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Tissue Sulfhydryl Groups

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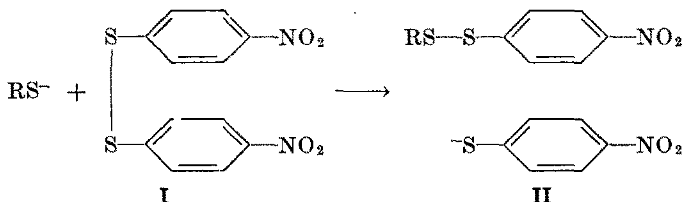
Introduction

While studying the effects of certain drugs on tissues (1), it seemed that the sulfhydryl content might have been changed. A method for measuring their concentration was devised.

In a prior publication (2), it was reported that bis(*p*-nitrophenyl) disulfide (I) reacts with aliphatic thiol compounds at pH 8.0 to produce one mole of *p*-nitrothiophenol anion per mole thiol. Since this anion is highly colored (ϵ_m = 13,600 at 412 mμ), it can be used to measure the thiol con-

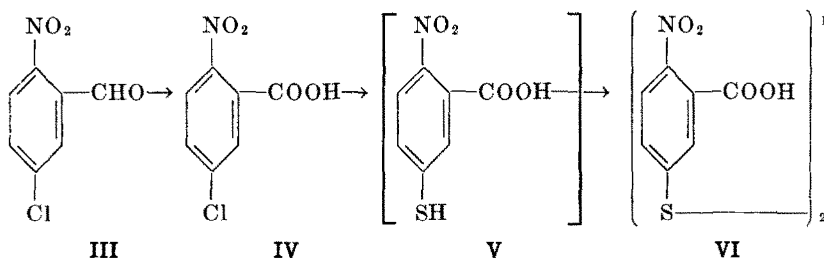


centration. The disulfide (I) is very insoluble in water; consequently, aqueous acetone solutions had to be used to dissolve the reactants. Although this is not a serious drawback for some compounds, it made the reaction with others rather more complex. For example, the reaction with cysteine or β-mercaptoethylamine is considerably slower than with β-mercaptoethanol or α-toluenethiol (2). Other objections to the use of acetone are its ability to precipitate and/or denature proteins, and the formation of schlieren lines in the photometer cell.

For these reasons, it seemed desirable to prepare a water-soluble derivative of bis(*p*-nitrophenyl) disulfide. Since the reaction must be performed in slightly alkaline medium, it seemed likely that the introduction of carboxyl groups would have the necessary solubilizing effect. Bis(3-carboxy-4-nitrophenyl) disulfide was prepared, and the expected water solubility of its alkali-metal salts was observed. Experiments were conducted to establish the usefulness of this reagent.

SYNTHESIS

2-Nitro-5-chlorobenzaldehyde (III) was obtained from Aldrich Chemical Company. It was oxidized by treatment with $KMnO_4$, the resulting acid (IV) treated with Na_2S, and the thiol (V) oxidized with iodine.



2-Nitro-5-chlorobenzoic acid (IV)

Thirteen grams of the aldehyde (III) was added to 200 ml. water in a flask and heated and stirred. When the aldehyde was melted and formed small drops (70°C.), a 5% solution of $KMnO_4$ was added dropwise until oxidation was complete. (This can most easily be detected by placing a drop of the reaction mixture on a piece of filter paper. The solution will diffuse outward from the spot and, if there is excess permanganate present, the purple color can be readily seen.) Then a few drops of concentrated sodium sulfite were added to remove the excess permanganate. The hot solution was filtered, and the black precipitate was washed with several hundred milliliters of hot water. The solution was cooled, acidified with concentrated HCl (pH 1 or less), and extracted three times with ethyl ether. The ether was evaporated, leaving 10.1 g. of pale yellow powder, m.p. 135–37°. Recrystallized from water, m.p. 137.5–138.2° [Holleman and DeBruyn (4), 139°]. Chloride: 18.1%; theory: 17.6%. The precipitated MnO_2 was dissolved in conc. HCl (in a hood), diluted to about 20% HCl, and cooled. The dark solution was extracted with ether. On evaporating the ether, an additional 0.8 g. of IV was obtained.

