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EFFECT OF THIOLS IN BIOLOGICAL SYSTEMS ON PROTEIN SULFHYDRYL CONTENT

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Abstract—An erythrocyte plasma system was incubated with various thiols (final concentration, 0·003 M) to study the effects on protein sulfhydryl content. After timed incubation, the plasma sulfhydryl concentration, i.e. the protein and nonprotein fractions, was determined by polarographic analysis. The results indicate that sulfhydryl groups of the thiol were transferred or converted to increase the protein sulfhydryl content in the presence of tissue sulfhydryl groups. An increase of protein sulfhydryl did not take place in the absence of cellular elements and was partially inhibited by first blocking the sulfhydryl groups of the cellular elements.

Studies *in vivo* were also conducted to determine whether the sulfhydryl groups of the thiols can be exchanged with, or converted to, tissue protein sulfhydryl groups. *N*-acetylcysteine was given intravenously in Swiss mice and the protein and nonprotein sulfhydryl content of various tissue fractions was assayed. Distribution studies after administration of the ^{35}S-labeled *N*-acetylcysteine were conducted utilizing autoradiographic procedures. A significant increase in the tissue protein sulfhydryl content was again achieved *in vivo*.

These findings support the hypothesis that thiols can be utilized to increase tissue sulfhydryl groups when the latter are deficient, as reported in various clinical disorders. The approach may, therefore, have therapeutic application.

PROTEIN sulfhydryl (SH) groups are important in a variety of biochemical reactions affecting enzyme systems,[1, 2] membrane transport,[3] hormonal action,[4] and conformational protein structure.[5] The serum protein sulfhydryl content of normal subjects is rather stable and rarely fluctuates below the 400–600 micromoles per liter (μmoles/l.) range.[6] Conversely, the serum† protein sulfhydryl content has been reported to be subnormal in subjects with a variety of clinical disorders.[7, 8] The administration of thiol compounds for therapeutic purposes has been reported to exert a beneficial clinical effect when given in situations where the serum sulfhydryl content is likely to be subnormal.[9–11] An increase in sulfhydryl content of subnormal serum has been achieved with the therapeutic administration of these agents.[6, 12, 13] Sheffner *et al.*,[14] have reported that the administration of thiols in experimental animals was not associated with a significant increase in the tissue or plasma sulfhydryl content; however, their observations were based on the nonprotein fractions.

The present investigation was undertaken in an attempt to determine: (1) whether an increase in tissue protein sulfhydryl content can be achieved with thiols under

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† Deficiency in serum protein SH content similarly affects plasma proteins.

controlled experimental conditions; (2) to characterize the mechanism by which the sulfhydryl groups of the nonprotein thiol contribute to the increase in protein sulfhydryl content; and (3) to relate the dynamics of these interactions to the tissue sulfhydryl content.

METHODS

The following compounds were used in this study: α-mercaptopropionyl glycine, Calbiochem; D-penicillamine, Merck & Co., Inc.; Dithiothreitol, Calbiochem; *N*-acetylcysteine (NAC), Mead Johnson & Co.; *P*-chloromercuriphenyl sulfonic acid (PCMBS), Sigma Chemical Co.; *N*-ethylmaleimide (NEM), Schwarz Bioresearch, Inc.; *N*-acetylcysteine ^{35}S (specific activity, 174 microcuries per gram), Mead Johnson & Co.

I. *Conversion* in vitro *of nonprotein SH to protein SH*

Five fresh specimens of 25-ml heparinized human venous blood were collected under sterile conditions. From each specimen a series of 5 polycarbamate tubes was prepared by adding 4 ml of the heparinized blood and 1 ml of the appropriate thiol to each tube. The thiols used were: α-mercaptopropionyl glycine, 0·015 M; D-penicillamine, 0·015 M; *N*-acetylcysteine, 0·015 M; Dithiothreitol, 0·0075 M. All thiol solutions were freshly prepared in 0·9% saline; the osmolarity was determined in a Fisk osmometer and adjusted, when necessary, with a 5% saline solution to 320–330 milliosmoles per liter. All tubes, including control specimens (containing 4-ml heparinized whole blood diluted with 1 ml 0·9% saline), were then incubated in a shaking water bath at 37°. One-ml aliquots were removed from each of the incubating specimens at intervals of 1, 2, 4 and 5 hr and immediately centrifuged for determination of plasma sulfhydryl content, i.e. protein and nonprotein fractions. These results are indicated in Table 1.

