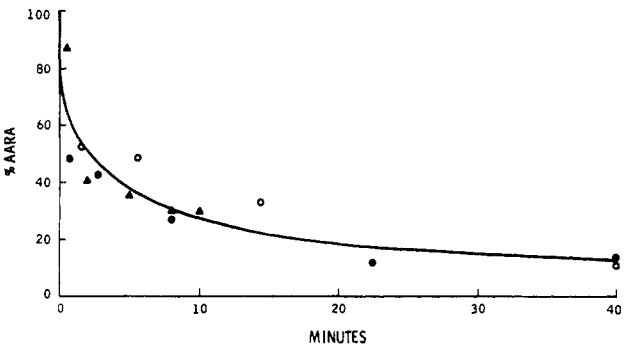

FIGURE 3: Kinetics of AARA inhibition by 0.63 mM Nbs_2 (0.5 mg of protein/mL; cacodylate buffer, pH 7.0): (○) no additions; (●) 10 mM Ca^{2+}; (▲) 1 mM EDTA. Nbs_2 stock solution was added to membrane suspension in corresponding buffer. GSH in concentrations equivalent to those of Nbs_2 was used to stop the reactions. The presence of GSH, up to 2 mM, in the membrane washing solutions had no effect on AARA.

Table III: AARA in Membranes from Nbs_2-Treated Platelets[a]

Nbs_2 (mM)	pH	time (min)	% AARA
4	7.1	20	79
4[b]	7.1	20	83
8.6	7.0	40	25

[a] Once-washed fresh or expired platelet concentrates were reacted with Nbs_2 (1.2×10^{10}–1.8×10^{10} platelets/mL). Reaction was stopped with equal volume of cold Tris-citrate buffer, pH 6.3. Platelets were collected by centrifugation and washed twice, and membrnaes were isolated. AARA assay as described under Experimental Procedures. [b] Fresh platelet concentrates.

Table IV: Effect of Nbs_2 on the Oxygen Consumption of Resting and Stimulated Platelets[a]

stimulus	nmol of O_2/min: control, unstimulated	nmol of O_2/min: control, stimulated	nmol of O_2/min: Nbs_2-treated, unstimulated	nmol of O_2/min: Nbs_2-treated, stimulated	oxygen burst % of control
thrombin (10 units/mL)	9.45	55.8	10.3	43.6	72
thrombin (5 units/mL)	10.3	40.5	10.9	36.6	85
collagen (32 μg/mL)	9.15	22.2	9.75	19.2	72

[a] Platelets were isolated from freshly collected blood from donors who had not ingested aspirin during the previous week. Once-washed platelets were reacted with 4 mM Nbs_2 (pH 7.1, 25 °C, 20 min, 1.2×10^{10} platelets/mL) and washed twice. Final platelet suspension was in 1.15% KCl (1.2×10^{10} platelets/mL). Control platelets were treated identically, except for omission of Nbs_2. Data are from one of two separate experiments. All determinations were done in duplicate.

Rates of oxygen consumption of untreated and Nbs_2-treated platelets are given in Table IV. Basal respiration was not affected by Nbs_2. Following stimulation with either thrombin or collagen, the rapid increase in oxygen uptake due to oxygenation of released arachidonic acid (Hamberg & Samuelsson, 1973; Fukami et al., 1976; Pickett & Cohen, 1976) was reduced in Nbs_2-treated platelets. As reported elsewhere (Harbury & Schrier, 1974; MacIntyre et al., 1977), we have also found that Nbs_2 treatment of platelets did not affect aggregation.

