solution was then concentrated to dryness. The resulting solid crystallized from 10 cc. of hot water in the form of needles, m. p. 208–210°.[12]

Anal. Calcd. for $C_{15}H_{23}NO_3S$: C, 60.6; H, 7.80. Found: C, 61.3; H, 7.97.

The sulfoxide of N-(β-benzylmercapto)-ethyl-DL-phenylalanine was prepared in exactly the same way except that the recrystallization in this case was from a large volume of aqueous acetone. The product melted at 219–220°.

Anal. Calcd. for $C_{18}H_{21}NO_3S$: C, 65.2; H, 6.39. Found: C, 64.9; H, 6.45.

Control Experiments. A.—In a series of experiments, DL-phenylalanine (68 mg.) was dissolved in 15 cc. of either 5 *N* hydrochloric acid, 30% (by weight) sulfuric acid, or a 1:1 mixture of 5 *N* hydrochloric acid and glacial acetic acid. Benzyl-H* (0.2–0.5 mg.) was added and the mixtures were heated at 100–110° for twelve to fifty-four hours. To each reaction mixture was added non-radioactive N-(β-benzylmercapto)-ethyl-DL-phenylalanine (Ia). The derivative Ia was isolated from the mixture, recrystallized, and analysed for radiosulfur. In no case did the isolated compound contain as much as 0.1% of the radiosulfur added as benzyl-H*.

B.—A mixture of N-benzoyl-DL-phenylalanine and benzyl-H* was heated in a 1:1 mixture of 5 *N* hydrochloric acid and glacial acetic acid. The phenylalanine derivative Ia was added, isolated from the mixture, and then analysed for radiosulfur. It contained less than 0.1% of the radiosulfur added as benzyl-H*.

C.—A mixture of 50 mg. of crystalline insulin and 1 mg. of butyl-H* was heated at 100° in 10 cc. of 5 *N* hydrochloric acid for forty-four hours. The mixture was diluted to 25 cc., and to an aliquot (2.5 cc.) were added 100 mg. of the phenylalanine derivative I and 2 cc. of 50% aqueous acetic acid. The derivative I was isolated from the mixture, purified, and analysed for radiosulfur. It contained no measurable amount of radiosulfur.

D.—The phenylalanine derivative Ia (0.53 g.) and benzyl-H* (0.38 g.) were heated under reflux for nineteen hours in 5 cc. of 5 *N* hydrochloric acid and 10 cc. of glacial acetic acid. When the reaction mixture was diluted with water to a volume of 100 cc. and cooled, the product separated. It was recrystallized from 45 cc. of 30% aqueous acetic acid to give 0.36 g. of product which contained 7.1% of the radiosulfur added to the reaction mixture as benzyl-H*.

A portion of this product (105 mg.) was converted to the sulfoxide by the procedure described above. After the sulfoxide had been recrystallized twice from hot water and once from 50% acetone, it had a melting point of 218–220°, and did not contain a detectable amount of radiosulfur.

(12) All melting points are corrected micro melting points.

Acknowledgment.—The authors wish to thank Mr. Roscoe C. Funk, Jr., for performing the microanalyses and for aid in the radioactivity measurements. They also wish to thank Dr. G. H. A. Clowes for supplying the crystalline insulin. They would also like to express their appreciation to Dr. Mary Elizabeth Wright for invaluable aid in the preparation of this manuscript.

Summary

Insulin which had been treated with a minute amount of *n*-butyl β-chloroethyl sulfide (butyl-H) containing radiosulfur was subjected to acid hydrolysis. By application of the "washing-out" technique, evidence was obtained for the presence of radiosulfur-containing N-(β-butylmercapto)-ethyl-phenylalanine in the hydrolysate. Various control experiments provided no evidence for the attachment of vesicant residues to the amino group of phenylalanine during the hydrolysis procedure. Therefore it was concluded that in the vesicant-treated insulin, a fraction of the vesicant had been attached to the free amino group of some of the phenylalanine moieties in the intact protein molecule.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, CORNELL UNIVERSITY MEDICAL COLLEGE]

Studies of the Effect of Mustard-type Vesicants on the Phenol Color Reaction of Proteins[1]