TABLE 1. PLASMA PROTEIN SH CONTENT AFTER INCUBATION OF ANTICOAGULATED WHOLE BLOOD WITH THIOL COMPOUNDS*

Compounds	% Increase of plasma protein SH (mean of five determinations)			
	1 hr	2 hr	4 hr	5 hr
N-acetylcysteine†	140	220	590	600
D-penicillamine†	80	200	300	420
α-Mercaptopropionyl glycine†	120	310	400	450
Dithiothreitol‡	200	350	390	390

* Initial protein SH content of plasma specimen: 390 ± 40 μmoles/l.
† Final concentration, 0·003 M.
‡ Final concentration, 0·0015 M.

Similar experiments were performed to compare the effects of thiols when incubated with heparinized venous blood and the plasma fraction of the identical blood specimen. Thirty ml of heparinized venous blood was collected; a 20-ml aliquot was centrifuged, and 8 ml of the supernatant plasma removed and introduced into polycarbamate tubes. An 8-ml aliquot of the heparinized venous blood was similarly introduced into paired tubes. Two ml of 0·015 M freshly prepared NAC was then added and both tubes

were incubated in a shaker bath at 37°. One-ml aliquots were removed at timed intervals; the whole blood specimen was first centrifuged to remove the red blood cell (RBC) mass. The sulfhydryl content of the protein and non-protein fractions of both plasma specimens obtained from five patients was assayed and compared in the manner described (Fig. 1).

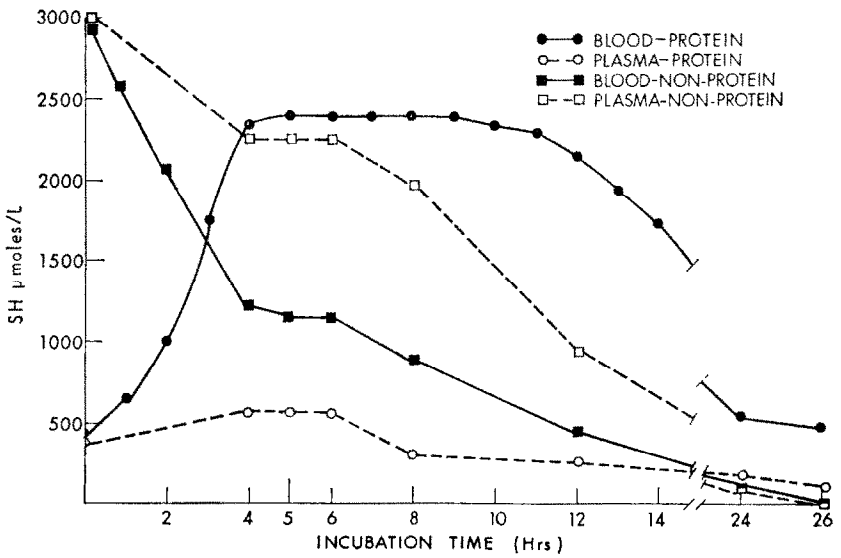

FIG. 1. Illustration of increase in plasma protein SH content observed on incubating anticoagulated human blood with thiols (NAC final concentration, 0·003 M) at 37°, ●—●. After timed incubation each tube was centrifuged and the SH content of the supernatant plasma specimen was determined. Open circles (○—○) indicate control specimens containing plasma specimen incubated with 0·003 M NAC. Squares indicate nonprotein SH; ■—■, anticoagulated blood specimen; □—□, plasma-incubated specimens. Each symbol represents the mean of five determinations.