Kinetics of Reaction of EtMal with Membrane Sulfhydryls—Effect on AARA. Inhibition of AARA by millimolar EtMal concentrations is shown in Figure 4. Marked inhibition was observed only at concentrations >0.5 mM. Figure 5 shows the kinetics of inhibition by 1.66 mM

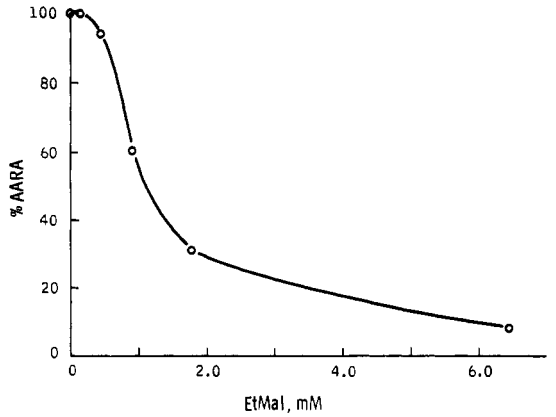

FIGURE 4: Effect of millimolar concentrations of EtMal on AARA (0.5 mg of protein/mL; phosphate buffer, pH 7.2, 1 mM EDTA; 10-min incubations). Reaction was stopped as indicated in Figure 3.

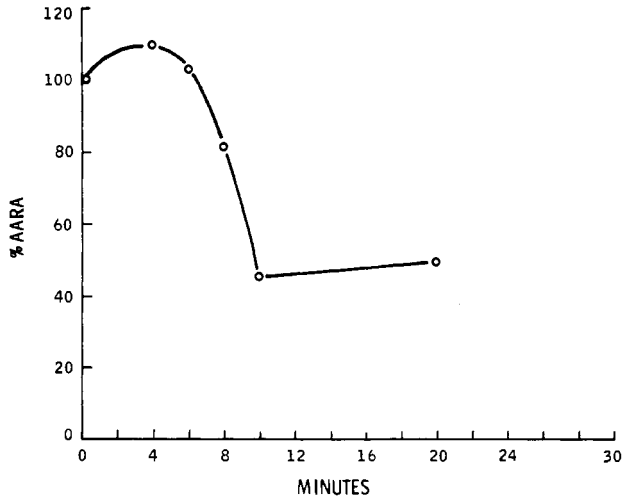

FIGURE 5: Kinetics of AARA inhibition by 1.66 mM EtMal at pH 7.2 (0.4 mg of protein/mL). Buffer contained 1 mM EDTA. Reaction was stopped as indicated in Figure 3.

EtMal. No pH effect was observed for 1 mM EtMal between pH 6.9 and 7.6 (0.1 pH unit intervals, duplicate 10-min incubations, data not shown).

Membranes previously incubated with 1.5 mM EtMal for 4 and 20 min had the AARA inhibited further by 10-min incubations at pH 7.2 with 2 mM Nbs_2. The activity of membranes treated in this manner went from 98% to 20% and from 61% to 11%, respectively.

Kinetic studies with [^{3}H]EtMal are given in Table V. In 1 h 21.1 nmol of membrane residues reacted with [^{3}H]EtMal. Of these residues, 59% reacted in the first 4 min. Approximately 1.8 nmol was alkylated during the time interval when most of the AARA inhibition took place (4–10 min, Figure 5). Using Nbs_2 to determine the residual number of sulfhydryls following incubations with 1.5 mM nonradioactive EtMal, we have found that between 14.1 and 22 nmol of SH reacted with EtMal during the first 4 min. Between 4 and 10 min, an additional 1.8–3.5 nmol of SH reacted. It appears, therefore, that under these conditions no significant number of residues other than sulfhydryls reacted with EtMal.

AARA in Membranes from EtMal-Treated Intact Platelets. No significant differences in inhibition were observed when either isolated platelet membranes or intact platelets prior to membrane isolation were incubated with 1 mM EtMal (pH 7.0, 5 min). The AARA of membranes isolated from these platelets was 89%, while isolated membranes incubated with EtMal under similar conditions possessed 98% AARA.

