inhibition of cell multiplication in the host epidermis.

1. Bullough, W. S., Biol. Rev., 1962, v37, 307.
2. Bullough, W. S., Laurence, E. B., Proc. Royal Soc. Series B, 1960, v151, 517.
3. Mazia, D., in The Cell; biochemistry, physiology, morphology, Brachet, J. & Mirsky, A. E., Ed., Academic Press, New York, 1961, vIII, 393.
4. Gross, J., Bogoroch, R., Nadler, N. J., Leblond, C. P., Amer. J. Roentgen. & Rad. Ther., 1951, v65, 420.
5. Messier, B., Leblond, C. P., Proc. Soc. Exp. Biol. and Med., 1957, v96, 7.
6. Weiss, P., Science, 1952, v115, 487.
7. Glinos, A. D., Gey, G. O., Proc. Soc. Exp. Biol. and Med., 1952, v80, 421.
8. Glinos, A. D., Science, 1956, v123, 673.
9. Saetren, H., Exp. Cell Res., 1956, v11, 229.
10. Stich, H. F., Florian, M. L., Canad. J. Biochem. & Physiol., 1958, v36, 855.
11. Swann, M. M., Cancer Res., 1957, v17, 727; ibid., 1958, v18, 118.
12. Abercrombie, M., Symp. Soc. Exp. Biol., 1957, v11, 235.

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Influence of Hydralazine on the Sulfhydryl Group in the Presence of Cupric Ion.* (30108)

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Two antirheumatic drugs, gold thiomalate (1) and chloroquine(2) have been reported to inhibit the reactivity of the sulfhydryl group. Sulfhydryl group reactivity in turn controls the extent of sulfhydryl-disulfide interchange reaction(3) which can denature proteins(4) such as human gamma globulin (5) by forming intermolecular and new intramolecular disulfide bonds. Fig. 1 illustrates one type of sulfhydryl-disulfide interchange reaction between 2 protein molecules. It is possible that such denaturation might render proteins antigenic and play a role in the inflammation associated with rheumatoid arthritis and systemic lupus erythematosus. This hypothesis would be supported if it were shown that drugs(6) reported to induce rheumatic complaints in patients, increase the reactivity of the sulfhydryl group and promote denaturation by sulfhydryl-disulfide interchange. A study was therefore devised to determine whether these rheumatogenic drugs can increase sulfhydryl group reactivity by removing cupric ion which suppresses sulfhydryl group reactivity(7). Normal human serum contains approximately 1.7 μM "free" non-ceruloplasmin copper(8).

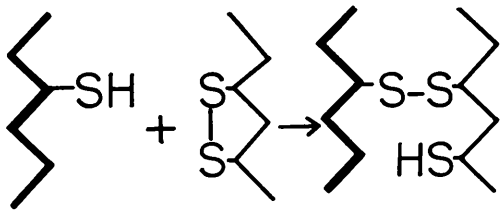

FIG. 1. Sulfhydryl-disulfide interchange reaction between 2 protein molecules.

Methods. Effect of hydralazine on reaction between bis(p-nitrophenyl)-disulfide and cysteine in presence of cupric ion. Bis(p-nitrophenyl)disulfide† reacts with cysteine by means of a sulfhydryl-disulfide interchange reaction to give rise to a yellow product that has been identified as p-nitrophenylsulfide(9). The concentration of this substance may be determined by its absorbency at 412 mμ. The parent compound bis(p-nitrophenyl)disulfide does not absorb significantly at this wave length. Reagents that react with the sulfhydryl group prevent the formation of the yellow color by reacting either with the sulfhydryl group of cysteine or the sulfhydryl group of p-nitrophenylsulfide(9). Accordingly, cysteine does not produce an increase in absorbency at 412 mμ when incubated with bis(p-nitrophenyl)disulfide in the presence of

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† Eastman Organic Chemicals, Rochester N. Y.