A series of experiments was also performed to determine whether and to what extent erythrocytes contributed to the increase in plasma protein sulfhydryl content observed with thiol incubation of heparinized blood. For these series of experiments RBC were allowed to sediment at low-speed centrifugation (2000 *g* for 15 min) and the supernatant plasma was removed. The cell count of the erythrocyte suspension was determined in a Coulter counter and the volume adjusted to yield a final concentration of 5×10^6 cells/mm^3. The specimens were then subjected to the following experimental conditions:

A. Sedimented RBC (2·0 ml) and plasma (2·0 ml) were then recombined. NAC 0·015 M (1·0 ml) was then added to 4 ml of the recombined RBC plasma suspension to study specific contribution of RBC.

B. The ratio of RBC to plasma was adjusted to contain 50% of the initial RBC content by using 1·0 ml of the sedimented RBC mass and increasing the plasma volume to 3·0 ml. NAC 0·015 M (1·0 ml) was added to yield a final concentration of 0·003 M.

C. The RBC mass (2·0 ml) was washed once with physiological saline. The erythrocytes were sedimented with low-speed centrifugation and the saline was removed.

Two ml of the initially removed plasma and 1 ml of NAC 0·015 M were added. (Final concentration of NAC was 0·003 M.)

D. Two appropriate controls were prepared from the same specimen by adding 4 ml of plasma to one tube and 4 ml of heparinized blood to another tube. To each control 1 ml of 0·015 M NAC was added to yield a final concentration of 0·003 M NAC.

Five different specimens were studied according to the described manner; all were incubated under sterile conditions at 37°. After incubation intervals of 2, 5 and 20 hr, the specimens were centrifuged, the supernatant plasma was removed, and the nonprotein sulfhydryl content was determined. The protein sulfhydryl content was then calculated by subtracting the nonprotein sulfhydryl content from the total sulfhydryl value.

E. The effect of sulfhydryl-blocking agents: This experiment was performed as described under Section I-A, except for the following changes: each sedimented RBC (2·0 ml) was first incubated with PCMBS or NEM (0·5 ml, 0·003 M) for 10 min at 20° and then centrifuged for 15 min at 2000 *g*. The supernatant was discarded and the RBC resuspended in a 2-ml volume of their respective plasma. To these reconstituted blood specimens, 1 ml of 0·015 M NAC was added, the specimens were incubated at the identical conditions described under Section I-A and assayed for sulfhydryl content.

II. *Conversion* in vivo *of nonprotein SH to protein SH*

A. Distribution of NAC ^{35}S was studied by intravenous injection into the tail vein of Swiss mice (20 g). A dose of 2 mg/g of body weight was given in a volume of 0·2 ml which represents approximately 5 μc. Under light anesthesia, sixteen mice were immersed 3 to 5 min in a hexane dry ice mixture. Sagittal sections (20 μ thick) of the whole animal were prepared as described by Ullberg[15] on a microtome at −15° by adhering on 3M mending tape No. 810. The mice were sacrificed 5 and 30 min and 1, 4 and 24 hr after injection.

B. The tissue sulfhydryl content was determined by polarographic determination after the intravenous administration of NAC. Animals were sacrificed by decapitation at timed intervals after injection. Each organ was isolated, removed, immediately frozen by packing in dry ice, triturated in the frozen state with a mortar and pestle that were packed in dry ice. This material was transferred to polycarbamate tubes, allowed to thaw, and then centrifuged at 5°, 28,700 *g* for 5 min. The soluble fraction was then assayed for protein and nonprotein sulfhydryl content. Appropriate control studies were conducted utilizing litter mates that had not received NAC. Tissues from these control animals were assayed under identical conditions. The possibility of nonspecific thiol binding of protein was also investigated. Tissues from control animals were triturated and incubated with NAC (2 mg/g of frozen tissue) for intervals varying from 30 min to 4 hr at 37°. The sulfhydryl content of the total soluble fraction and the acid soluble (nonprotein fraction) was then assayed.[16]

III. *Sulfhydryl determination*

Protein sulfhydryl content was determined by a modified polarographic technique previously described.[6]

A. The protein sulfhydryl content was then calculated by subtracting the nonprotein from the total sulfhydryl level.

B. The nonprotein sulfhydryl content was determined after metaphosphoric acid precipitation,[16] then assayed for sulfhydryl content, as described in paragraph III.