Table V: Rate of Reaction of [^{3}H]EtMal with Membranes[a]

incubation time (min)	nmol of [^{3}H]EtMal reacted/ mg of protein
4	12.5 ± 1.8
10	14.3 ± 1.8
20	14.6 ± 2.0–17.5 ± 2.6
60	21.1 ± 1.7

[a] Results of two sets of experiments (1 mg of protein/experiment; 1.5 mM [^{3}H]EtMal; pH 7.2, 1 mM EDTA). In the first set, 4-, 10-, and 20-min incubations were carried out in duplicate; in the second set 20- and 60-min incubations were done in triplicate. The range in values listed for 20-min incubations indicates the discrepancy between different experiments. The low value belongs to the first set.

Cross-Linking of Membrane Sulfhydryls and Its Effect on AARA. The effect of sulfhydryl cross-linking alone on AARA was studied with diamide (Kosower et al., 1969; Harris & Biaglow, 1972). At pH 9.5 (10 min, 0 °C) 5 mM diamide reduced the number of free SH from 51.4 to 5.3 nmol/mg. The AARA of diamide-treated membranes increased by 6–11%. Subsequent incubations of these membranes with 2 mM Nbs_2 (pH 7.2, 20 min) or with 1.5 mM EtMal (pH 7.2, 10 min) reduced their AARA by 92% and 83%, respectively.

Discussion

Results of these studies indicate that platelet membrane AARA is dependent upon the integrity of one or more essential sulfhydryls. AARA was inhibitable by both Nbs_2 and EtMal but not by diamide. Analysis of the inhibition studies combined with sulfhydryl reaction kinetics provided evidence for this conclusion.

At pH 7.0, 51.4 nmol of membrane SH/mg of protein reacted with Nbs_2 as four kinetically distinct classes. From the pseudo-first-order reaction rate constants (Table I), the calculated second-order constants for classes I–III were 41.5, 446, and 2500 M^{-1} min^{-1}, respectively. Two of these constants are in good agreement with those obtained by Ando & Steiner (1973). They reported 31 nmol of SH/mg of protein reacting as two classes with reaction rate constants of 390 and 1332 M^{-1} min^{-1}. Differences in membrane preparation and in reaction conditions could account for the discrepancies. As was pointed out by these authors, their method of analysis precluded identification of the very fast reacting group—class IV of our study. Also, the continuous method used in our experiments assured attainment of equilibrium and thus identification of class I, which constituted 40% of the total sulfhydryls (Table I). Robey et al. (1979), using methodology similar to that of Ando and Steiner, reported a range of 12.5–24.3 nmol of SH/mg. The reasons for this difference are not readily apparent.

Cross-linking of membrane sulfhydryls was demonstrated by the spectrophotometric titrations of membranes with Nbs_2 (Table II). This was not merely a phenomenon related to high pH, since similar results were obtained at pH 7.4 (results not shown). Although the reactions of membrane sulfhydryls with excess Nbs_2 could not be analyzed in a similar fashion, the difference of 19 nmol in the total number of sulfhydryls determined by the continuous and indirect methods can be attributed to disulfide formation. We have also determined a loss of both free sulfhydryls and mixed disulfides upon pH 9.5 incubations of Nbs_2-treated membranes (data not shown)—thus indicating additional sulfhydryl cross-linking. Although Ando & Steiner (1973) obtained consistent results by both the direct and indirect techniques, they reported cross-linking in their $NaDodSO_4$–polyacrylamide gel electrophoretic studies

of Nbs_2-treated membranes (Ando & Steiner, 1976).

The effect of Nbs_2 on platelet membrane AARA could be due to (1) mixed disulfides, (2) the presence of low concentrations of Nbs during the assay, and (3) Nbs_2-induced inter- and/or intraprotein disulfide cross-linking. Possible Nbs inhibition was excluded by AARA assays carried out in the presence of trace amounts of this reagent. No inhibition was observed by 2.8–46 μM Nbs and a maximum of 4 μM Nbs_2 (data not shown). Cross-linking of membrane sulfhydryls per se did not inhibit, since diamide cross-linked more than 80% of the membrane sulfhydryls without inhibiting AARA. Similar diamide cross-linking has also been reported in erythrocytes (Haest et al., 1977). The sulfhydryls essential to AARA were not cross-linked by diamide, since Nbs_2 or EtMal could subsequently inhibit AARA in such membranes. We therefore conclude that AARA inhibition was due only to the formation of mixed disulfides.