BY CARL M. STEVENS,[2] HERBERT MCKENNIS, JR.,[3] AND VINCENT DU VIGNEAUD

In the course of the wartime studies on the reaction of H (bis-(β-chloroethyl) sulfide) and H-type vesicants ($RSCH_2CH_2Cl$) with proteins, the action of Folin's phenol reagent[4] on vesicant-treated proteins was investigated. Herriott, Anson and Northrop[5] found that proteins which had been treated with vesicant at *p*H 6 gave less color with the phenol reagent at *p*H 8 than the corresponding untreated proteins. These investigators also noted that in most instances the chromogenic power of the vesicant-treated proteins toward the phenol reagent returned to normal after treatment with alkali for various lengths of time.

One possible interpretation of this phenomenon was that a chemical reaction had occurred between the vesicant and the tyrosine or tryptophan groups in the intact proteins, the subsequent action of the alkali being to cleave the linkages thus formed and to free the phenolic or indolyl groups for reaction with the phenol reagent.

(1) The work described in this paper was carried out under Contract OEMsr-144 between the Office of Scientific Research and Development and Cornell University Medical College and is described in Progress Reports to the National Defense Research Committee, December, 1942, to September, 1943.

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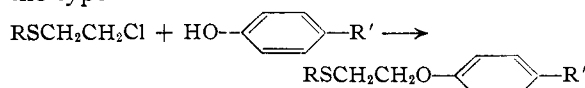
(4) Olcott and Fraenkel-Conrat [*Chem. Rev.*, **41**, 151 (1947)] have presented a synoptic critique of the Folin phenol method, including references to the literature regarding its use in studies on proteins.

(5) Northrop, *et al.*, Informal Progress Reports to NDRC Section B4C (1942); for the published results of this work, see Herriott, Anson and Northrop, *J. Gen. Physiol.*, **30**, 185 (1946).

During the course of our wartime researches on the reaction of H-type vesicants with proteins,[6] we had prepared numerous derivatives of amino acids by their reaction with the one-handed vesicants, *n*-butyl β-chloroethyl sulfide (butyl-H) and benzyl β-chloroethyl sulfide (benzyl-H).[6b] In these studies we had found no evidence for a reaction of the vesicants with the indolyl group of tryptophan or with the phenolic group of tyrosine when the *p*H of the reaction mixture was in the range 6–8. However, in strongly basic solution the vesicants did react appreciably with the phenolic group of tyrosine to yield phenolic ethers of the type



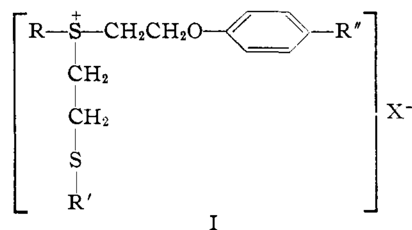
Phenolic ethers are in general very stable.[7] The stability of the O,N-disubstituted benzyl-H derivative of tyrosine to alkali was tested. After this tyrosine derivative was boiled for ten minutes in 0.5 *N* sodium hydroxide, the reaction mixture produced no color upon treatment with the phenol reagent. This result indicated that the linkage between the vesicant and the phenolic group of tyrosine was stable to alkali. In view of this stability, it was difficult to attribute the recovery of chromogenic power which followed alkali treatment of vesicant-treated proteins to cleavage of phenol-vesicant linkages.

In exploring other possible explanations for the behavior of the vesicant-treated proteins toward the phenol reagent, it occurred to us that the methods used by Miller[8] in studying the loss of chromogenic power toward the phenol reagent of tobacco mosaic virus upon treatment with various acylating agents might be applied to the study of the vesicant-treated proteins. Miller found that following treatment of the virus derivatives with sodium dodecyl sulfate in acid solution there was partial recovery of the chromogenic power toward the phenol reagent at *p*H 8 without any significant cleavage of acyl groups from the protein. He concluded that the amount of color produced by tobacco mosaic virus or its derivatives on treatment with the phenol reagent at *p*H 8 depended to a large extent on the degree of denaturation of the virus protein.

