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SULFHYDRYL AND DISULFIDE GROUPS OF PLATELET MEMBRANES

I. DETERMINATION OF SULFHYDRYL GROUPS

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SUMMARY

Methods were evaluated for the quantitative measurement of free thiols in isolated platelet membranes. Two techniques were found applicable utilizing either the retentive capacity of nitrocellulose filters for ^{203}Hg-labeled *p*-chloromercuribenzoate adducts of membrane proteins or the spectrophotometric assay of the thiol or thione anions released in the formation of mixed disulfides between membrane thiols and the disulfide reagents, 5,5′-dithiobis(2-nitrobenzoic acid) or 6,6′-dithiodinicotinic acid. Complete or partial solubilization of membranes with urea, sodium dodecyl sulfate or phospholipase A resulted in exposure of additional thiols to reaction with 5,5′-dithiobis(2-nitrobenzoic acid) or 6,6′-dithiodinicotinic acid. Approximately one half of the total thiols was found to be masked in native non-solubilized membranes. Kinetic studies on the reaction rates of the various SH reagents tested indicate the presence of at least two classes of thiols in the platelet membrane. The reproducibility and simplicity of the methods proved their general usefulness in quantitative and qualitative thiol assays of membrane proteins.

INTRODUCTION

Platelet membranes not only preserve the structural intactness of these blood elements but also mediate adhesion and aggregation[1]. Reactive free sulfhydryl groups play an essential role in maintaining these functions[2–4]. As quantification of sulfhydryl and disulfide groups has proved to be of crucial importance for the elucidation of the molecular structure of proteins so has the determination of the reactivity of free thiols in proteins substantially contributed to an understanding of the mechanism of their function. This investigation represents the beginning of a long-range effort to determine the role of free thiols and disulfides in membrane-mediated platelet functions and to identify the specific membrane proteins carrying these groups thus gaining insight into the molecular basis of such platelet functions as aggregation and adhesion.

Abbreviations: PCMB, *p*-chloromercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); CPDS, 6.6′-dithiodinicotinic acid; CNTP, 3-carboxylato-4-nitrothiophenolate.

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As the proteins of plasma membranes containing free thiols and disulfides are embedded in a lipid matrix, a quantitative analysis of such groups faces certain special problems which merit a more detailed description of the methods used. Both penetrating and non-penetrating sulfhydryl reagents were examined and the effect of agents capable of solubilizing membrane proteins was investigated. Reaction kinetics and optimal experimental conditions were evaluated for the various reagents and their relative value compared. Sulfhydryl groups were determined in membrane vesicles obtained from human thrombocytes.

METHODS AND MATERIALS

Isolation and preparation of platelet membranes

Blood was collected from normal, healthy male volunteers into plastic bags containing acid–citrate–dextrose anticoagulant (ACD, U.S.P. formula "A"). Platelet-rich plasma was obtained from whole blood by centrifugation at $1600 \times g$ for 5 min. Red and white cell contamination was reduced by brief (2 min) centrifugation at $550 \times g$. Plateles were then separated by spinning the platelet-rich plasma at $2250 \times g$ for 30 min. The resultant platelet pellet was washed 3 times with 0.01 M Tris–HCl buffer, pH 7.4, containing 0.154 M NaCl and 0.001 M EDTA and was finally suspended in the same buffer adjusting the platelet concentration to approximately 10^{10} platelets per ml. All centrifugations were performed at 10 °C. This platelet suspension was layered onto a linear glycerol gradient prepared from 40% (w/v) glycerol in 0.154 M NaCl and from 0.1 M Tris–HCl (pH 7.4) containing 0.154 M NaCl and 0.01 M EDTA[5]. The platelets were initially sedimented very slowly through the glycerol gradient at $1465 \times g$ for 30 min and then at $5860 \times g$ for 10 min at 4 °C. The resultant glycerol-loaded platelets were suspended in approximately 5–10 vol. of cold 0.01 M Tris–HCl buffer, pH 7.5, containing 0.25 M sucrose and the platelets were lysed by vigorous mixing on a Vibromixer. The platelet lysate was layered onto a solution of 27% (u/v) sucrose and the membrane vesicles banded by centrifugation at $63500 \times g$ for 180 min at 4 °C. After removing this fraction from the gradient it was repelleted by centrifugation at $105000 \times g$ for 30 min at 4 °C and subsequently suspended in 0.154 M NaCl. Membrane suspensions of 0.5–1 mg protein per ml were stored at —20 °C.