5,5′-Dithiobis(2-nitrobenzoic acid) (VI) (DTNB)

Six grams of the acid (IV) was suspended in 150 ml. water, and conc. NaOH was added to bring the pH to 7.2. To this solution was added 7.4 g. $Na_2S.9H_2O$ in 30 ml. water. This solution was stirred and heated (50°) for 2 hr. The reaction mixture was oxidized with I_2 in KI. Iodine solution (4.0 g./100 ml. of 5% KI) was added until the orange-yellow color of the thiol anion disappeared and was replaced by the pale yellow color of the disulfide. When this solution was acidified, an oil, which crystallized on further stirring, was formed. Yield: 5.4 g. VI from 6.0 g. IV. This was recrystallized from dimethylformamide or glacial acetic acid; m.p. 237–8°, dec.; neut. equiv. 197; theory 198.

APPLICATIONS

In general, the method used was to add a small volume (0.01–0.05 ml.) of the reagent to a larger volume of a buffered (pH 8.0) solution of the thiol compound. This was frequently done in the photometer cell in order to follow the progress of the reaction.

[1] Patent applied for.

Reagent

Dissolve 39.6 mg. of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in 10 ml. phosphate (μ = 0.1) buffer (pH 7.0).

Example

Unknown solution	3.0 ml.
Buffer (phosphate, μ = 0.1, pH 8.0)	2.0
Water	5.0

The reagent (0.02 ml.) was added to 3.0 ml. of this mixture in a photometer cell. The color developed rapidly (2 min.), and the absorbance at 412 mμ was 0.271.

Calculations

$$C_0 = \frac{A}{\epsilon} D$$

where C_0 = original concentration
A = absorbance at 412 mμ
ϵ = extinction coefficient
= 13,600/M/cm.
D = dilution factor
In this example,

$$C_0 = \frac{0.271}{13{,}600} \times \frac{10}{3} \times \frac{3.02}{3}$$
$$= 0.669 \times 10^{-4}\ M$$

Blood

Fresh or citrated blood (human, dog, or rabbit), 0.02 ml., was added to 9.0 ml. distilled water. Then 1.0 ml. of phosphate buffer (pH 8.0) was added. Three milliliters of this solution was placed into each of two Beckman 1-cm. cells, using one to adjust the absorbance to zero. To the other, 0.02 ml. reagent was added. The absorbance was

TABLE I
Whole Blood Sulfhydryl Content

	mmolar	±1 S.D.[a]
GLE	5.74	0.3
	5.18	0.3
LCH	6.55	0.2
	6.57	0.2
PS	5.45	0.3
DLD	6.58	0.3
	6.47	0.2
WBN	6.55	0.2
	6.26	0.4
JAF	5.85	0.2
DL	5.32	0.2

[a] Due to variation in photometer.

TABLE II

Typical Results

Material	Sulfhydryl Content	Time to develop full color
	mmoles/g. wet wt.	*min.*
Liver (rabbit) extracted with		
pH 8.0 buffer	0.6	25
Hot ethanol (80%)	1.9	1
5% trichloroacetic acid (TCA)	8.1	<1
Heart (rabbit) extracted with		
{KCl, 3.5% KHCO$_3$	0.6	25
Hot ethanol (80%)	0.5	<1
5% TCA	15.2	<1
Kidney (rabbit) extracted with hot ethanol (80%)	10.1	2
Lung (rabbit) extracted with hot ethanol (80%)	2.2	1
Bovine serum albumin	0.0054	15
	mmoles/l.	
Urine, human, random sample, uncontrolled diet	0.038	20
Plasma, dog, fresh	0.133	25

determined at 420 mμ after 1 hr. Results are expressed as mmoles (SH)/l. blood:

$$C_0 = \frac{A}{\epsilon}D = 36.8 \text{ A}.$$

Results of a series of such determinations on human finger-tip blood are shown in Table I.

Urine

Fresh urine (human) usually will form a precipitate if adjusted to pH 8.0. The mixture was filtered, and the determination was performed on the filtrate. Three milliliters of filtrate was placed in each of two cells; one was adjusted to $A = 0$; 0.02 ml. reagent was added to the other. The difference in absorbance was determined. See Table II for a typical result. The data in this table are intended to indicate the applicability of the method and are not intended to be a complete study of sulfhydryl content per se.

Tissues

Tissue was extracted by grinding in a mortar with washed sand, and the appropriate solvents were added. The suspension was filtered, and the filtrate was treated with the disulfide reagent as described above for urine, after adjusting the pH to 8.0. See Table II.