IV. *Protein determination*

Total protein concentration was determined by a standard biuret method.[17] If any specimen was lipemic or hemolyzed, background was corrected by tartrate–iodide incubated blank.

RESULTS

Increases in plasma protein sulfhydryl content (initial values, 400 μmoles/l.) were observed after incubating sedimented human erythrocytes and their respective plasma with thiol compounds of 0·003 M final concentration for varying intervals (Table 1). *N*-acetylcysteine, D-penicillamine, α-mercaptopropionyl glycine and Dithiothreitol yielded increases ranging from 390–600 per cent after 4 hr incubation. Subsequent studies were conducted with NAC because it is exceptionally well tolerated in experimental animals and human subjects; thus, it may have considerable therapeutic potential.

Maximum increase in protein sulfhydryl content was observed after 5 hr incubation of the described RBC and plasma system with NAC 0·003 M (Fig. 1). A 6-fold increase in protein sulfhydryl content was achieved (Table 2). Removal of 50 per cent of the RBC mass did not significantly affect the time required to achieve the indicated increase in protein sulfhydryl content nor the final protein SH/nonprotein SH ratio (Table 2). Conversely, a low yield in the conversion of nonprotein thiol to protein SH

TABLE 2. CONVERSION OF THIOL SH TO PLASMA PROTEIN SH IN THE PRESENCE OF HUMAN ERYTHROCYTES*

RBC mass 10^6 cells/mm³ (ml)	Plasma (ml)	NAC 0·015 M (ml)	SH-blocking agent	Total SH (μmoles/l.)	Nonprot. SH (μmoles/l.)	Protein SH (μmoles/l.)	Molar ratio Prot. SH/ Nonprot. SH
2·0	2·0			441	50 ± 7	390 ± 40	7·80
2·0†	2·0	1·0		3427	1118 ± 110	2309 ± 227	2·07
1·0‡	3·0	1·0		3200	1050 ± 81	2150 ± 150	2·05
2·0§	2·0	1·0		3036	2440 ± 245	596 ± 64	0·25
	4·0	1·0		2952	2327 ± 216	625 ± 61	0·27
2·0	2·0	1·0		3551	1183 ± 120	2368 ± 254	2·01
2·0	2·0	1·0	PCMBS	3109	1207 ± 117	1902 ± 187	1·58
2·0	2·0	1·0	NEM	2760	1700 ± 150	1060 ± 90	0·62

* The values express mean of 5 determinations ± 1 standard deviation after 5 hr 37° incubation.
† Sedimented RBC.
‡ Fifty per cent RBC mass.
§ Saline-washed RBC.

was observed on incubating the plasma rather than the whole blood with the thiol under otherwise identical conditions. Similarly, a single washing of the RBC mass with physiological saline followed by recombination of the red cells with the plasma and NAC incubation almost totally abolished an increase in protein SH.

An attempt was made to further elucidate the mechanism for conversion of nonprotein thiol to protein SH utilizing this sytem. The role played by the sulfhydryl

groups of the erythrocyte's membrane was studied by subjecting sedimented RBC to sulfhydryl-blocking agents prior to incubation with the thiol and plasma system. The decline in conversion of thiol to protein SH was observed after this procedure, thus indicating that the reaction was partially inhibited by PCMBS. This agent, because of its high diffusion gradient, blocks the outer membrane of the sulfhydryl groups.[18] NEM, which is more diffusible under identical conditions, i.e. 10-min exposure, caused greater inhibition.[19] The soluble intracellular erythrocyte sulfhydryl content determined after hypotonic lysis, utilizing the described polarographic procedure, was not affected by these agents under these conditions. The molar ratio of protein SH/nonprotein SH was 1·58 for PCMBS, 0·62 for NEM. The control specimen not subjected to sulfhydryl-blocking agents yielded a ratio of 2·0. Thus, the presence of sulfhydryl groups on the surface and within the RBC membranes may be implicated in this reaction (Table 2, Fig. 2).