Direct and indirect methods of quantification of SH groups in platelet membranes

(*a*) *Direct methods*

(*1*) *Filtration of ^{203}Hg-labeled p-chloromercuribenzoate (PCMB) platelet membrane adducts.* Nitrocellulose filters of 0.45-μm pore size were presoaked in a 0.02 M Tris–HCl buffer, pH 7.8, containing 0.05 M NaCl for no less than 15 min at room temperature. Stock solutions of ^{203}Hg-labeled PCMB were prepared fresh daily by dissolving 5 mg of ^{203}Hg-labeled PCMB (spec. act. 17.7 mCi/g) in 1 ml 0.04 M NaOH and diluting with 0.01 M Tris–HCl buffer, pH 7.8, to the desired volume.

In general the reaction mixtures contained 40–60 μg membrane protein (0.1 ml) and 120 nmoles of ^{203}Hg-labeled PCMB (0.1 ml). The volume was made up to 2 ml with 0.2 M Tris–HCl buffer, pH 7.8. Unless otherwise indicated these mixtures were incubated for 60 min at 37 °C with gentle agitation in a Dubnoff metabolic shaking incubator. Incubation was terminated by rapidly filtering the reaction mixtures

through the nitrocellulose filters under weak negative pressure. This was followed by washing the membrane protein adsorbed onto the filters with several aliquots (totaling 10 ml) of 0.02 M Tris–HCl buffer, pH 7.8, containing 0.05 M NaCl.

The radioactivity of the filters was measured with a liquid scintillation spectrometer using 2,5-diphenyloxazole (PPO) as the primary and 1,4-bis(2-(4-methyl-5-phenyloxazolyl))-benzene (POPOP) as the secondary fluor both dissolved in toluene.

(*2*) *Spectrophotometric determination with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).* 0.1 ml of 1.0 M phosphate buffer, pH 8.1. and 0.1 ml of 3 mM DTNB in 10 mM phosphate buffer, pH 8.1, were added to 1.0 ml of sample containing 200–250 μg membrane protein. The test tubes were allowed to stand for 30 min at room temperature (22 °C). The absorbance was then read at 412 nm against blanks consisting of reagent mixtures without DTNB. The readings were corrected for the absorbance of the reagent blank. The concentration of the colored thiol anion, 3-carboxylato-4-nitrothiophenolate (CNTP), released into the reaction mixture was calculated by the use of a molar extinction co-efficient of $1.36 \cdot 10^4$. The results were expressed as moles per 10^5 g membrane protein. Protein was determined by the method of Lowry *et al.*[6].

(*3*) *Spectrophotometric determination with 6,6'-dithiodinicotinic acid (CPDS).* Preparation of the reaction mixture and the incubation procedures were identical to those described above for DTNB. Absorbance was measured at 344 nm and a molar extinction coefficient of $1 \cdot 10^4$ was used.

(*b*) *Indirect methods*

Spectrophotometric determination with DTNB or CPDS followed by dithiothreitol. 0.1 ml of 1.0 M phosphate buffer, pH 8.1, and 0.1 ml of 3 mM DTNB or CPDS were added to 200–250 μg of platelet membranes suspended in 1.0 ml 0.154 M NaCl. The reaction was allowed to proceed 60 min at room temperature (22 °C). The incubation was terminated by addition of 10 ml ice-cold 10 mM phosphate buffer, pH 8.1, containing 0.154 M NaCl. The membranes were then pelleted by centrifugation at $105000 \times g$ for 30 min at 4 °C. After centrifugation the supernate was discarded and the button was washed 3 times to remove excess reagent as well as the thiol or thione (in the case of CPDS) released in the reaction.

The treated membranes were resuspended in 2 ml of 0.154 M NaCl containing 0.01 M phosphate buffer, pH 8.1, to which was added 0.5 ml of 10 mM dithiothreitol. The reaction mixtures were incubated for 60 min at 37 °C with gentle agitation. Incubation was terminated by centrifugation at $105000 \times g$ for 30 min at 4 °C. The concentration of the thiol or thione released from the platelet membranes by dithiothreitol was determined spectrophotometrically at 412 or 344 nm in membrane suspensions reacted with DTNB or CPDS, respectively. The membrane button was washed once with 0.154 M NaCl and the protein content determined as described above.