Purified Protein

Bovine serum albumin (Pentex, Incorporated, Kankakee, Ill., Lot B 12016 P) was dried overnight in a vacuum desiccator over P_2O_5. The white powdery material was

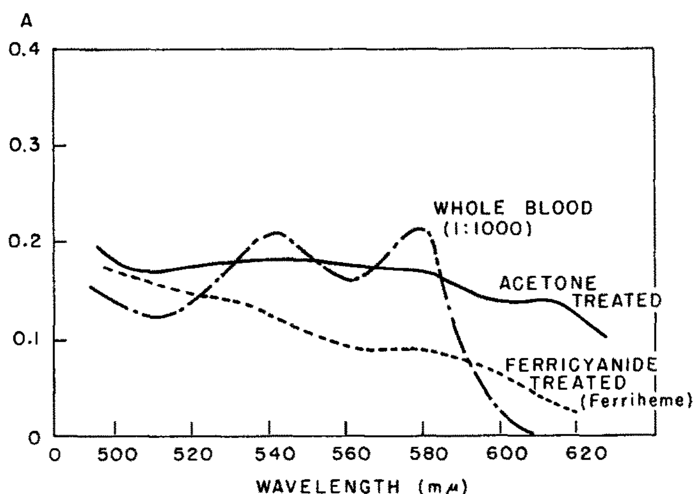


FIG. 1. Changes of absorption caused by treating whole blood as described.

dissolved in distilled water (0.3462 g. in 10 ml.). Five milliliters of distilled water and 2 ml. of pH 8.0 buffer was added to 3 ml. of this solution. It was placed in a Beckman 1-cm. cell, and the reagent (0.02 ml.) was added to it. Results are presented in Table II.

Discussion

These results on blood samples are considerably less than those reported previously (2). It was noted then that addition of acetone to the blood–buffer mixtures caused a change in the normal bright color to a paler brown shade. When the determination is done in water there is no color change. The spectral changes involved are shown in Fig. 1. These changes suggest oxidation of the heme iron to the ferric state. When DTNB is added to blood that has been treated with acetone, the amount of colored anion released is 2–3 times that produced in water alone. If one waits 20 min. after adding the acetone to the blood, however, most of the additional thiol groups have disappeared (see Fig. 2). When the reaction was done under nitrogen, the increased absorption in acetone was still observed. Blood treated with dimethylformamide displays the same phenomenon. An aqueous blood solution saturated with *n*-butanol, however, does not. When bovine serum albumin or unhemolyzed plasma was studied, no enhancement of the thiol levels was observed when 50% acetone or 8.5 *M* urea was the solvent, although the rate of reaction is increased. On the other hand, washed and hemolyzed red cells do show the same sort of enhancement as whole blood. These experiments are summarized in Table III.

These results suggest that the values reported earlier are in part an artifact due to reduction of protein disulfide bonds. If they were entirely

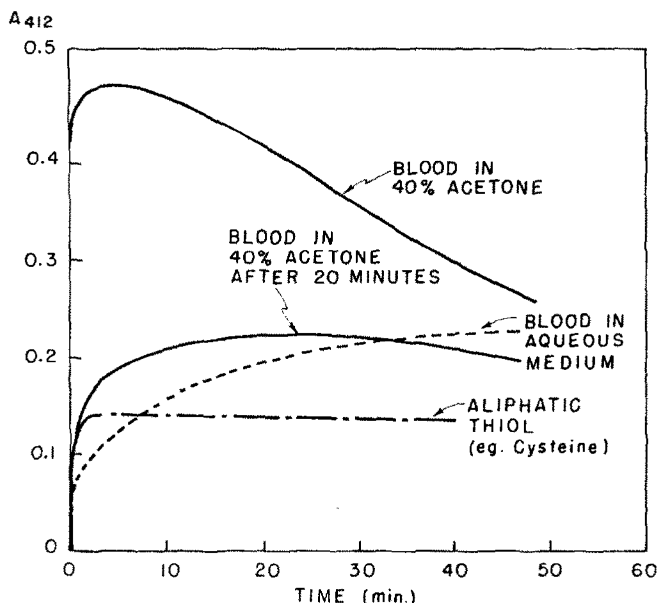


FIG. 2. Absorbance vs. time for whole blood and cysteine treated with DTNB.

