S, 11.78; Na, 8.45. Found: C, 39.06, 39.30; H, 6.06, 5.74; S, 11.38; Na, 8.22.

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Summary

A sulfonic acid analog of desthiobiotin was synthesized. The compound showed inhibitory activity toward *S. cerevisiae*, which was more pronounced against *d,l*-O-heterobiotin and *d,l*-desthiobiotin than *d*-biotin. The compound had no effect on *L. casei*.

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

The Reaction of Some Radioactive Mustard-type Vesicants with Purified Proteins[1]

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In a collaborative attack on the problem of the mechanism of action of mustard-type vesicants, we had undertaken to study the reaction of vesicants with proteins. A study of the reactions of a large number of amino acids with vesicants has already been described.[4] The second phase of our work was the study of the interaction of vesicants with certain well characterized, highly purified proteins. Preliminary experiments indicated that treatment of certain proteins with relatively large amounts of vesicant resulted in a chemical reaction. The reaction products differed from the original proteins in physical properties and had a higher sulfur content. These observations supplemented earlier evidence[5] of chemical reactions between vesicants and proteins and indicated the desirability of a detailed study.

Kistiakowsky, Moritz, Henriques and co-workers[6] had already demonstrated that an extremely small amount of mustard gas (H) is bound in the tissue at the site of a burn produced by a minimum amount of H. At the same time, all indications were that the vesicants were capable of reacting with a large number of different types of groups presumably present in proteins. Reactions at the site of the burn must involve only a small percentage of these groups and possibly only certain types. It was, therefore, of particular interest to study the reaction of H-type vesicants with proteins *in vitro* at correspondingly low ratios of vesicant to protein in an effort to determine the most reactive groups under these conditions. It was decided to utilize the radioactive tracer technique in approaching this question.

(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, January, 1942, to November, 1943.

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(4) du Vigneaud, Stevens, McDuffie, Wood and McKennis, THIS JOURNAL, **70**, 1620 (1948).

(5) (a) Berenblum and Wormall, *Biochem. J.*, **33**, 75 (1939); (b) unpublished British Reports: Berenblum (1940), Pirie (1941), Peters (1941).

(6) Progress Reports to NDRC Section B4C (1942).

Benzyl β-chloroethyl sulfide (benzyl-H*) and *n*-butyl β-chloroethyl sulfide (butyl-H*), containing S^{35} of 87-day half-life,[7] were synthesized from benzyl mercaptan* and butyl mercaptan*, respectively. The general scheme for the synthesis of the vesicants is shown

$$RMgX \xrightarrow{S^*} RS^*MgX \xrightarrow[H^+]{HOH} RS^*H \xrightarrow[ClCH_2CH_2OH]{NaOH}$$

$$RS^*CH_2CH_2OH \xrightarrow{HCl} RS^*CH_2CH_2Cl$$

A number of syntheses of these compounds containing isotopic sulfur have already been described.[8] The procedure employed by us in this investigation contains technical features which facilitated the handling of the small amounts of materials involved, and is, therefore, presented in some of its details.

The estimations of radioactivity were carried out essentially by the method of Henriques and coworkers.[9] The radioactivity of the sulfur in the vesicants used to treat the proteins was sufficient to allow the detection of as little as 5×10^{-6} mg. of benzyl-H* or butyl-H* residues per milligram of protein sulfur in the protein derivative or its hydrolysis products.

The proteins utilized were crystalline insulin, crystalline pepsin and crystalline tobacco mosaic virus.

Insulin.—Insulin was selected for study particularly because of its unique physiological activity, and because a large amount of chemical data on the molecule is available. Furthermore, it has no known organic constituents other than amino acids.

Insulin was treated with benzyl-H* or butyl-H* in amounts ranging from 0.25 to 4.0 mg. of vesicant per 100 mg. of protein. This resulted in insulin-vesicant* preparations containing from 0.3 to

(7) An asterisk (*) is used to indicate the presence of radiosulfur in a compound.

(8) Tarver and Schmidt, *J. Biol. Chem.*, **146**, 69 (1942); Seligman, Rutenburg and Banks, *J. Clin. Investigation*, **22**, 275 (1943); Kilmer and du Vigneaud, *J. Biol. Chem.*, **154**, 247 (1944).