an equimolar concentration of cupric ion. If a substance is added to this system which is capable of ligating the cupric ion an increase in absorbency at 412 mμ develops due to a reaction between bis(p-nitrophenyl)disulfide and the cysteine liberated from the copper-cysteine complex by the ligating agent. Ten ml aliquots were prepared to contain 50% acetone, 50% water, 0.01 M phosphate (pH 7.4), 250 μM bis(p-nitrophenyl)disulfide, 200 μM cupric ion and 200 μM cysteine. To each aliquot a test compound such as hydralazine was added where possible as a 0.1 M solution to a final concentration of 200 μM and the solution incubated at 37°C for 20 minutes. An equal volume of water was added to the control solution. Each aliquot was then centrifuged at 3000 rpm for 20 minutes to remove excess copper-cysteine complex. The absorbency of the supernatant was determined at 412 mμ in a Coleman Universal spectrophotometer. For each substance control experiments were performed in which no cupric ion or cysteine or no bis(p-nitrophenyl)disulfide was added and occasional minor appropriate corrections made. All experiments were performed in triplicate.

Effect of hydralazine on denaturation of human gamma globulin in presence of cupric ion. The denaturation of human gamma globulin by heat has been shown to proceed in part by means of the sulfhydryl-disulfide interchange reaction between protein molecules(5). The extent to which this reaction has proceeded and the protein molecules thereby crosslinked can be determined by measuring the solubility of the denatured product in 83% acetic acid. The solubility in 83% acetic acid is inversely proportional to the extent to which the sulfhydryl-disulfide interchange reaction between protein molecules has proceeded(5). Using a method previously described(5) a solution was prepared which contained 0.5 g per 100 ml human gamma globulin,‡ 0.1 M phosphate buffer at pH 7.4, 0.05 M sodium chloride, 0.6 mM ammonium chloride and 30 μM copper sulfate. One ml aliquots of this solution were incubated in triplicate for 16 hours with a test substance such as hydralazine over a range of concentrations of the drug from 0 to 10 mM in increments of 2 mM. The test substance was added where possible as a 0.1 M solution. An equal volume of water was added to the control solution. Each aliquot was heated to 87°C for 6 minutes and then thoroughly mixed with 5 ml of glacial acetic acid. The concentration of the human gamma globulin remaining insoluble in 83% acetic acid was determined as previously described(5) or alternatively by measuring the turbidity of the suspension at 540 mμ in a Coleman Universal spectrophotometer before and after centrifugation at 3000 rpm for 2 hours. No significant difference could be detected between the values obtained by these two methods.

Failure of hydralazine to reduce cupric sulfate to metallic copper. To determine whether cupric ion is reduced to metallic copper by hydralazine or isoniazid 10 ml aliquots were prepared to contain 0.1 M aqueous phosphate buffer at pH 7.4, 33 μM copper sulfate and graded concentrations of hydralazine or isoniazid from 0 to 2 mM. Incubation was carried out at 37°C or 90°C for 30 minutes to test for a possible oxidation-reduction reaction between copper and the drug at both high and low temperatures. The concentration of ionic copper remaining after incubation was determined by addition of diethyldithiocarbamate to a concentration of 10 mM and determination of the absorbency of the solution after 30 minutes at 440 mμ according to the method of Gubler and associates(10).

Results. Effect of hydralazine on reaction between bis(p-nitrophenyl)-disulfide and cysteine in presence of cupric ion. In a 10 ml solution (50% acetone, 50% water, 0.01 M phosphate, pH 7.4, 250 μM bis(p-nitrophenyl)disulfide, 200 μM cupric ion, and 200 μM cysteine) to which 200 μM hydralazine had been added and the mixture incubated for 30 minutes, 14% of the cysteine reacted with the bis(p-nitrophenyl)disulfide as measured by the absorbency of the solution at 412 mμ. In the absence of hydralazine no cysteine reacted with the bis(p-nitropenhyl)disulfide. This indicates that hydralazine is capable of

‡ American Red Cross, Washington, D. C.