TABLE III

Experiments on Blood Sulfhydryls

Material	Content
	mmoles/l.
Blood	5.36
Blood in acetone (50%)	16.3
Blood in acetone (50%) under N_2	16.1
Blood in dimethylformamide	14.8
Blood in aqueous *n*-butanol (8%)	5.39
Plasma	0.133
Plasma in acetone (50%)	0.129
Plasma in urea (8.5 *M*)	0.131
Washed, hemolyzed red blood cells	5.21
Washed, hemolyzed red blood cells in acetone (50%)	16.3

due to "unfolding" of the proteins, it is difficult to explain the simultaneous oxidation of the heme with the appearance of additional thiol (Fig. 3).[2] Comparison of the kinetics of the reaction of simple thiol compounds and

[2] If the reaction of disulfide with heme proceeds as follows: heme-Fe^{+++} + disulfide → heme-Fe^{++} + sulfhydryl, then the rate of disappearance of heme-Fe^{++} is proportional to the rate of appearance of sulfhydryl. Or approximately, $\Delta A_{578} = k(\Delta SH)$. The change in sulfhydryl is proportional to the absorbance change at 412 mμ after adding DTNB; therefore $\Delta A_{578} = k' \Delta A_{412}$. The data in Fig. 3 fit this equation well.

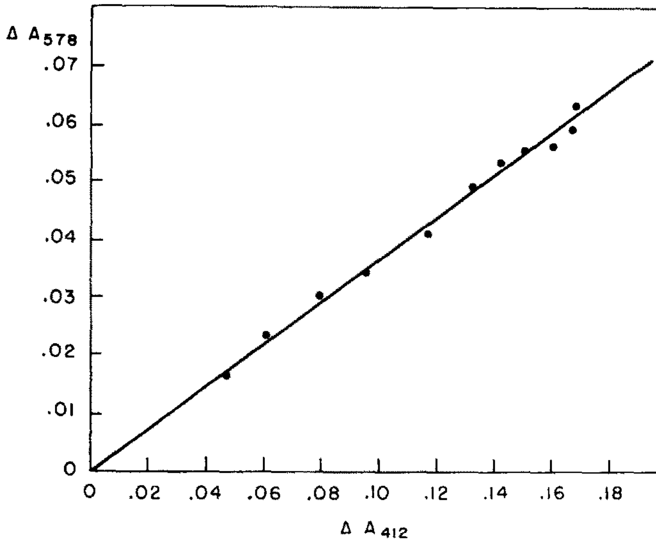


FIG. 3. Abscissa: Change of absorbance at 578 mμ after addition of blood to acetone–water (1:1); ordinate: change of absorbance at 412 mμ due to addition of DTNB (i.e., sulfhydryl concentration).

of blood in water (Fig. 2) indicates that some of the blood sulfhydryl groups are slow to react with DTNB. The effect of the acetone is to make some sulfhydryl groups more "reactive" and to make disulfide links available to the heme. A direct test of this possibility was attempted, but maintaining a reduced heme in the absence of a reducing agent proved to be very difficult. An analogous reaction, however, was shown to occur: if one mixes Versene®, ferrous ammonium sulfate, and DTNB, a steady production of the colored anion can be observed at pH 8.0.

There are at least two possible sources of the disulfide which the heme-iron might reduce: the adjacent globin portion of the molecule, or disulfide links in other red-cell proteins (3). We have observed that the sedimentation constant as determined in an ultracentrifuge was reduced from 4.06*S* to 1.755*S* in blood treated with 50% acetone. This change is consistent with an appreciable decrease in molecular weight, as might be brought about by cleavage of disulfide bonds. The alternate possibility that disulfide bonds in other proteins are being cleaved cannot be ruled out.

Those tissue extracts which contain reduced heme and proteins can undergo the same sort of changes. Thus, aqueous extracts of liver when treated with acetone show enhanced sulfhydryl content. Extracts in hot 80% ethanol, however, show no such changes and react immediately, indicating that such thiols are fully reactive (i.e., like cysteine, etc.).

Acknowledgments

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Summary

A water-soluble (at pH 8) aromatic disulfide [5,5′-dithiobis(2-nitrobenzoic acid)] has been synthesized and shown to be useful for determination of sulfhydryl groups.

Several applications have been made to show its usefulness for biological materials.

A study of the reaction of this disulfide with blood has produced some evidence for the splitting of disulfide bonds by reduced heme.

References

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