Although there is a correlation at pH 7.0 between the rate of reaction of Nbs_2 with membrane sulfhydryls and its inhibitory effect on AARA (Figures 1 and 3), at pH 9.5 no such correlation was observed. Thus, membranes with a given number of free sulfhydryls had greater AARA when the reaction with Nbs_2 took place at pH 9.5 than at pH 7.0 (Figures 2 and 3; Table II; free sulfhydryls at pH 7.0 were calculated by using parameters in Table I). This suggests that AARA inhibition was caused by modification of only certain sulfhydryls, whose reaction rates with Nbs_2 relative to the rest of membrane sulfhydryls differed with pH.

Nbs_2 inhibition of AARA in isolated platelet membranes was much greater than in membranes from Nbs_2-treated intact platelets. Incubations (20 min) of the membranes with 4 mM Nbs_2 produced 92% inhibition of AARA, while similar incubations of intact platelets resulted in only 20% inhibition. Membranes isolated from platelets preincubated with 8.6 mM Nbs_2 still possessed 25% of AARA, although in isolated membranes such inhibition can be achieved with only 0.5 mM Nbs_2 (Figure 2, Table III). It appears that some of the essential sulfhydryls are not accessible to Nbs_2 in whole platelets, possibly because of the poor membrane-penetrating properties of this reagent (Smith & Ellman, 1973). In isolated membranes, however, the essential sulfhydryls are more accessible to Nbs_2.

Nbs_2 inhibition of the oxygen "burst" in thrombin- and collagen-stimulated platelets was similar in magnitude to the AARA inhibition in membranes from Nbs_2-treated platelets (Tables III and IV). The increase in oxygen consumption (oxygen burst) upon platelet stimulation depends upon sequential activities of the arachidonic acid releasing enzyme(s) and of the cyclooxygenase. Since platelet cyclooxygenase is not inhibited by sulfhydryl reagents (Ho et al., 1976), the reduction of the burst in the Nbs_2-treated platelets may be attributable to inhibition of arachidonic acid release. These results suggest that the enzymes involved in AARA in the platelet membrane are the same as those responsible for arachidonic acid mobilization in stimulated intact platelets.

The results of the Nbs_2 studies may be interpreted in several ways. The isolated membrane vesicles could have been "inside out" or "open" to nonpenetrating reagents. The membrane proteins could also have been altered as a consequence of the isolation procedure. In any of the above instances, additional sulfhydryls could have been exposed to Nbs_2. Had major changes in the microenvironment of the sulfhydryls taken place so as to alter Nbs_2 inhibition of AARA from 20% to 92%, one would expect such changes to also affect EtMal inhibition. This was not the case.

The effect on AARA in EtMal-treated membranes was similar to that in membranes from EtMal-treated intact platelets. Since EtMal, unlike Nbs_2, is considered to be a membrane-penetrating reagent (Smith & Ellman, 1973; Muenzer et al., 1975), its reactivity with membrane residues should be independent of whether the vesicles are closed or open. Thus, it appears likely that no major structural changes took place during membrane isolation. Therefore, we may be dealing with either open or inside-out vesicles. Our results are compatible with a model wherein most of the essential sulfhydryls may be facing the interior of the platelet. It is of interest that Derksen & Cohen (1975) have suggested that phospholipase A_2 in human platelets is an endoenzyme.

The kinetics of EtMal inhibition of AARA in platelet membranes was different from that of Nbs_2. For an equivalent inhibitory effect higher EtMal concentrations were required (Figures 2–5). This finding is not unusual, for selectivity of a reagent for a particular residue in a membrane protein does not necessarily correlate with its membrane-penetrating properties (Carraway & Shin, 1972; Brown et al., 1975).