(9) Henriques, Kistiakowsky, Margnetti and Schneider, *Ind. Eng. Chem., Anal. Ed.*, **18**, 349 (1946).

5.3 vesicant residues[10] per molecule of insulin, as shown by the data of Table I (Preparations 1–9). It may be pointed out that the amount of vesicant which combined with the protein depended upon the amount of vesicant applied. In fact, despite the sixteenfold variation in the amount of vesicant applied, the per cent. of the applied vesicant which combined with the insulin was approximately constant (*ca.* 50%).

Using a method which is essentially that employed by Scott[11] for the crystallization of insulin, it was possible to obtain from an insulin-benzyl-H* preparation crystals of the alkylated protein. After recrystallization, this product contained an average of 1.1 vesicant residues per molecule of protein. It is of interest that the product, when tested in rabbits, displayed considerable hypoglycemic activity.

Pepsin.—Crystalline pepsin was dissolved in 0.05 *M* borate buffer (*p*H 7.4) at a concentration of 20 mg./cc. and treated with benzyl-H*. From the data of Table I (Preparation 10), it can be seen that pepsin combined with approximately 35% of the vesicant.

Tobacco Mosaic Virus Protein.—The early reports of Berenblum[5b] on the susceptibility of nucleoproteins to precipitation by mustard gas indicated the desirability of studying the nature of the reaction of vesicants with nucleoproteins.[12] As a protein for study, we selected tobacco mosaic virus (TMV). This material contains 6% of nucleic acid. The protein has a unique amino acid composition, being devoid of histidine as well as methionine, and possessing a small content of cysteine, which, however, is not detectable chemically except after denaturation.[13] Furthermore, the intact protein has a unique and readily measurable biological activity which might be of help in characterizing the changes brought about by vesicant treatment.

Treatment of TMV with various amounts of benzyl-H* or butyl-H* (Table I, Preparations 11–14) resulted in the attachment of 25–40% of the applied vesicant to the TMV. Because of the enormous molecular weight of the virus, even the lowest level of vesicant applied (0.25 mg./100 mg. of TMV) resulted in the substitution of approximately 260 vesicant residues per virus molecule.

Through the kindness of Dr. W. M. Stanley and Dr. L. O. Kunkel, we were able to obtain tests of the biological activity of Preparations 11 and 13.

The determination of the virus activity was carried out on 34 half-leaves of *N. glutinosa*, and the samples were inoculated at a concentration of 10^{-5} g./cc. As a control, buffered solutions of a sample of virus and a sample of benzyl-H* were stirred separately and then combined and dialyzed. This control sample was found to be as active as the original virus and was used as the standard. Preparation 11 possessed 93% of the activity of the control sample, while Preparation 13 showed 52% of the activity of the control.

From these results, it may be concluded that Preparation 11 was not appreciably inactivated, while Preparation 13 had only about half of the activity of the control. These samples contained, respectively, 1500 and 3200 vesicant residues per virus molecule. Apparently, then, treatment leading to the addition of approximately 1500 vesicant residues to reactive groups of the protein caused no appreciable inactivation, while treatment resulting in the substitution of an additional 1700 groups caused 50% inactivation.

Preparation 11 was also tested for the production of mutants. Each of 225 plants was inoculated with material from a different lesion produced in *N. glutinosa* by the control sample of virus, and each of 225 more plants with material from a different lesion produced by benzyl-H*-treated virus. Of the plants inoculated with the control virus, 196 became diseased, and 4 of these were atypical, resulting apparently from mutants. Of the plants inoculated with the vesicant-treated virus, 174 became diseased, and 5 of these appeared atypical. Thus, the vesicant treatment which resulted in the substitution of approximately 1500 vesicant residues caused neither inactivation nor mutation of the virus to an extent detectable by the tests employed.

Experimental

Benzyl β-Chloroethyl Sulfide (Benzyl-H*) Containing Radiosulfur.—Barium sulfate*[14] was reduced to barium sulfide* by a procedure similar to those which have already been described.[15] Dried barium sulfate* (116 mg.) was spread in a thin layer on a platinum boat and placed in a Vycor tube. The air was expelled by a stream of hydrogen and the Vycor tube was heated at 900–1000° for two hours and then allowed to cool, a slow stream of hydrogen being maintained throughout the reduction. The issuing gases were bubbled through an absorption train consisting of 6 cc. of 0.5 *N* sodium hydroxide in a small test-tube and 1 cc. in a second tube.