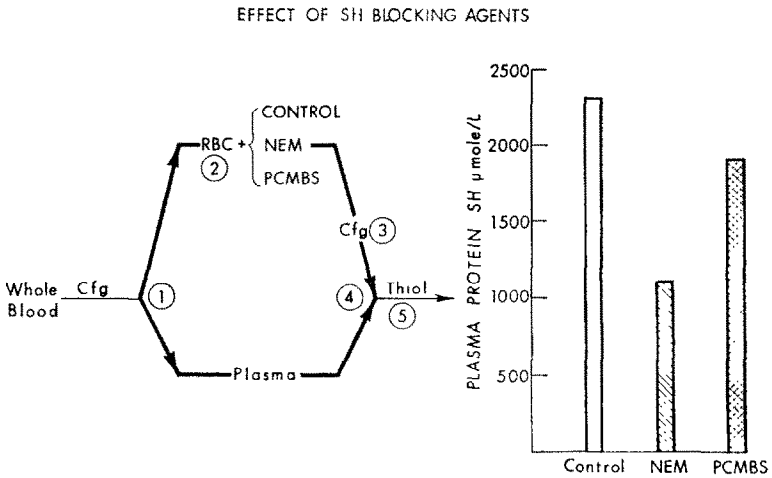

FIG. 2. Red blood cells (RBC) membrane SH groups were partially blocked with PCMBS or NEM. RBC were then recombined with their respective plasma and incubated with 0·003 M thiol. This partial blocking of RBC membrane SH groups inhibited the increase in plasma protein SH content that would otherwise occur with thiol incubation of this system. Circled numbers represent the following: 1 and 3, centrifugation steps; 2, RBC membrane SH groups partially blocked (total blockage causes RBC lysis); 4, recombination of RBC with their respective plasma; 5, RBC and plasma recombined and incubated with 0·003 M thiol for 5 hr at 37°.

The action of thiol given intravenously was further studied in Swiss mice by autoradiographic technique. Tissues were assayed for sulfhydryl content of the soluble extracted protein fraction. Whole body autoradiographic studies disclosed that after intravenous injection of NAC ^{35}S the radioactivity in the blood did not appear to clear rapidly. The compound was estimated to be distributed quite evenly over most of the body within 5 min (Fig. 3). In the 5-min autoradiograph the kidney shows the highest activity followed by the skin and smooth muscle layers of the intestines which are slightly more radioactive than the blood. Cardiac and skeletal muscles are much less active.