The possibility that EtMal inhibition was due to alkylation of residues other than sulfhydryls (Brewer & Riehm, 1967) was investigated. Since there was no pH effect on EtMal inhibition between 6.9 and 7.6, reaction with a histidine with a normal pK (Steinhardt & Beychok, 1962) was ruled out. Comparing the total number of alkylated residues with the number of alkylated sulfhydryls (Table V and Results) in the time period when AARA inhibition took place (4–10 min; Figure 5), we obtained 1.8 nmol of total residues and 1.8–3.5 nmol of alkylated sulfhydryls. Had EtMal reacted with residues other than cysteines during that time, the values obtained with [^{3}H]EtMal would have been greater than those obtained by means of Nbs_2. Therefore, inhibition due to modification of a lysine or a histidine with an abnormally high pK can be considered unlikely and was probably due to sulfhydryl modification.

Extensive studies of EtMal inhibition of arachidonic acid mobilization in stimulated, intact platelets are not feasible. EtMal rapidly inhibits aggregation and release, induces lysis, and totally abolishes basal platelet respiration (Harbury & Schrier, 1974; MacIntyre et al., 1977; Muenzer et al., 1975). Although prolonged incubation with EtMal was reported to stimulate prostaglandin production (Stuart et al., 1975; Jafari et al., 1976), the same effect seems to be produced by platelet lysis (Nordøy et al., 1978). We suggest that the effect of EtMal on platelet prostaglandin synthesis is related to lysis.

The EtMal and Nbs_2 inhibition of AARA may involve one or several enzymes. These might include one or more phospholipases, lipase zymogen(s), or an "activating" enzyme. Whether the inhibition observed was due to active-site modification, conformational changes, or stearic hindrance remains to be elucidated. By radiolabeling of diamide cross-linked membranes with EtMal or Nbs_2, identification and possible isolation of these enzymes might be achieved.

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References

Ando, Y., & Steiner, M. (1973) *Biochim. Biophys. Acta 311*, 26–37.

Ando, Y., & Steiner, M. (1976) *Biochim. Biophys. Acta 419*, 51–62.

Apitz-Castro, R. J., Mas, M. A., Cruz, M. R., & Jain, M. K. (1979) *Biochem. Biophys. Res. Commun. 91*, 63–71.

Bell, R. L., Kennerly, D. A., Stanford, N., & Majerus, P. W. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3238–3241.

Bills, T. K., Smith, J. B., & Silver, M. J. (1977) *J. Clin. Invest. 60*, 1–6.

Bressler, N. M., Broekman, M. J., & Marcus, A. J. (1979) *Blood 53*, 167–178.

Brewer, C. F., & Riehm, J. P. (1967) *Anal. Biochem. 18*, 248–255.

Broekman, M. J., Westmoreland, N. P., & Cohen, P. (1974) *J. Cell Biol. 60*, 507–519.

Broekman, M. J., Handin, R. I., Derksen, A., & Cohen, P. (1976) *Blood 47*, 963–971.

Brown, P. A., Feinstein, M. B., & Sha'afi, R. I. (1975) *Nature (London) 254*, 523–525.

Butler, W. L. (1972) *Methods Enzymol. 24B*, 3–25.

Butler, W. L., & Norris, K. H. (1960) *Arch. Biochem. Biophys. 87*, 31–40.

Butterworth, P. H. W., Baum, H., & Porter, J. W. (1967) *Arch. Biochem. Biophys. 118*, 716–723.

Carraway, K. L., & Shin, B. C. (1972) *J. Biol. Chem. 247*, 2102–2108.

Cuatrecasas, P. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 1264–1268.

Derksen, A., & Cohen, P. (1975) *J. Biol. Chem. 250*, 9342–9347.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70–77.

Fales, H. M., Jaouni, T. M., & Babashak, J. F. (1973) *Anal. Chem. 45*, 2302–2303.

Flashner, M., Hollenberg, P. F., & Coon, M. J. (1972) *J. Biol. Chem. 247*, 8114–8121.

Fukami, M. H., Holmsen, H., & Bauer, J. (1976) *Biochim. Biophys. Acta 428*, 253–256.