In the present publication, experiments are described which applied this concept to the vesicant-treated proteins. Pepsin and tobacco mosaic virus which had been treated with butyl-H gave a much smaller amount of color with the phenol reagent at *p*H 8 than did the untreated proteins. However, if the vesicant-treated or untreated proteins were first submitted to the action of Duponol C (sodium dodecyl sulfate) in acid solution and then subjected to the phenol reagent at *p*H 8, the same amount of color was produced from both the vesicant-treated and untreated proteins. Furthermore, it was possible to show that no significant amount of vesicant residues was liberated from vesicant-treated proteins by the action of Duponol C. These results demonstrate that the increase in the amount of phenol color from vesicant-treated proteins after the action of Duponol C is in all probability not due to the cleavage of vesicant residues from the tyrosine or tryptophan groups of the proteins. Thus it appears that the decreased chromogenic power of vesicant-treated proteins toward the phenol reagent at *p*H 8 is not due to the reaction of the vesicant with tyrosine or tryptophan groups in the protein molecule.[9]

This conclusion is in harmony with the results of the studies of Herriott, Anson and Northrop[5] on the nature of the alkali-labile linkages between vesicants and proteins. They were able to show that the number of free carboxyl groups lost from the protein upon treatment with H equalled, in several cases, the number of alkali-labile H residues bound to the vesicant-treated protein. Thus, not enough alkali-labile H residues were attached to the vesicant-treated protein to have combined both with the free carboxyl groups and the tyrosine phenolic groups of the protein.

Rydon,[10] in considering the decreased determinable phenolic groups of a vesicant-treated protein and the reversibility of this effect by alkali, suggested the possibility of the formation of sulfonium compounds[11] of the type I and their subsequent cleavage with release of tyrosine phenolic groups.



We have prepared such a compound (I, R = CH_3, R′ = C_4H_9, R″ = H) by the reaction of butyl-H with methyl β-phenoxyethyl sulfide and isolated it as the diliturate. This compound gave almost no color with the phenol reagent at *p*H 8 either before or after treatment with Duponol C.

(6) (a) du Vigneaud and Stevens, THIS JOURNAL, **69**, 1808 (1947); (b) du Vigneaud, Stevens, McDuffie, Wood and McKennis, *ibid.*, **70**, 1620 (1948); (c) Wood, Rachele, Stevens, Carpenter and du Vigneaud, *ibid.*, **70**, 2547 (1948); (d) Carpenter, Wood, Stevens and du Vigneaud, *ibid.*, **70**, 2551 (1948); (e) Stevens, Wood, Rachele and du Vigneaud, *ibid.*, **70**, 2554 (1948).

(7) Lüttringhaus and Sääf, *Angew. Chem.*, **51**, 915 (1938).

(8) Miller, *J. Biol. Chem.*, **146**, 339, 345 (1942).

(9) In a report from this Laboratory (Informal Progress Report to NDRC Section B4C, December 21, 1942) to the coöperating groups it was suggested that "... a decrease in phenol color value of a protein resulting from treatment with [H] or related vesicants may not involve any reaction directly with the tyrosine or tryptophan groups, but may be due rather to an effect of substitution on the stability of the molecule to denaturation."

(10) Rydon, unpublished British Report, February 19, 1943.

(11) For studies involving other sulfonium compounds of the S-mustards, see: Stahmann, Fruton and Bergmann, *J. Org. Chem.*, **11**, 704 (1946); Stein and Moore, *ibid.*, **11**, 681 (1946).

TABLE I

PHENOL COLOR VALUES[a]

Sample	Method A (Duponol + acid)	Method B (no treatment)	Method C (Duponol)	Method D (acid)	Method E (alkali-20°)	Method F (alkali-50°)
Tobacco mosaic virus	132	70	78	70	125[p]	135[p]
Butyl-H-treated tobacco mosaic virus	132	30[p]	52[p]	30[p]	38[p]	113[p]
Pepsin	191	190	195		200	204
Butyl-H-treated pepsin	188	106	179		199	102[p]
Dimethyl β-phenoxyethyl sulfonium iodide[b]	24	20[p]			335	
Methyl β-phenoxyethyl β-butylmercaptoethyl sulfonium diliturate[b]	21	20[p]			293	

[a] The figures given are the actual colorimeter readings. Under these conditions, a sample containing 0.02 mg. of tyrosine gave a reading of 75. [b] One cubic centimeter of an 0.001 M aqueous solution was used. [p] Samples thus marked indicate that a precipitate formed. In order that colorimetric determinations could be made, the precipitates were removed as completely as possible by centrifugation. It seems possible that precipitation may in some cases involve loss of reactive groups.