Materials

^{203}Hg-labeled *p*-chloromercuribenzoate (PCMB) was obtained from Amersham/Searle Corp., Arlington Heights, Ill. Dithiothreitol and 5,5-dithiobis(2-nitrobenzoic acid) were purchased from Calbiochem, Los Angeles, Calif. and 6,6'-dithiodinicotinic acid was the product of Aldrich Chemical Company Inc., Milwaukee, Wisc. Phospholipase A was purchased from Sigma Chemical Co., St. Louis, Mo. Nitro-

cellulose membrane filters (No. B-6, pore size 0.45 μm, 24 mm diameter) were obtained from Schleicher and Schuell Inc., Keene, N.H.

RESULTS

(*a*) *Filtration method using* ^{203}*Hg-labeled PCMB as SH reactant*

This method which has proved to be a rapid and simple technique for the assay of labeled PCMB bound to SH groups of soluble proteins[7] was adapted for use with membrane vesicles. Up to 100 μg protein (the largest amount tested) a perfectly linear relationship could be shown to exist between the amount of ^{203}Hg-labeled PCMB retained on the nitrocellulose filters and the amount of membrane protein applied (Fig. 1). The pH of the reaction mixture filtered through the nitrocellulose membranes was found to be of critical importance. Relatively constant values of

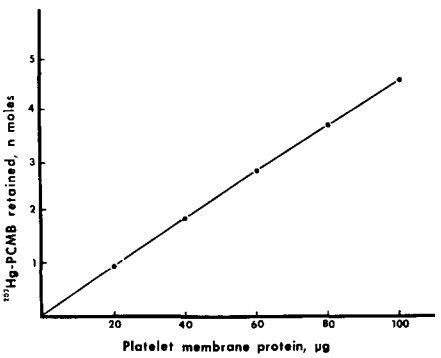

Fig. 1. Effect of increasing platelet membrane protein concentration on the amount of ^{203}Hg-labeled PCMB retained by nitrocellulose filters. Experimental conditions were standard.

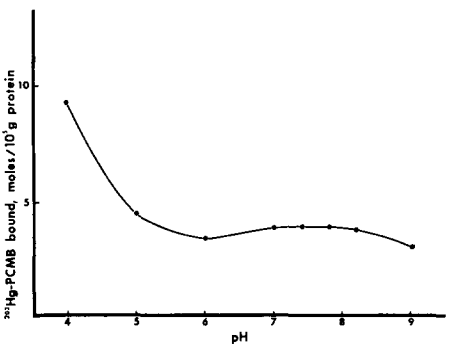

Fig. 2. Effect of pH on retention of ^{203}Hg-labeled PCMB adducts of platelet membrane protein by nitrocellulose filters. Three different buffer systems were used in the pH range from 4.0 to 9.0: from pH 4 to 5, 0.2 M acetate; from pH 6 to 7, 0.2 M phosphate; and from pH 7.4 to 9.0, 0.2 M Tris–HCl. Incubation and filtration procedures were as described in Methods and Materials. Washing of the adsorbed membrane proteins was performed with the respective buffer solutions used for adjusting the pH.

PCMB adducts were obtained in the pH region between 7 and 8 (Fig. 2). At pH less than 6 ^{203}Hg-labeled PCMB was increasingly retained by the filter in an unspecific, *i.e.* non protein-bound manner. Titration of the reactive membrane protein SH groups with the mercurial reagent could be performed by this method as can be seen from Fig. 3. The end point at the intersection of the 2 straight lines occurs at 4 nmoles SH per 90 μg protein.

When platelet membrane vesicles were allowed to react with excess PCMB a fast initial rate of mercaptide formation was followed by a slower one extending over longer periods of time. A semilogarithmic plot of the remaining free non-reacted SH groups as a fraction of the total monothiols present (ΔC) *vs* time showed a linear portion at reaction times exceeding 45 min and displayed a positive deviation which increased as the incubation time decreased (Fig. 4). This type of plot suggests

the occurrence of two simultaneous reactions with rate constants of considerably differing magnitude. A composite semilograithmic plot was constructed in the usual manner, *i.e.* by drawing a straight line corresponding to the slow reaction and subtracting for each observed time from the measured value of ΔC the extrapolated ΔC predicted by the straight line. From these straight-line plots the pseudo first-order rate constants k_{1obs} and k_{2obs} were obtained. There were 3.7 moles SH per 100000 g protein which were fast-reacting ($t_{1/2}$ 7.5 min) and 1.0 mole SH which formed mercaptides at a slower rate ($t_{1/2}$ 37 min).