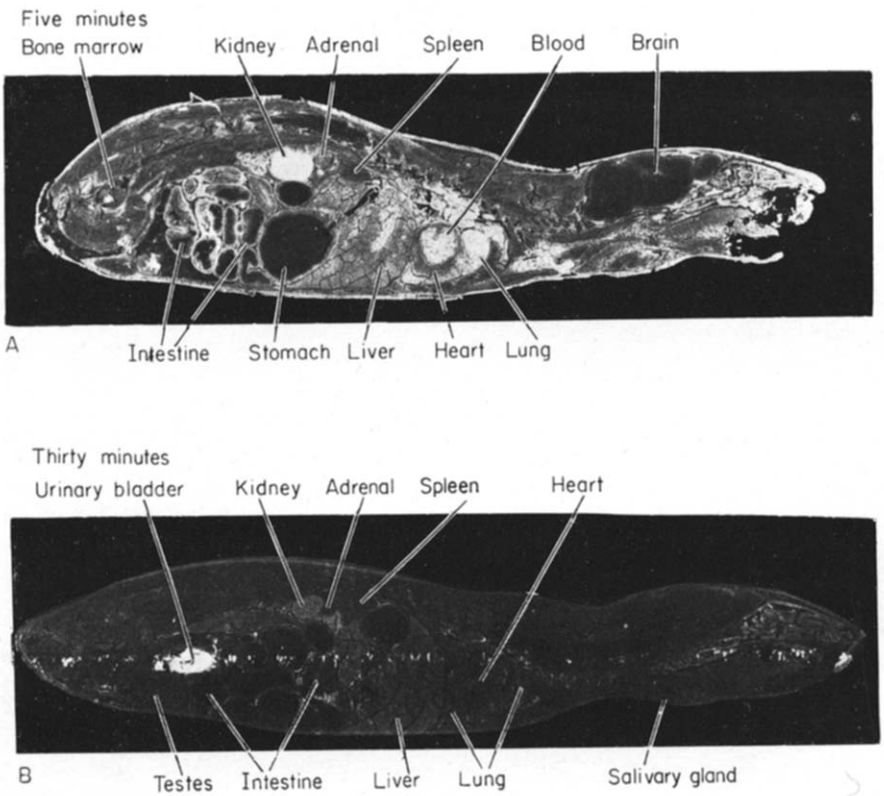

FIG. 3. Distribution of ^{35}S NAC after intravenous injection, 2 mg/g in Swiss mice. A. Distribution 5 min after injection. B. Distribution 30 min after injection.

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Between 30 and 60 min after injection the radioactivity of the blood decreased rapidly. Those organs estimated to contain the highest activity in sequence are: pancreas, blood vessels, liver, kidney, smooth muscle layers of the stomach, intestine, salivary glands and bone marrow. At this time all other structures, including the brain, have approximately the same activity as the blood. The activity in the lung appears to reflect that found in the blood.

Distribution studies with NAC ^{35}S yielded important information for proceeding with the assay for organ sulfhydryl content. The high uptake of label in kidney, spleen, liver and lung suggested that sulfhydryl assay in these tissues would likely yield information relating to the fate and possible conversion of the administered thiol. The protein sulfhydryl content of liver and kidney in the control animals, i.e. those not receiving NAC, was observed to be somewhat greater than the nonprotein SH content as indicated by the molar ratios (protein SH/nonprotein SH) of approximately 1·5 (Table 3). The protein sulfhydryl content of the soluble tissue organ extracts

TABLE 3. COMPARISON OF TISSUE SULFHYDRYL (SH)* CONTENT BEFORE AND 5 min AFTER INTRAVENOUS ADMINISTRATION OF NAC IN MICE†

Tissue		Nonprotein (SH)	Protein (SH)	Protein SH increase (%)	Protein SH‡ / Nonprot. SH
Liver	Control	20·1 ± 3·0	28·4 ± 4·7		1·41
	NAC (2 mg/g)	23·0 ± 4·1	58·0 ± 8·1	104	2·52
Kidney	Control	12·2 ± 1·4	18·8 ± 3·1		1·54
	NAC	51·5 ± 5·8	58·7 ± 7·7	212	1·14
Spleen	Control	11·0 ± 1·1	23·8 ± 4·1		2·16
	NAC	12·0 ± 1·4	50·1 ± 6·8	111	4·17
Lung	Control	2·1 ± 0·3	18·9 ± 2·7		9·01
	NAC	8·0 ± 1·0	33·3 ± 3·2	77	4·17
Muscle	Control	3·7 ± 0·4	11·7 ± 2·0		3·16
	NAC	3·3 ± 0·3	11·7 ± 2·2	0	3·55
Skin	Control	3·1 ± 0·3	5·0 ± 0·9		1·61
	NAC	5·6 ± 0·6	5·4 ± 1·1	8	0·96

* (SH) is expressed in μmoles per g of soluble protein.
† The values express mean of five determinations ± 1 S. D.
‡ Molar ratio.