Experimental

Stability of the Benzyl-H Derivative of Tyrosine.—O,N-Di-(β-benzylmercaptoethyl)-L-tyrosine[6b] gave a negative test with Folin's phenol reagent[12] in alkaline solution. After the derivative had been allowed to stand in 0.5 N sodium hydroxide for one week at room temperature, or had been heated in the alkali for ten minutes at 100°, it still gave no color with the phenol reagent.

Reaction of Tobacco Mosaic Virus with Butyl-H.—A dialyzed solution of tobacco mosaic virus[13] (7.5 cc.) containing 300 mg. of virus was added to 7.5 cc. of 0.5 M sodium bicarbonate. To this solution was added 0.4 cc. of butyl-H and the mixture was stirred gently for seven hours. The resulting solution was shaken with ether and the aqueous layer was dialyzed against running water. This solution was diluted with 3 volumes of water; 0.2-cc. aliquots were removed and diluted to 1 cc. for use in the phenol color determinations.

Reaction of Pepsin with Butyl-H.—To 10 cc. of an aqueous solution of pepsin (1.2 mg. N/cc.)[14] was added 0.2 cc. of butyl-H. The mixture was stirred for one and one-half hours, the *p*H being kept at approximately 7 by the dropwise addition of 1 cc. of 0.5 M sodium bicarbonate. The aqueous layer was decanted and stirred gently with an equal volume of ether. The aqueous layer was then dialyzed, diluted to 20 cc., and 0.2-cc. aliquots were removed and diluted to 1 cc. for use in the phenol color determinations.

Determination of "Phenol Color Values."—The modified procedures utilized for the phenol color determinations were developed from the methods of Miller,[8] who used sodium dodecyl sulfate as a denaturing agent.

Method A.—One cubic centimeter of the unknown (containing a suitable amount of the protein or other material) was placed in a test-tube containing 0.2 cc. of a 10% solution of Duponol C.[15] One-tenth cubic centimeter of 0.2 N hydrochloric acid was added and the mixture was allowed to stand for fifteen minutes. Then 0.1 cc. of 0.2 N sodium hydroxide was added, followed by 0.6 cc. of water, 1 cc. of diluted phenol reagent and 2 cc. of phosphate buffer. The buffer was prepared by adding 10 cc. of 10% sodium hydroxide to 90 cc. of 0.5 M disodium hydrogen phosphate. The phenol reagent[12] was diluted so that 1 cc. mixed with 2 cc. of phosphate buffer and 1 cc. of water had a *p*H of 7.7 at the end of thirty minutes.

Method B.—The same as Method A except that 0.2 cc. of water was added in place of the Duponol C solution, and 0.2 cc. of 0.1 M sodium chloride was added in place of the acid and alkali.

(12) Folin and Ciocalteu, *J. Biol. Chem.*, **73**, 627 (1927).

(13) This sample of virus was kindly supplied by Dr. W. M. Stanley.

(14) The pepsin was kindly supplied by Dr. R. M. Herriott.

(15) This Duponol C (du Pont) was stated to be 92% sodium dodecyl sulfate and 8% sodium sulfate. The suspension obtained upon dissolving the Duponol C in water was allowed to settle, and the clear liquid was decanted for use.

Method C.—The same as Method A except that 0.2 cc. of 0.1 M sodium chloride replaced the acid and alkali.

Method D.—The same as Method A except that 0.2 cc. of distilled water was used in place of the Duponol C solution.

Method E.—One cubic centimeter of the unknown was added to 0.1 cc. of 0.2 N sodium hydroxide, and the mixture was allowed to stand for fifteen minutes at 20°. Then 0.1 cc. of 0.2 N hydrochloric acid, 0.8 cc. of water, 1 cc. of diluted phenol reagent, and 2 cc. of phosphate buffer were added in this order.