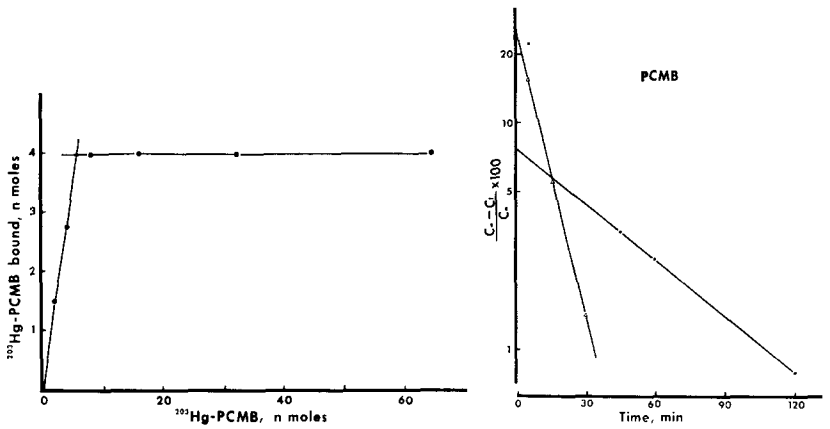

Fig. 3. Thiol titration of platelet membrane protein with ^{203}Hg-labeled PCMB. In a total volume of 2 ml the reaction mixtures contained 90 μg of platelet membrane protein, ^{203}Hg-labeled PCMB as indicated in the figure and 0.2 M Tris–HCl, pH 7.8. Incubation and filtration techniques were standard.

Fig. 4. Semilogarithmic plot of the number of non-reacted free thiols ($C_\infty - C_t$) expressed as percentage of the total available thiols (C_∞) *vs* time, showing the reaction of two types of sulfhydryl groups. Conditions of incubation and filtration were standard. The solid circles represent the observed values and the two straight line portions indicate the reaction of the two groups of membrane thiols. The method used for graphic evaluation of the observed values by a composite semilogarithmic plot is described in the text.

Solubilization and denaturation of membrane proteins with sodium dodecyl sulfate completely prevented their adsorption onto nitrocellulose filters (Table I). The addition of urea in concentrations up to 8 M did not influence significantly either rate of formation or total number of mercaptides. This denaturant did not decrease the retention of the membrane proteins by nitrocellulose filters. Partial solubilization of platelet membranes could be attained by incubation with phospholipase A (activity 8 units/mg protein). Increasing concentrations of enzyme plotted logarithmically were related linearly but inversely to the quantity of ^{203}Hg-labeled PCMB membrane adducts retained by the filters (Fig. 5). The addition of phospholipase A did not decrease the retention of membrane protein by nitrocellulose filters. This was demonstrated by measuring the radioactivity of ^{203}Hg-labeled PCMB-treated platelet membranes adsorbed onto the filters before and after incubation with varying concentrations of phospholipase A.

TABLE I

EFFECT OF UREA AND SODIUM DODECYL SULFATE ON RETENTION OF PROTEIN-BOUND PCMB BY NITROCELLULOSE FILTERS

Platelet membranes were incubated with ^{203}Hg-labeled PCMB for 60 min at 37 °C. After removal of excess reagent and repeated washing by centrifugation the membrane vesicles were resuspended in 0.1 M Tris–HCl (pH 7.4) containing 0.154 M NaCl and 0.01 M EDTA containing varying concentrations of urea or sodium dodecyl sulfate, filtered and washed as described in Methods and Materials.

Addition	*Concentration*	*Protein-bound ^{203}Hg-labeled PCMB (cpm)*
None		11 500
Urea	2 M	11 270
Urea	6 M	11 753
Urea	8 M	11 707
Sodium dodecyl sulfate	0.02%	4210
Sodium dodecyl sulfate	0.05%	555
Sodium dodecyl sulfate	0.5%	302
Sodium dodecyl sulfate	1.0%	260

(b) Reaction of membrane SH groups with the disulfide reagents DTNB or CPDS

The reaction of DTNB or CPDS with free SH groups leads to mixed disulfides with the concomitant release of a colored thiol or thione anion. We have estimated the number of reactive free SH groups either directly by determining the amount of thiol or thione anion released (reaction I) or indirectly by measuring its release by dithiothreitol from the mixed disulfide linkage between protein and reagent thiol (reaction sequence II).

$$\text{R–SH} + \text{DTNB} \rightarrow \text{R–S–CNTP} + \text{CNTP} \qquad \text{(I)}$$

$$\text{R–SH} + \text{DTNB} \rightarrow \text{R–S–CNTP} + \text{CNTP} \qquad \text{(II)}$$

$$\text{R–S–CNTP} + \text{dithiothreitol} \rightarrow \text{R–SH} + \text{dithiothreitol–CNTP}$$

$$\text{dithiothreitol–CNTP} \rightarrow \text{oxidized dithiothreitol} + \text{CNTP}$$

The formation of CNTP as a function of the amount of protein titrated was linear up to 200 μg, the maximum quantity tested (Fig. 6).