before and after administration of NAC is compared in the table. The efficient conversion of thiol (NAC) to protein SH is indicated in these soluble organ fractions by the sustained high molar protein SH/nonprotein SH ratio, though some decline in this ratio was recorded after NAC administration. This ratio in the control was observed to be somewhat higher in the spleen, 2·16, and noted to be greatest in pulmonary tissue, 9·01. Five min after intravenous administration of NAC a marked increase in sulfhydryl content was noted in the extracts of these organs, thus reflecting the high uptake of NAC ^{35}S observed in the autoradiographs. The marked increase in sulfhydryl content of these organs is largely reflected in the protein sulfhydryl fractions expressed in micromoles per liter: liver, 104%; kidney, 212%; spleen, 111%; lung, 77%; muscle and skin, 0% and 8% (Fig. 4).

The increase in protein sulfhydryl content of these organs achieved with intravenous

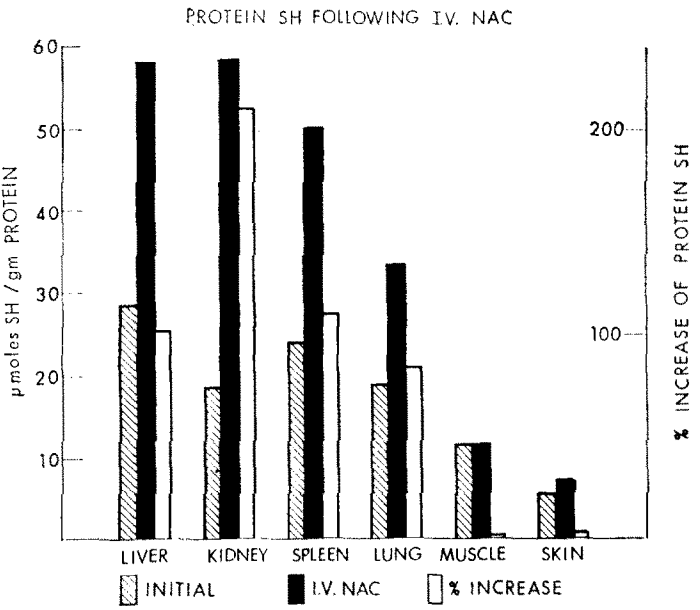

FIG. 4. Increase in SH content in the soluble protein extracted from various tissues of five Swiss mice after intravenous administration of 2 mg NAC/g of body weight. Assays were performed 5 min after the injection. Values of the bars are the mean of five determinations. The bars with diagonal lines represent initial tissue protein SH content. The solid bars indicate tissue protein SH content after intravenous injection of NAC. The open bars indicate the percentage increase.

thiol injection declined rather rapidly (Fig. 5). Four hr after intravenous administration the protein sulfhydryl values of various organ fractions had almost returned to pre-injection values. The rapid decline in protein sulfhydryl content might be attributable to metabolic degradation rather than oxidation of protein sulfhydryl groups. This conclusion is based on the autoradiographic studies that demonstrate a sharp decline of the ^{35}S organ labeling. The transient incorporation of the thiol could explain the increase in protein sulfhydryl content and the uptake of the label (see scheme under Discussion). The subsequent decline in sulfhydryl content and that of the label may suggest a reversal of this process as it occurs in normal tissues. It is noteworthy, however, that incorporation of the label and increase in protein sulfhydryl content were not observed when plasma or triturated tissue was incubated with the thiol. These findings indicate that the presence of intact tissue is required if an increase in protein sulfhydryl content is to be achieved with thiol incubation.