Method F.—Identical with Method E except that the treatment with alkali was at 50° rather than 20°.

In all cases the color was allowed to develop for thirty minutes at room temperature and then was estimated in a Klett–Summerson photoelectric colorimeter using the No. 54 (green) filter. The data are recorded in Table I.

Stability of Protein-Vesicant Linkages to Sodium Dodecyl Sulfate.—If vesicant residues were split from butyl-H-treated proteins by the action of Duponol C, the expected hydrolysis product[3d] would be *n*-butyl β-hydroxyethyl sulfide ($C_4H_9SCH_2CH_2OH$) (IV). Preliminary experiments showed that this compound could be extracted almost quantitatively from water by an equal volume of ether; after removal of the ether the amount of IV could be determined by Northrop's procedure[15] for the determination of mustard gas and related compounds. Since, in the case of the vesicant-treated proteins, no other ether-soluble compounds were likely to be present in the system, this method was considered to be quite specific for vesicant residues.

Samples (2 cc.) of the solutions of the vesicant-treated and untreated pepsin were added to 0.4 cc. of 10% Duponol C and 0.2 cc. of 0.2 N hydrochloric acid, and the resulting solutions were allowed to stand for twenty minutes. At the end of this period the liberated butyl-H residues were determined. The solutions were shaken for one minute with 2 volumes of peroxide-free ether; 1-cc. aliquots of the ether solutions were added to 2 cc. of water, and the ether was evaporated *in vacuo* at room temperature. One cubic centimeter of 2 M sulfuric acid and 2 cc. of water were added, and the solution was titrated by the procedure described by Northrop.[16] The untreated pepsin gave a titration value which was equivalent to approximately 0.01 mg. of butyl-H residues per 7 mg. of protein; this value was used as a blank. The vesicant-treated pepsin gave a corrected titration value equivalent to less than 0.02 mg. of vesicant residues per 7 mg. of vesicant-treated pepsin. If the increase in the phenol color value of the vesicant-treated pepsin after treatment with Duponol C were due to cleavage of the linkages between tyrosine and vesicant residues, it would require the liberation of approximately 0.2 mg. of butyl-H residues per 7 mg. of protein, a value ten times that actually found.

(16) Northrop, Informal Progress Report to NDRC Section B4C, July 23, 1942; for published method, see Ref. 5.

Identical experiments with solutions of vesicant-treated and untreated tobacco mosaic virus gave similar results. After the action of Duponol C on the vesicant-treated virus, a value of 0.01 mg. of vesicant residues per 7 mg. of virus was obtained.

These results demonstrate that the restoration of phenol color value in vesicant-treated pepsin and tobacco mosaic virus by the action of Duponol C is not accompanied by any appreciable degree of cleavage of vesicant residues from the proteins.

Dimethyl β-Phenoxyethyl Sulfonium Iodide.—This salt was prepared by treatment of methyl β-phenoxyethyl sulfide with methyl iodide under the conditions described by Crane and Rydon.[10,17]

Methyl β-Phenoxyethyl β-Butylmercaptoethyl Sulfonium Diliturate.—Methyl β-phenoxyethyl sulfide (1.68 g.) and butyl-H (3.0 g.) were dissolved in 20 cc. of 95% ethanol. After four days, the mixture was diluted with 3 volumes of water and centrifuged. The upper layer was removed and treated with 3 volumes of a saturated aqueous solution of dilituric acid (5-nitrobarbituric acid). The light yellow prisms which separated were collected and washed with cold methanol and cold acetone. The product (1.5 g.) was purified by one recrystallization from acetone and two recrystallizations from methanol. The recrystallizations were carried out at a maximum temperature of 25° on account of the instability of the compound. The purified sulfonium salt melted on the hot stage with decomposition and evolution of gas at 120–130°.

Anal. Calcd. for $C_{15}H_{25}OS_2 \cdot C_4H_2O_5N_3$: N, 9.18; S, 14.01. Found: N, 9.18; S, 13.85.