The boat containing the barium sulfide* was placed along with 5 mg. of zinc dust in a 125-cc. ⅋ 24/40 Erlenmeyer flask equipped with a separatory funnel and a delivery tube. The delivery tube was attached to the sodium hydroxide absorption train used with the reduction of the barium sulfate* and the apparatus was swept with oxygen-free nitrogen. Twenty cubic centimeters of 6 *N* phosphoric acid, which had been boiled with about 5 mg. of zinc dust to expel air and cooled somewhat, was placed in the separatory funnel. This acid was dropped onto the barium sulfide* at such a rate as to produce a slow evolution of hydrogen sulfide*, which was absorbed in the

(10) These values represent a statistical average of the number of vesicant residues per molecule in the particular preparation, and are not intended to indicate that every molecule contains this number of residues.

(11) Scott, *Biochem. J.*, **28**, 1592 (1934).

(12) For other studies of reactions of vesicants with nucleoproteins, see Banks, Boursnell, Francis, Hopwood and Wormall, *Biochem. J.*, **40**, 745 (1946); Young and Campbell, *Can. J. Research*, **25B**, 37 (1947).

(13) Stanley and Lauffer, *Science*, **89**, 345 (1939); Ross, *J. Biol. Chem.*, **136**, 119 (1940).

(14) Samples of barium sulfate containing radiosulfur were kindly supplied by Dr. M. Kamen and Dr. F. C. Henriques, Jr.

(15) Cooley, Yost and McMillan, THIS JOURNAL, **61**, 2970 (1939); Boursnell, Francis and Wormall, *Biochem. J.*, **40**, 743 (1946); Henriques and Margnetti, *Ind. Eng. Chem., Anal. Ed.*, **18** 476 (1946).

sodium hydroxide scrubbers. When all the acid had been added, the reaction mixture was warmed slowly to its boiling point, allowed to cool in a stream of nitrogen and swept with nitrogen for one hour.

Fifteen cubic centimeters of 0.1 *N* iodine in potassium iodide and 1 cc. of concentrated hydrochloric acid were placed in a 50-cc. centrifuge cone. The 6 cc. of sodium sulfide* solution from the first scrubber was introduced at the bottom of the solution by means of a long slender pipet. The 1 cc. of solution from the second scrubber was used to wash the first and the washings were added to the iodine solution. The scrubbers were washed further with small portions of water until a nitroprusside or lead acetate test for the sulfhydryl group on the washings was negative. The portion of the transfer pipet coated with sulfur* was then broken off and placed in the iodine solution. After fifteen minutes the excess iodine was destroyed with a few drops of a freshly prepared solution of stannous chloride in 5 *N* hydrochloric acid. When the resulting suspension was allowed to stand overnight, the free sulfur* coagulated and was then collected by centrifugation. The precipitate was washed with water by centrifugation and decantation.

Ten cubic centimeters of purified *m*-xylene was added and the sulfur* was dissolved by boiling the mixture cautiously. The xylene solution was transferred to a 15-cc. centrifuge cone, washed first with a few cc. of the acid stannous chloride solution to remove traces of iodine and then with water. The traces of water in the xylene solution were removed by azeotropic distillation. The xylene solution was transferred to a 50-cc. centrifuge cone with xylene, evaporated to a volume of 5 cc. and cooled to room temperature under nitrogen. Five cubic centimeters of a 0.4 *N* solution of benzylmagnesium bromide in benzene was added. The tube was stoppered and allowed to stand overnight at room temperature.

