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A Colorimetric Method for Determination of Sulfhydryl Groups in Tissue Homogenates by 1-(4-Chloromercuriphenylazo)-Naphthol-2. (17868)

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Recently Bennett and Yphantis(1) reported the synthesis of 1-(4-chloromercuriphenylazo)-naphthol-2, henceforth referred to as red -SH reagent, because it forms a red precipitate with compounds containing free -SH groups. While the reagent appeared to be suitable for histochemical demonstration of -SH compounds(2), its low molecular extinction coefficient limited its use as a colorimetric -SH reagent(3). We have found that the addition of a strong mineral acid greatly intensifies the light absorption of the -SH reagent, thus making it possible to develop a simple colorimetric -SH estimation in tissue homogenates.

Experimental. The method is based on the observation that when an aqueous solution (or suspension) of -SH compounds is shaken with an amyl acetate solution of the red -SH reagent, a red precipitate forms in the aqueous layer. The amount of precipitate is directly proportional to the amount of -SH groups and can be determined by centrifugation, washing it several times, redissolving it in concentrated sulfuric or hydrochloric acid, and reading the extinction in a colorimeter.

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1. Bennett, H. S., and Yphantis, D. A., *J. Am. Chem. Soc.*, 1948, v70, 3522.

2. Bennett, H. S., *Anat. Rec.*, 1948, v100, 640.

3. Bennett, H. S., personal communication.

It is more convenient, however, to determine colorimetrically the decrease in concentration of the dye in the amyl acetate supernatant. The amount of -SH groups present can be read from a calibration curve obtained by shaking the amyl acetate solution of the -SH reagent with known amounts of an -SH compound (glutathione).

Reagents. 1. 1-(4-chloromercuriphenylazo)-naphthol-2. Three mg of the finely pulverized dye, prepared as described elsewhere (1), are dissolved in 100 ml of amyl acetate with continuous stirring until most of it is in solution. Heating to facilitate solution should be avoided because this produces a solution which is unstable. After filtration, the amyl acetate solution may be stored for a long period in a dark, glass stoppered bottle at 4°C. without appreciable change in its reactivity toward -SH groups. When an aliquot of this golden brown solution is mixed with half its volume of concentrated hydrochloric acid and allowed to stand for 15 minutes, the light absorption of the dye increases and gives a reading (log Io/Ix) of 220-320 in the Klett-Summerson colorimeter with filter 54. Maximum absorption occurs at 520 mμ. The color is stable for several days. 2. Concentrated HCl. 3. Glutathione standard‡ solution (10-100 mg glutathione freshly dissolved in 100 ml of distilled water).

Procedure. Five ml samples of the amyl acetate solution of the red -SH reagent are measured into glass stoppered centrifuge tubes. The tissues to be analyzed are chilled in an ice bath immediately upon removal from the freshly killed animal and weighed rapidly. They are then homogenized in ice cold medium with glass homogenizers(4). The homogenates are diluted to a final tissue concentration of 5%. One-tenth and 0.2 ml (5 and 10 mg) are pipetted into the amyl acetate solution of the red -SH reagent. The centrifuge tubes are stoppered and mechanically shaken for 20 minutes with an oscillation of 250 per minute. In preliminary

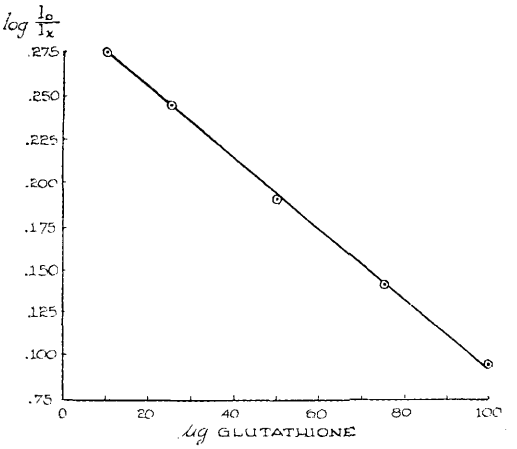

FIG. 1.
Calibration of –SH reagent with glutathione solution.