The time course of the reaction of excess DTNB or CPDS with free membrane SH groups was evaluated in a manner identical to that described above for PCMB. The semilogarithmic plots again were typical of the occurrence of two simultaneous reactions (Fig. 7). Rate constants of the pseudo first order reactions were determined for platelet membranes incubated with or without sodium dodecyl sulfate. Dividing each k_{obs} by the concentration of the sulfhydryl reagent yields a value for the apparent second order rate constant, k_{app}. This follows from the equation describing a bimolecular reaction between protein SH groups and sulfhydryl reagent.

$$-\frac{\mathrm{d}[\text{R–SH}]}{\mathrm{d}t} = k_{app}[\text{PCMB}]\,[\text{R–SH}]$$

$$k_{app} = \ln \frac{[\text{R–SH}]_t}{[\text{R–SH}]_\infty} \Big/ t[\text{PCMB}] = k_{obs}/[\text{PCMB}]$$

The second order rate constants are tabulated for the various SH reagents used and the solubilizing agent (sodium dodecyl sulfate) employed (Table II).

Solubilization with this ionic detergent increased the second order rate constants for CPDS and DTNB 3- and 10-fold, respectively. The reaction rate of DTNB with membrane monothiols was measured over a range of reagent concentrations. A double reciprocal plot according to Lineweaver and Burk (Fig. 8) allowed calculation

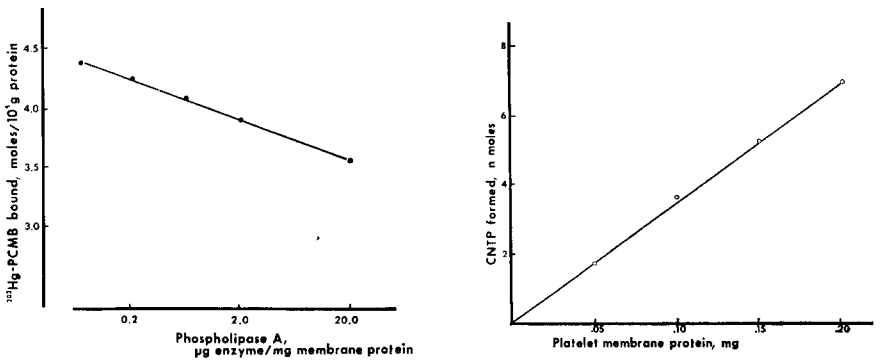

Fig. 5. Effect of phospholipase A concentration on the number of mercaptides retained by nitrocellulose filters. The enzyme concentration was plotted logarithmically. Conditions of incubation and filtration were standard.

Fig. 6. Quantitative relationship between membrane protein and CNTP release. Experimental conditions are described in Methods and Materials.

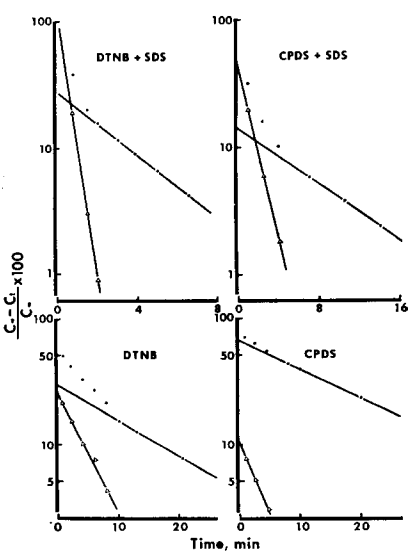

Fig. 7. Semilogarithmic plots of the number of non-reacted free thiols ($C_\infty - C_t$) expressed as percentage of total available thiols (C_∞) *vs* time. Two disulfide reagents, DTNB and CPDS, were used with or without addition of 1% sodium dodecyl sulfate (SDS). The curves representing the observed values (solid circles) could be resolved into two straight line portions indicating the presence of at least two types of free thiols in platelet membrane proteins. The construction of the composite semilogarithmic plots is described in the text. Experimental conditions were standard.