TABLE I. Reagents Which Appear to Compete with the Sulfhydryl Group for the Cupric Ion.

Reagent	Bis(p-nitrophenyl)-disulfide method. % of cysteine reacting	Gamma globulin method. % reversal of copper effect per mM reagent*
Hydralazine (Apresoline)	14	29
Isoniazid	4	26
Ephedrine sulfate	0	7
Methoxamine (Vasoxyl)	0	4
Procainamide (Pronestyl)	0	2
None	0	0

* Avg SD ± 1.3%.

removing copper from a copper-cysteine complex. Tables I and II indicate the results obtained with 50 other compounds. Results are reported as the percentage of the cysteine present in the solution that has reacted with the bis(p-nitrophenyl)disulfide. Test substances were selected arbitrarily to include drugs reported to induce rheumatic disease and drugs commonly used by physicians. Of the 51 compounds tested, only hydralazine and isoniazid yielded values above 1%. Both have been reported to induce a disease resembling systemic lupus erythematosus.

Effect of hydralazine on denaturation of human gamma globulin in presence of cupric ion. In a solution of 0.5 g per 100 ml human gamma globulin at pH 7.4 (0.1 M phosphate, 0.05 M sodium chloride, 0.6 mM ammonium chloride) to which cupric ion (30 μM) had been added, the coagulum formed after heating to 87°C for 6 minutes was 50% soluble in 83% acetic acid. In the absence of cupric ion only 8% of the denatured human gamma globulin was soluble in 83% acetic acid. The effect of the presence of hydralazine on the extent to which cupric ion inhibits the sulfhydryl-disulfide interchange reaction and thereby renders the denatured coagulum soluble in acetic acid is indicated in Fig. 2. The value of 29% for the reversal of the cupric ion effect by hydralazine is derived from the expression:

$$\% \text{ reversal} = \frac{(20-8)(100)}{50-8} = 29\%$$

In this expression 20 represents the per cent gamma globulin soluble in 83% acetic acid in the presence of hydralazine and 30 μM cupric sulfate, 8 represents the per cent gam-

TABLE II. Substances with No Measurable Effect on the Sulfhydryl Group in Presence of Cupric Ion as Measured by Either Bis(p-nitrophenyl)disulfide or Denaturation of Human Gamma Globulin.

acetazolamide	neomycin†
acetone	N-ethylmaleimide
ammonium chloride	nicotinamide
antipyrine	ethyl alcohol
arsenic pentoxide	o-iodosobenzoic acid
beta-aminopropionitrile	paraldehyde
benzyl alcohol	para-aminohippurate
caffeine sodium benzoate	p-aminosalicylic acid*
chloroform	5-(p-hydroxyphenyl)5-phenylhydantoin
chlorothiazide	penicillin G*
chloramphenicol	phenobarbital
coumadin	propylene glycol
diphenylhydantoin (Dilantin)†	pyrazinoic acid
epsilon aminocaproic acid	pyruvic acid
erythromycin lactobionate*	sodium borate
ethyl alcohol	sodium citrate
5-ethyl-3-methyl-5-phenylhydantoin (Mesantoin)†	sodium salicylate
hydrochlorothiazide	sulfamethoxypyridazine (Kynex)
5,5-hydroxyphenylethylhydantoin	toluene
indomethacin	trimethadione (Tridione)*
iodoacetamide	tris(hydroxymethyl)aminomethane (Tris)
lidocaine	urea
lithium carbonate	uric acid†

* Not tested on denaturation of human gamma globulin because of instability at 87°C.
† Not tested on denaturation of human gamma globulin because of insolubility in concentrations required.