measurements 20 minutes' shaking was sufficient to complete the reaction between the -SH groups and the red -SH reagent. After centrifugation for 15 minutes at 2000 r.p.m., an aliquot of 4 ml of the clear amyl acetate supernatant is transferred to a test tube. Upon the addition of 2 ml of concentrated HCl, the red color develops immediately. The tubes are thoroughly shaken and allowed to stand for 15 minutes. The color is measured in the Klett-Summerson photoelectric colorimeter, with filter 54, against a blank containing 2 parts of amyl acetate and 1 part of HCl. An -SH standard curve is obtained by plotting the extinction of the acidified red -SH reagent against the amount of -SH groups (in the form of glutathione). Since the -SH reagent is insoluble in water, the values obtained are not measurably altered by the amount of water in which the tissue is homogenized or the glutathione dissolved. As a rule all tissue samples and -SH standards were made up to 0.5 ml with the homogenizing medium. In agreement with histochemical findings, the reaction of -SH groups with the red -SH reagent is completely abolished when iodoacetamide or mercuric chloride is added to the samples before the analysis.

Results and discussion. The apparent -SH content of tissue homogenates was found to depend on the nature of the homogenizing medium. Analyses of samples of the same mouse liver, homogenized in 0.9% NaCl,

‡ Obtained from Schwartz Laboratories, Inc., 202 E. 44th St., New York City.

4. Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, v114, 495.

TABLE I.
-SH Content of Homogenates of Rabbit, Rat, and Mouse Livers (in γ/100 mg wet weight).

Liver of	Anson's method	Author's method
Rabbit	46.5	45.2
Rat	46.5	47.5
"	48.4	53.8
"	35.6	36.7
"	38.9	36.7
"	47.5	48.6
"	44.3	43.2
Mouse	34.5	35.6
"	31.3	36.5
"	52.9	59.4

Tissues were homogenized in 0.9% NaCl and diluted to a final tissue concentration of 0.5% for Anson's method and to 5% for the present method.

TABLE II.
-SH Content of Kidney, Heart, and Brain Homogenates (in γ/100 mg wet weight).

Tissue	Anson's method	Author's method
Rat kidney	25.2 (18.2-26.6)	56.4 (53.8-60.2)
" heart	20.1 (17.2-26.6)	48.9 (45.2-56.9)
" brain	15.6 (11.8-18.2)	59.2 (55.9-62.3)
Mouse kidney	20.4 (14.0-22.6)	54.3 (35.0-61.3)
" heart	17.2 (10.8-19.4)	41.9 (36.6-47.3)
" brain	14.0 (9.7-16.0)	52.7 (44.1-53.8)

Each figure represents the avg of determinations obtained in 6 animals. Min. and max. values in parentheses. Every determination carried out in duplicate on 2.5, 5, and 7.5 mg tissue samples with Anson's method and on 5 and 10 mg samples with the present method.

0.88 M sucrose, distilled H_2O, 10% urea (in H_2O), and 10% Duponol PC (Na-laurylsulphate), gave 29, 31, 47, 77, and 89 γ of -SH, respectively, per 100 mg of wet tissue. In all tissues analyzed, the lowest -SH values were obtained when NaCl or sucrose solutions were used as homogenizing media, an increase being noted with distilled H_2O or protein denaturing agents. The liberation of -SH radicals in protein denaturation is described by Anson(5).

Comparison showed acceptable agreement with results obtained by the ferricyanide procedure of Anson(6) on liver tissue (Table I), but higher values on kidney, brain, and heart (Table II). The discrepancy may be explained by the protein denaturing effect of amyl acetate on tissue proteins other than liver. The resistance of liver proteins to amyl acetate remains unexplained at present. Native egg white which showed no free -SH radicals with Anson's method(6) reacted with the red -SH reagent, indicating that protein denaturation had occurred during treatment with the amyl acetate solution.

The convenient procedure described in this paper offers the possibility of determining -SH groups in tissue homogenates. The optically clear solutions used for colorimetric measurements give it an advantage over procedures where the color of a turbid suspension has to be estimated. In further experiments, correlation between enzyme activities and -SH content of tissue homogenates is being investigated.

Summary. A colorimetric procedure was developed for the determination of -SH groups. The method involved the estimation of the amount of an organic mercury salt removed from its amyl acetate solution by shaking with tissue homogenates. The dye removed was found proportional to the -SH content of the sample analyzed.

5. Anson, M. L., *Adv. Protein Chem.*, 1945, v2, 361.

6. Anson, M. L., *J. Gen. Physiol.*, 1941, v24, 399.

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Hemolysis of Erythrocytes Incubated in Salines. (17869)

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The increase in fragility of erythrocytes caused by incubation of blood at 37°C for 12 to 32 hours, after which treatment the corpuscles may hemolyse even in serum or isotonic saline, has been shown to have differential value between bloods from normal