TABLE II

OBSERVED AND APPARENT REACTION RATE CONSTANTS OF SH REAGENTS WITH PLATELET MEMBRANE PROTEIN SH GROUPS

Platelet membrane vesicles were reacted with various SH reagents for periods of time ranging between 30 s and 60 min. The observed rate constants for the pseudo first-order reactions, $k_{1\ obs}$ and $k_{2\ obs}$ were determined from composite semilogarithmic plots. Apparent second order rate constants $k_{1\ app}$ and $k_{2\ app}$ were calculated as described in the text.

Reagent	*Concn* $\times 10^4$ (M)	*Fast reacting SH groups*		*Slow reacting SH groups*	
		$k_{1\ obs}$ (s^{-1})	$k_{1\ app}$ ($M^{-1} \cdot s^{-1}$)	$k_{2\ obs}$ (s^{-1})	$k_{2\ app}$ ($M^{-1} \cdot s^{-1}$)
PCMB	0.6	0.00156	25.9	0.00031	5.1
DTNB	1.67	0.00371	22.2	0.00108	6.5
DTNB with sodium dodecyl sulfate	1.67	0.0384	229.9	0.00423	25.3
CPDS	1.67	0.00452	27.1	0.00088	5.3
CPDS with sodium dodecyl sulfate	1.67	0.01342	80.2	0.00205	12.3

of V ($3.51 \cdot 10^{-9}$ mole/min per mg protein) and K_m ($0.87 \cdot 10^{-5}$ M) for the formation of mixed disulfides between membrane thiols and thiol anions of DTNB.

Solubilization of membrane proteins increased the number of DTNB-accessible thiols substantially (Table III). Both sodium dodecyl sulfate and urea produced identical CNTP release. The combined use of the two denaturants did not result in an increase in the number of reactive thiols over and above that obtained with either one alone. Incubation of membrane vesicles with phospholipase A resulted in more

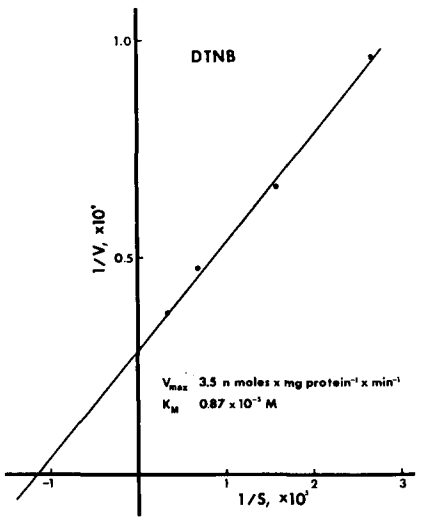

Fig. 8. Lineweaver–Burk plot of the reaction of DTNB with sulfhydryl groups of platelet membranes. Experimental conditions were standard.

TABLE III

EFFECT OF MEMBRANE SOLUBILIZERS ON THE NUMBER OF REACTIVE SH GROUPS IN PLATELET MEMBRANES

Membrane monothiols were quantified at room temperature by direct spectrophotometric assay with excess DTNB or CPDS in the presence or absence of membrane-solubilizing agents.

Solubilizer	*Concentration*	*SH groups (moles/10^5 g protein)*	
		DTNB	*CPDS*
None		3.1	3.6
Urea	6.8 M	6.8	
Sodium dodecyl sulfate	1.0%	6.9	8.4
Urea + sodium dodecyl sulfate	6.0 M, 1.0%	6.8	
Phospholipase A	2.0 μg enzyme protein/mg membrane protein	4.1	

thiols accessible to reaction with DTNB compared to the control but did not equal the effect of urea or sodium dodecyl sulfate.

Measurement of free, reactive SH groups in the platelet membrane by the indirect method utilizing dithiothreitol according to reaction sequence II shown above was performed with CPDS as the thiol reagent. The number of reactive protein thiols found by this technique was in the same range as with the direct method utilizing DTNB as the reagent. As with the other methods of assay a biphasic reaction response could be observed. About 80% of the CPDS-reactive SH groups formed

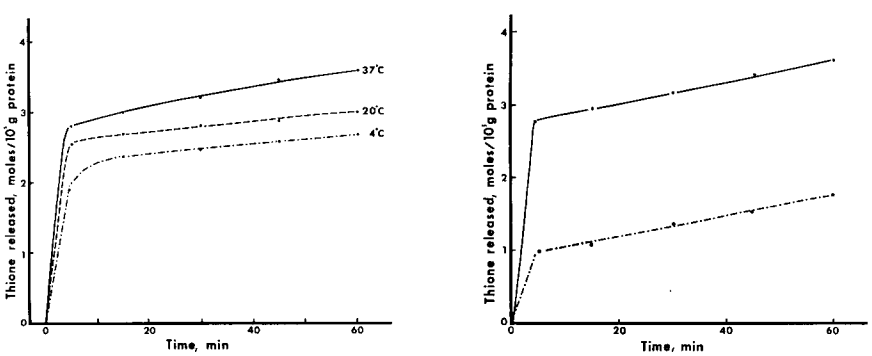

Fig. 9. Effect of temperature on the reaction of free thiols of membrane proteins with CPDS determined at varying time intervals. The thiols released were measured by the indirect method utilizing dithiothreitol as described in Methods and Materials.