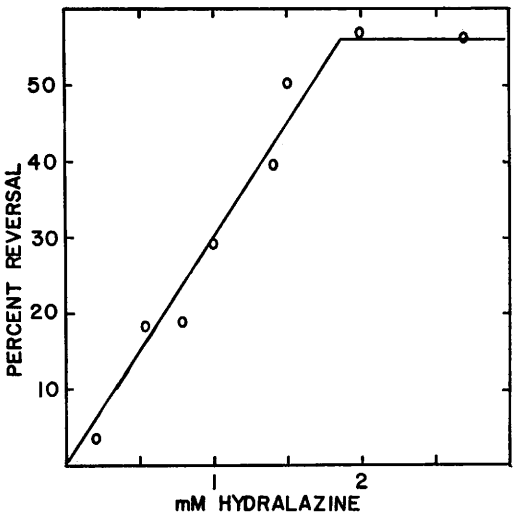

FIG. 2. Influence of hydralazine on effect of 30 μM cupric ion on the heat denaturation of 0.5 g per 100 ml human serum gamma globulin at pH 7.4. Abscissa indicates concentration of hydralazine (O) in mmoles per liter in a solution of human gamma globulin denatured at 87°C for 6 min. Ordinate indicates percent reversal of the effect of 30 μM cupric ion on heat denaturation of human gamma globulin. Effect of cupric ion was determined by the change in solubility of denatured protein in 83% acetic acid.

ma globulin soluble in the absence of drug or copper and 50 represents the per cent of gamma globulin soluble in 83% acetic acid in the presence of 30 μM cupric sulfate. The maximum effect produced by hydralazine (1.9 mM) was a 56% reversal of the cupric ion effect. In the presence of 1 mM isoniazid 26% of the effect of 30 μM cupric ion was reversed. This was the maximum effect produced by isoniazid and also occurred at a concentration of 0.9 mM. The effect of all the reagents studied was linearly related to the concentration of the reagent present during denaturation so long as submaximally effective concentrations of test reagent were used. The compounds with a measurable effect when present in concentrations of 10 mM or less are included in Table I. Results are reported as the per cent reversal of the effect of 30 μM copper produced by 1 mM of reagent on the solubility of the denatured human gamma globulin in 83% acetic acid. Table II contains those compounds with no measurable effect in concentrations of 10 mM or less.

Control experiments in which the compound under investigation was added after heat denaturation or in which heat denaturation was omitted produced negative results in all cases. Qualitatively similar results with hydralazine were obtained when 0.44 g per 100 ml bovine serum albumin in 0.1 M sodium acetate and 0.1 N sodium chloride at pH 5.6 was heated to 100°C for 3 minutes. Under these conditions the inhibitory effect of 136 μM cupric ion was approximately halved in the presence of 4 mM hydralazine.

Failure of hydralazine to reduce cupric sulfate to metallic copper. Cupric sulfate (33 μM) incubated with graded concentrations of hydralazine or isoniazid to 2 mM at 37°C or 90°C for 30 minutes in 0.1 M phosphate buffer showed no measurable loss in concentration of ionic copper as determined by the absorbency of the solution at 440 mμ on the addition of 10 mM diethyldithiocarbamate. This method would not detect an oxidation-reduction reaction resulting in the formation of cuprous ion.

Discussion. Hydralazine forms a complex with many divalent cations including Cu^{++}, Fe^{+++}, Mn^{++} and Fe^{++}. Of these cations cupric ion is the most strongly bound to hydralazine(11). Of the 51 compounds evaluated in this study 6 have been reported to induce a disease resembling systemic lupus erythematosus in certain individuals. These compounds are hydralazine, isoniazid, procainamide, diphenylhydantoin, trimethadione and 5-ethyl-3-methyl-5-phenylhydantoin. The best documented of these compounds is hydralazine(6) which was the most potent agent evaluated in this study in terms of its ability to reverse the inhibitory effect of cupric ion on the sulfhydryl group. Less well documented for their effect in inducing a disease resembling systemic lupus erythematosus are isoniazid(12) and procainamide(13). These compounds were also notable in their ability to compete with cysteine for cupric ion although less active in this respect than hydralazine. Anticonvulsant compounds(14) have been reported to induce systemic lupus erythematosus but do not appear to compete with cysteine for the cupric ion. Diphenylhydantoin, however, has been reported to in-

crease the urinary content of copper in patients receiving the drug(15). Ephedrine sulfate and methoxamine were weakly active in the gamma globulin system in competing with cysteine for copper but have not been reported to induce rheumatic disease.