DISCUSSION

This investigation was intended to study the feasibility of utilizing thiol compounds to increase or restore the protein sulfhydryl content in various tissues. A significant gain in protein sulfhydryl groups was achieved with these agents during controlled studies *in vitro* and *in vivo*. These findings would suggest the feasibility of a similar approach for correcting the subnormal protein sulfhydryl content reported in patients with connective tissue disorders.[6] We postulate that the deficiency in sulfhydryl

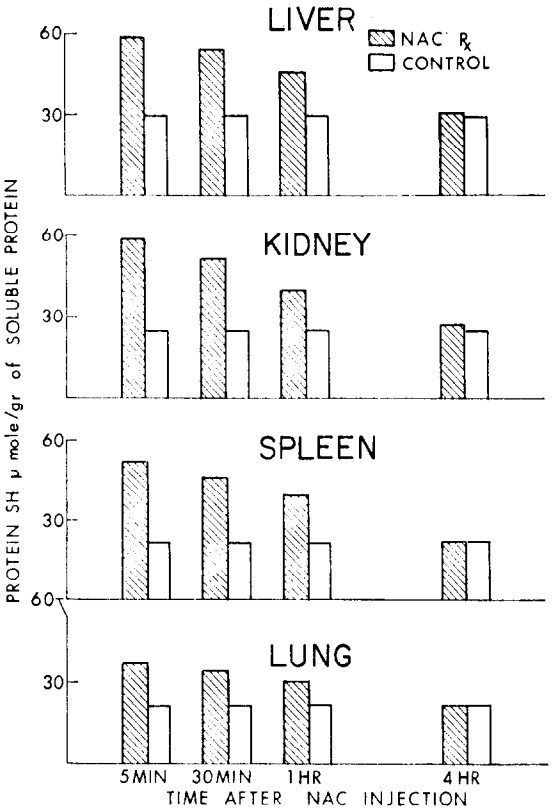

FIG. 5. Tissue protein SH content after thiol administration. Marked increase in tissue extractable protein SH content was observed after administration of intravenous NAC (bars with diagonal lines). These values declined rapidly during the ensuing 4-hr period approximating control values (open bars). The vertical height of each bar represents the mean value of five determinations.

groups observed in these patients may reflect a disseminated metabolic disturbance involving the regeneration of sulfhydryl groups, a reaction requiring the expenditure of biochemical work.[20] It is also noteworthy that a high incidence of serum protein alteration has been reported to coincide with the deficiency in serum protein sulfhydryl content.[21] Thus, several deleterious effects could ensue from the failure to maintain the normal protein sulfhydryl content: (1) conformational changes in protein structure; (2) immunological reactions directed toward the altered or denatured proteins; (3) inactivation of essential sulfhydryl-dependent enzymes. The suggested therapeutic approach for increasing subnormal protein sulfhydryl content could eliminate these untoward possibilities. Our data support the hypothesis that thiols can be utilized at the tissue level to increase protein sulfhydryl content as illustrated in Fig. 6. Our findings also indicate that intact cells, most likely the membrane sulfhydryl groups, may be an essential requirement for the oxidation reduction scheme (Fig. 6).

The question that next must be asked is whether these investigational studies have relevance to clinical situations where deficiency in tissue sulfhydryl content is manifested. This condition is frequently expressed by the marked depression in serum

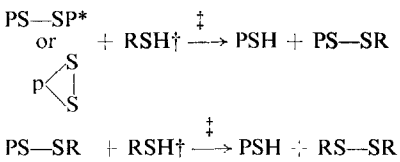

FIG. 6. Oxidation reduction scheme.
* Protein interchain (S—S).
† RSH = thiol compounds concentration, 0·003 M.
‡ Membrane or tissue SH present.

protein sulfhydryl content that occurs in patients with connective tissue disorders.[6] We studied the effect of thiol incubation on the sulfhydryl content of plasma and erythrocyte membranes manifesting such deficiency in sulfhydryl groups.[8] We were still able to achieve an increase in protein sulfhydryl content with thiol incubation utilizing the system *in vitro* described. Moreover, an increase of the subnormal serum protein sulfhydryl values was also achieved through the therapeutic use of D-penicillamine or *N*-acetylcysteine when these agents were given for therapeutic purposes in human subjects.[12, 22] The increase in subnormal serum protein sulfhydryl values in these patients was correlated with clinical improvement.

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