Fig. 10. Effect of *N*-ethylmaleimide alkylation on the reaction of free membrane thiols with CPDS over varying periods of incubation. The interrupted line denotes membranes pretreated for 30 min at 37 °C with 2 mM *N*-ethylmaleimide. The solid line indicates non-pretreated control membranes. The thione anions released were measured by the indirect method utilizing dithiothreitol as described in Methods and Materials.

TABLE IV

MONOTHIOL CONTENT OF NATIVE AND DENATURED PLATELET MEMBRANES

Figures in parentheses denote the number of experiments performed.

Reagent	*Monothiol content (moles/10^5 g protein)**	
	Native membrane proteins	*Denatured membrane proteins*
PCMB (9)	4.7 ± 0.5	4.7 ± 0.5
DTNB (8)	3.1 ± 0.9	6.1 ± 1.1
CPDS (3)	3.6	8.4
CPDS with dithiothreitol (3)	3.7	

* Mean values $\pm$ 1 S.D.

mixed disulfides with the reagent at a fast rate while the remainder reacted more slowly (Fig. 9). Both reactions were found to be temperature-dependent.

The specificity of the reaction of CPDS with membrane thiols was examined by reacting membranes with *N*-ethylmaleimide before incubating them with CPDS (Fig. 10). Approximately one-third of the rapidly-reacting SH groups were not alkylated by *N*-ethylmaleimide pre-treatment as were all of the thiols which formed mixed disulfides with CPDS at a slower rate.

A comparison of the number of monothiols reactive with the different SH reagents used in this study in native and denatured membrane proteins is shown in Table IV.

DISCUSSION

This study dealt principally with methods of assay for membrane thiols and represents to our knowledge the first direct determination of reactive thiols in isolated membrane vesicles of any cell type. Their quantitative assay is made difficult by the particulate nature of the substrate. Spectrophotometric determination of mercaptides necessitates low suspension densities of the membranes or the use of a double-beam spectrophotometer. The application of radioactive SH reagents on the other hand makes extensive washing procedures mandatory. This difficulty was circumvented by the adaptation of a filtration method for the measurement of mercurial adducts. The technique which was found applicable to soluble proteins[7] proved to be quantitative also with plasma membranes. The platelet membranes adsorbed onto nitrocellulose filters could be washed free of non-specifically adsorbed excess radioactivity sufficiently fast to allow kinetic studies on the formation of mercurial adducts. Ionic strength as demonstrated by Krakow and Goolsby[7] and variation of pH in the region of neutrality as shown in this study did not influence the retention of mercaptides but addition of the ionic detergent sodium dodecyl sulfate produced complete loss of the filters' retentive capacity. The disappearance of free SH groups of membrane proteins, the result of mercurial adduct formation, was not a simple exponential function as would be expected if a single type of thiol were reacting but could be resolved into two logarithmic components. The apparent second order rate constants

for the two mercaptide reactions were in the range observed with masked SH groups of oxyhemoglobin (α104 and β112)[8] and those of a variety of other soluble proteins[9,10]. As all the sulfhydryl groups were measured by conventional and not by stopped flow techniques it is probable that very fast reacting thiols may have been missed in our studies. The failure of urea to unmask any additional PCMB-reactive membrane SH groups is difficult to explain. As there appeared to be no urea-induced loss of PCMB membrane adducts through the nitrocellulose filters it must be assumed that the lack of an unmasking effect by denaturation of the proteins is due to an alteration in the reactivity of the mercurial reagent with the protein thiols under the experimental conditions employed. This assumption is based on the fact that urea was able to uncover additional SH groups reactive with the disulfide reagent DTNB thus possibly incomplete solubilization of the membrane proteins cannot be responsible for the observed effect. The inverse proportionality which we have observed between phospholipase A concentration and formation of mercaptides by membrane proteins may have bearing on the explanation of a possible mechanism for this effect of urea. The enzyme catalysed hydrolysis of non-polar fatty acyl residues from phospholipids in membranes may curtail the available conduits for membrane penetration of the mercurial reagent and thus limit access to certain SH groups. Titration of reactive membrane SH groups with PCMB showed that only a small excess of reagent was sufficient to take mercaptide formation to completion. On the other hand a 5-fold excess of DTNB was needed to react all available membrane thiols, attesting presumably to its much lower affinity for free membrane SH groups compared to that of the organic mercurial.