The resulting suspension of benzylmercaptomagnesium bromide* was centrifuged and the excess Grignard solution was decanted. The solid was washed 3 times with 5-cc. portions of petroleum ether (b. p. 35°) by centrifugation and decantation. It was then suspended in petroleum ether (5 cc.) and cooled in an ice-bath under nitrogen. (A nitrogen atmosphere was maintained as long as benzyl mercaptan* was present.) One cubic centimeter of 5 *N* hydrochloric acid was added and the mixture was shaken until free of solids. The acid layer was separated by means of a pipet. The petroleum ether layer containing the benzyl mercaptan* was washed with two 1-cc. portions of water and the washings were added to the acid layer. The combined aqueous layers were extracted with 1-cc. portions of peroxide-free ether until the nitroprusside test for the sulfhydryl group was negative. The combined ether extracts were added to the petroleum ether layer. Then 1 cc. of an ether solution containing 36 mg. of ethylene chlorohydrin and 1 cc. of 1 *N* sodium hydroxide were added. The two layers were thoroughly mixed by bubbling the alkali through the organic layer with the aid of a pipet. The mixture was heated gently in a hot water-bath until the organic solvents had evaporated. If the aqueous solution was not alkaline or not free of mercaptan, additional alkali or additional ethylene chlorohydrin or both were added and warming was continued until all the mercaptan had reacted.

The benzyl β-hydroxyethyl sulfide* was extracted with one 5-cc., one 3-cc. and three 1-cc. portions of ether. In a 15-cc. centrifuge tube, the combined ether extracts were washed with 1 cc. of water. The ether solution was transferred to a Carius tube which was heated on a water-bath to remove the ether. The last traces of ether were removed by attachment to a water pump. Two cubic centimeters of petroleum ether and 3 cc. of concentrated hydrochloric acid were added to the residue. The tube was cooled, sealed and shaken at 65–70° for twenty-four hours; 2 cc. of petroleum ether was added and the petroleum ether layer was separated. The acid layer was extracted with three 2-cc. portions of petroleum ether. The combined petroleum ether solutions were concentrated at −40° and 10^{-3} mm. Toward the end of the concentration, crystals generally appeared. The last traces of petroleum ether were removed at 0° (10^{-3} mm.). If the product on melting was not perfectly colorless, it was distilled at 10^{-3} mm. and 40° onto a cold finger which was cooled to *ca.* −70° with a Dry Ice–cellosolve mixture. The apparatus was so arranged that after completion of the distillation the colorless product dropped into a cup. The product adhering to the cold finger was rinsed into a cup with 2 cc. of petroleum ether and this solution was transferred to a small weighing tube. The tube was connected to a distilling apparatus and cooled to −70°. The benzyl β-chloroethyl sulfide* crystallized and the petroleum ether was removed *in vacuo* at gradually rising temperatures up to 10°. The yield was 70–72 mg. (75–77%).

Anal. Calcd. for $C_9H_{11}ClS$: Cl, 19.0. Found: Cl, 18.9.

Butyl β-Chloroethyl Sulfide (Butyl-H*) Containing Radiosulfur.—Barium sulfate* (116 mg.) was converted to sulfur* and the sulfur* was dissolved in xylene in the manner described previously. To the dried, cooled solution in a 50-cc. centrifuge cone was added 40 cc. of a 0.6 *N* solution of *n*-butylmagnesium bromide in benzene. After three to four hours the tube was filled with petroleum ether (b. p. 50–60°) and the mixture was centrifuged. The supernatant liquid was decanted into a 100-cc. centrifuge tube. The residue of butylmercaptomagnesium bromide* was stirred with 25 cc. of petroleum ether and centrifuged. This supernatant liquid was added to the solution in the 100-cc. centrifuge tube and the volume was increased to 100 cc. with petroleum ether, whereupon an additional amount of precipitate was obtained. This was collected and added to the main residue. The combined precipitates were washed with 25 cc. of petroleum ether by centrifugation.

The butylmercaptomagnesium bromide* was suspended in a few cc. of petroleum ether, cooled in an ice-bath under nitrogen and treated with 2 cc. of 5 *N* hydrochloric acid. The mixture was transferred to a 30-cc. glass-stoppered flask with a little petroleum ether. The flask was shaken violently until the magnesium salts had dissolved. The hydrochloric acid was separated and the petroleum ether layer was washed with 2 cc. of water. The combined aqueous solutions were extracted with small portions of petroleum ether until a test for the sulfhydryl group was negative. To the combined petroleum ether layers in the 30-cc. flask were added 1 cc. of water containing 40 mg. of ethylene chlorohydrin and 1 cc. of 1 *N* sodium hydroxide.