Phenol Color Reactions of the Sulfonium Compounds.—The phenol color reactions of the sulfonium compounds are summarized in Table I. A very small amount of color was produced by the action of the phenol reagent at *p*H 8 on the sulfonium compounds; the amount of color produced remained unchanged after preliminary treatment with Duponol C. However, considerable color was produced with the phenol reagent after the compounds had been treated with alkali.

Acknowledgment.—The authors would like to take this opportunity to express their appreciation to Dr. Mary Elizabeth Wright for invaluable aid in the preparation of this manuscript.

Summary

A study has been made of the decreased chromogenic power toward Folin's phenol reagent at *p*H 8 displayed by pepsin and tobacco mosaic virus which had been treated with *n*-butyl β-chloroethyl sulfide (butyl-H). After treatment with sodium dodecyl sulfate (Duponol C), the vesicant-treated and untreated proteins give the same amount of color with the phenol reagent. Moreover, no significant amount of vesicant residues is liberated by the action of Duponol C on the vesicant-treated proteins.

It is concluded that the increase in the amount of phenol color from these vesicant-treated proteins after the action of Duponol C is not due to the cleavage of vesicant residues from the tyrosine or tryptophan groups of the proteins. It is further concluded that the decreased chromogenic power of vesicant-treated proteins toward the phenol reagent at *p*H 8 is not due to reaction of the vesicant with the tyrosine or tryptophan groups in the proteins.

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(17) Crane and Rydon, *J. Chem. Soc.*, 766 (1947).

[CONTRIBUTION NO. 78 FROM THE GENERAL LABORATORIES OF THE UNITED STATES RUBBER COMPANY]

The Reaction of α-Methylstyrenes with Thioglycolic Acid

BY CHEVES WALLING, DEXTER SEYMOUR AND KATHERINE P. WOLFSTIRN

Studies of the relative reactivities of meta- and para-substituted styrenes[1] and α-methylstyrenes[2] with free radicals derived from copolymerizing monomers have been useful in determining the nature of the "alternating effect" in copolymerization. An entirely similar approach may be made to the study of the nature of the attack of solvent radical on monomer in the chain transfer reaction.[3] This paper presents an investigation of the relative reactivities of six meta and para substituted α-methylstyrenes toward the radical $\cdot SCH_2COOH$ derived from thioglycolic acid.

Since the transfer constant for an α-methylstyrene with thioglycolic acid is very large,[4] when two α-methylstyrenes are heated with thioglycolic acid in the presence of a free-radical catalyst, virtually the only reaction by which styrenes will be consumed will be by reaction with the $\cdot SCH_2CO$-OH radical (to give, eventually, β-phenylpropylmercaptoacetic acid), and the kinetic equations will be identical with those for the system of two α-methylstyrenes and maleic anhydride.[2] *I.e.*, $d[M_1]/d[M_2] = k_1[M_1]/k_2[M_2]$ where M_1 and M_2 represent the two styrenes and k_1 and k_2 the rate constants for their reaction with the mercaptide radical.[5] Calculations of relative reactivities including the determination of experimental errors were, accordingly, carried out as described previously.[2]

Experimental

Materials.—Thioglycolic acid was obtained by fractionating commercial material. Its physical constants were b. p. 79–80 (1 mm.), m. p. −17.5 to −15.5°. The α-

(1) Walling, Briggs, Wolfstirn and Mayo, THIS JOURNAL, **70**, 1537 (1948).

(2) Walling, Seymour and Wolfstirn, *ibid.*, **70**, 1544 (1948).

(3) Mayo, *ibid.*, **65**, 2324 (1943).

(4) The styrene radical reacts with ethyl thioglycolate 58 times as readily as with styrene. *Cf* Gregg, Alderman and Mayo, *ibid.*, in press. Since α-methylstyrene shows relatively little tendency to polymerize with itself, its transfer constant with thioglycolic acid is presumably even larger.

(5) It is of interest that the equation describing chain transfer, in general, is a special case of the copolymerization equation in which the "monomer reactivity ratio" for the solvent is zero and the "transfer constant" is the reciprocal of the "monomer reactivity ratio" for the monomer considered.