The observations here reported suggest that a drug like hydralazine which induces rheumatic like disease, activates the sulfhydryl group in the presence of copper and may thereby facilitate sulfhydryl-disulfide exchange between protein molecules causing protein denaturation.

Summary. Fifty-one compounds were studied for their ability to compete with the sulfhydryl group for cupric ions. The sulfhydryl-disulfide interchange reactions occurring during the reaction between cysteine and bis(p-nitrophenyl)disulfide and during the course of the denaturation of human serum gamma globulin by heat were used as models for this reaction. Hydralazine, a compound which has been reported to induce a disease resembling systemic lupus erythematosus was exceptionally active in reactivating the sulfhydryl-disulfide interchange reaction when this reaction was studied in the presence of cupric ion.

1. Gerber, D. A., J. Pharmacol. Exp. Therap., 1964, v143, 137.
2. ———, Arth. & Rheum., 1964, v7, 193.
3. Jensen, E. V., Science, 1959, v130, 1319.
4. Steinrauf, L. K., Dandliker, W. B., J. Am. Chem. Soc., 1958, v80, 3833.
5. Gerber, D. A., J. Immunol., 1964, v92, 885.
6. Harvey, A. M., Textbook of Medicine, Beeson, P. B., McDermott, W., Ed., W. B. Saunders Co., Phila. and London, 1963, 476.
7. Klotz, I. M., Urquhart, J. M., Klotz, T. A., Ayers, J., J. Am. Chem. Soc., 1955, v77, 1919.
8. Scheinberg, I. H., van den Hamer, C. J. A., Morell, A. G., Windsor, J., Ann. Intern. Med., 1963, v58, 719.
9. Ellman, G. L., Arch. Biochem., 1958, v74, 443.
10. Gubler, C. J., Lahey, M. E., Ashenbrucker, H., Cartwright, G. E., Wintrobe, M. M., J. Biol. Chem., 1952, v196, 209.
11. Schroeder, H. A., Hahnemann Symposium on Hypertensive Disease, W. B. Saunders Co., Phila. and London, 1959, 332.
12. Zingale, S. B., Minzer, L., Rosenberg, B., Lee, S. L., Arch. Intern. Med., 1963, v112, 63.
13. Hahn, A. L., Missouri Med., 1964, v61, 19.
14. Jacobs, J. C., Pediatrics, 1963, v32, 257.
15. Peters, H. A., Fed. Proc., 1961, v20 suppl. 10, 190.

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Free Amino Acids of Developing Skeletal Musculature of Normal and Genetically Dystrophic Chickens.* (30109)

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Muscular dystrophy, inherited as a recessive trait in New Hampshire chickens, is manifested by the inability of the birds to raise their wings and to perform the normal righting reflex when placed on their backs(1). Hypertrophy of the superior pectoral muscle occurs followed in some birds by a loss of muscle fibers. The breast muscle of dystrophic chickens has a higher fat(2) and free amino acid content(3), a higher water to nitrogen ratio(3) and a higher lysosomal enzyme activity(4) than that of normal chickens. Carnosine and anserine levels are lower than in normal birds(3).

Elevated levels of taurine and serine ethanolamine phosphate (SEP) are especially characteristic of dystrophic breast muscle(3). In 2 lines of dystrophic birds selected for low and high fat content SEP appeared in the breast muscles no earlier than 4 weeks of age. It was not detected in the breast muscle of 2-, 4-, and 6-week-old normal chicks. An unidentified ninhydrin-positive compound (hereafter referred to as CX) was present

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