Haest, C. W. M., Kamp, D., Plasa, G., & Deuticke, B. (1977) *Biochim. Biophys. Acta 469*, 226–230.

Hamberg, S., & Samuelsson, B. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 899–903.

Harbury, C. B., & Schrier, S. L. (1974) *Thromb. Diath. Haemorrh. 31*, 469–484.

Harris, J. W., & Biaglow, J. E. (1972) *Biochem. Biophys. Res. Commun. 46*, 1743–1749.

Ho, P. P. K., Walters, C. P., & Sullivan, H. R. (1976) *Prostaglandins 12*, 951–970.

Jafari, E., Saleem, A., Shaikh, B. S., & Demers, L. M. (1976) *Prostaglandins 12*, 829–835.

Jesse, R. L., & Cohen, P. (1976) *Biochem. J. 158*, 283–287.

Jesse, R. L., & Franson, R. C. (1979) *Biochim. Biophys. Acta 575*, 467–470.

Kosower, N. S., Kosower, E. M., Wertheim, B., & Correa, W. S. (1969) *Biochem. Biophys. Res. Commun. 37*, 593–596.

Lands, W. E. M. (1979) *Annu. Rev. Physiol. 41*, 633–652.

MacIntyre, D. E., Grainge, C. A., Drummond, A. H., & Gordon, J. L. (1977) *Biochem. Pharmacol. 26*, 319–323.

Marcus, A. J. (1978) *J. Lipid Res. 19*, 793–826.

Mauco, G., Chap, H., & Douste-Blazy, L. (1979) *FEBS Lett. 100*, 367–370.

McKean, M. L., Smith, J. B., & Silver, M. J. (1979) *Abstracts of Papers*, 178th National Meeting of the American Chemical Society, Washington, D.C., September 1979, American Chemical Society, Washington, D.C.

Miller, G. L. (1959) *Anal. Chem. 31*, 964.

Muenzer, J., Weinbach, E. C., & Wolfe, S. (1975) *Biochim. Biophys. Acta 376*, 243–248.

Mürer, E. H. (1968) *Biochim. Biophys. Acta 162*, 320–326.

Murphy, A. J. (1976) *Biochemistry 15*, 4492–4496.

Mustard, J. F., & Packham, M. A. (1977) *Br. Med. Bull. 33*, 187–193.

Nordøy, A., Svensson, B., & Hoak, J. C. (1978) *Thromb. Res. 12*, 597–608.

Pickett, W. C., & Cohen, P. (1976) *J. Biol. Chem. 251*, 2536–2538.

Rittenhouse-Simmons, S. (1979) *J. Clin. Invest. 63*, 580–587.

Rittenhouse-Simmons, S., & Deykin, D. (1977) *J. Clin. Invest. 60*, 495–498.

Rittenhouse-Simmons, S., Russell, A., & Deykin, D. (1977) *Biochim. Biophys. Acta 488*, 370–380.

Robey, F. A., Jamieson, G. A., & Hunt, J. B. (1979) *J. Biol. Chem. 254*, 1114–1118.

Russell, F. A., & Deykin, D. (1976) *Am. J. Hematol. 1*, 59–70.

Schafer, A. I., & Handin, R. I. (1979) *Progr. Cardiovasc. Dis. 22*, 31–52.

Smith, R. P. P., & Ellman, G. L. (1973) *J. Membr. Biol. 12*, 177–188.

Steinhardt, J., & Beychok, S. (1962) *Proteins 2*, 224–233.

Stuart, M. J., Murphy, S., & Oski, F. A. (1975) *N. Engl. J. Med. 292*, 1310–1313.

Telegdi, M., & Straub, F. B. (1973) *Biochim. Biophys. Acta 321*, 210–219.

Vallee, E., Gougat, J., Navarro, J., & Delahayes, J. F. (1979) *J. Pharm. Pharmacol. 31*, 588–592.

Vanderhoek, J. Y., & Feinstein, M. B. (1978) *Mol. Pharmacol. 16*, 171–180.