DTNB and CPDS are two SH reagents with very limited membrane penetrability. The direct spectrophotometric assay of the thiol or thione anion released by the reaction of these two disulfide reagents with free SH groups was made possible by the fact that the absorption peaks of the colored anions were sufficiently removed from the ultraviolet end of the spectrum to allow their measurement without too high absorption reading of the blank. Membrane suspensions exceeding 200 μg protein per ml were, however, found unsuitable for this type of direct assay. Primarily to circumvent this difficulty the indirect method utilizing dithiothreitol was adopted. This method is based on the observation that the reaction of DTNB or CPDS with monothiols forming mixed disulfides is reversible in the presence of thiol compounds such as cysteine, glutathione or dithiothreitol. While the total number of reactive SH groups was found to be the same whether the primary or the secondary thione anion, *i.e.* the one resulting from the mixed disulfide of protein thiol and reagent sulfhydryl, was assayed it was found to be difficult if not impossible to conduct accurate kinetic studies on the reaction of these disulfide reagents with the indirect method. Although the release of the thione anion was quantitative with dithiothreitol the time involved in the separation of the membrane vesicles was too long for accurate short term experiments.

The inability of *N*-ethylmaleimide pretreatment of membrane suspensions to block completely their subsequent reaction with disulfide reagents probably reflects both a lack of specificity of the alkylation by *N*-ethylmaleimide[11] and the existence of membrane thiols of considerable diversity in their reaction characteristics toward different SH reagents[12]. A direct comparison of the total number of SH groups in platelet membranes found in our studies with that of membranes of other cell

types is made difficult by the different means of expressing results, the different reagents and methods used and by the varying purity of the biological systems employed. The number of free thiols in nondenatured platelet membranes that formed mixed disulfides with DTNB or CPDS was approximately in the same range, however, as that found by Mehrishi and Grassetti[13]. Although more SH groups were able to react with PCMB, a highly nonpolar reagent, than with DTNB and CPDS the difference in the number of thiols reactive with these two types of SH reagents was small compared to the large difference in the number of SH groups observed when membranes were first denatured. Significant differences were found even between reagents of similar chemical structure and reactivity. As yet no satisfactory explanation for these results has been advanced.

Our studies have indicated possible methods for the quantitative analysis of sulfhydryl groups in membranes. Although platelets have been our sole membrane source the techniques described can be assumed to be equally applicable to membranes of any cell type.

ACKNOWLEDGEMENT

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REFERENCES

1 Michal, F. and Firkin, B. G. (1969) *Annu. Rev. Pharmacol.* 9, 95–118
2 Robinson, Jr, C. W., Mason, R. G. and Wagner, R. H. (1963) *Proc. Soc. Exp. Biol. Med.* 113, 857–861
3 Al-Mondhiry, H. and Spaet. T. H. (1970) *Proc. Soc. Exp. Biol. Med.* 135, 878–882
4 Aledort, L. M., Troup, S. B. and Weed, R. I. (1968) *Blood*, 31, 471–479
5 Barber, A. J. and Jamieson, G. A. (1970) *J. Biol. Chem.* 245, 6357–6365
6 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* 193, 265–275
7 Krakow, J. S. and Goolsby, S. A. (1971) *Biochem. Biophys. Res. Commun.* 44, 453–458
8 Chiancone, E., Currell, D. L., Vecchini, P., Antonini, E. and Wyman, J. (1970) *J. Biol. Chem.* 245, 4105–4111
9 Hasinoff, B. B., Madsen, N. B. and Avramovic-Zikic, O. (1971) *Can. J. Biochim.* 49, 742–751
10 Madsen, N. B. (1966) in *Metabolic Inhibitors* (Hochster, R. M. and Quastel, J. H., eds), Vol. II, pp. 119–143, Academic Press, New York
11 Smyth, D. G., Nagamatsu, A. and Fruton, J. S. (1960) *J. Am. Chem. Soc.* 82, 4600–4604
12 Shapiro, B., Kollmann, G. and Martin, D. (1970) *J. Cell. Physiol.* 75, 281–292
13 Mehrishi, J. N. and Grassetti, D. R. (1969) *Nature* 224, 563–564