The flask was shaken at room temperature for three to four hours until all the mercaptan had reacted. The aqueous layer was separated and the organic layer was washed with 2 cc. of water. The combined aqueous layers were extracted repeatedly with 1-cc. portions of petroleum ether. The combined petroleum ether layers were centrifuged and then were passed through an 8 × 50 mm. column of Permutit (Permutit according to Prof. Otto Folin, Eimer and Amend No. 901194). The product was then eluted with 25 cc. of dry benzene. The benzene solution was concentrated at room temperature under reduced pressure (water pump). The residue was distilled at 0° and 10^{-3} mm. onto a cold finger at −70°. The butyl β-hydroxyethyl sulfide* was transferred to a Carius tube with 2 cc. of petroleum ether, and 3 cc. of concentrated hydrochloric acid was added. The tube was sealed and shaken for twenty-four hours at 65°. The butyl β-chloroethyl sulfide* was then isolated in the manner described for the benzyl analog except that in this case the temperature was not raised above −40° (10^{-3} mm.) for removal of the last traces of petroleum ether. The product was usually analytically pure without distillation. The yield of butyl β-chloroethyl sulfide* was 33–46 mg. (44–62%).

Anal. Calcd. for $C_6H_{13}ClS$: Cl, 23.2. Found: Cl, 23.1.

Treatment of Insulin with Vesicants*.—Crystalline zinc insulin (500 mg.) was dissolved in 10 cc. of water by the slow addition of 4.5 cc. of 0.1 *N* hydrochloric acid. Five

cubic centimeters of 0.25 *M* borate buffer (*p*H 7.4) was added, followed by the dropwise addition with stirring of 5.5 cc. of 0.1 *N* sodium hydroxide. The final solution had a *p*H of 7.4.

Procedure A.—Samples (0.25–4.0 mg.) of benzyl-H* or butyl-H* were added to 5-cc. aliquots of the protein solution and the mixtures were stirred gently for twelve to twenty-four hours. The stirring was carried out in such a way as to disperse the vesicant in fine droplets throughout the protein solutions with minimal disturbance of the surface of the solutions. The reaction mixtures were then extracted with peroxide-free ether or were dialyzed against running water. Either treatment was found to remove practically all of the radiosulfur-containing material not precipitated by trichloroacetic acid. This procedure was used for Preparations 1–7 (Table I).

TABLE I

VESICANT*-TREATED PROTEINS

Prepn.	Protein	Vesicant*	Mg. ves. applied / 100 mg. protein	% Applied vesicant attached to protein	*M* Vesicant / *M* Protein[a]
1	Insulin	Benzyl-H*	0.25	55	0.3
2	Insulin	Benzyl-H*	2.0	55	2.4
3	Insulin	Benzyl-H*	4.0	50	4.3
4	Insulin	Benzyl-H*	4.0	50	4.3
5	Insulin	Benzyl-H*	2.0	55	2.4
6	Insulin	Butyl-H*	4.0	50	5.3
7	Insulin	Butyl-H*	0.25	55	0.4
8	Insulin	Butyl-H*	2.1	40	2.2
9	Insulin	Butyl-H*	1.6	50	2.1
10	Pepsin	Benzyl-H*	2.0	35	1.3
11	TMV	Benzyl-H*	2.0	35	1500
12	TMV	Butyl-H*	0.25[b]	40	260
13	TMV	Benzyl-H*	6.0	25	3200
14	TMV	Benzyl-H*	2.0	40	1700

[a] These ratios are calculated on the basis of the following molecular weights: insulin, 40,000; pepsin, 35,000; tobacco mosaic virus, 40,000,000. [b] In this experiment, the concentration of the protein solution was 40 mg./cc.; in all other experiments it was approximately 20 mg./cc.

Procedure B.—In this procedure, dilute, ethanolic solutions of the vesicant* (1.6–2.1 mg.) were added portionwise with stirring over a period of several hours to 5-cc. aliquots of the protein solution. The solutions were then dialyzed. Preparations 8 and 9 (Table I) were obtained in this manner.

Crystallization of Benzyl-H*-treated Insulin.—Preparation 5, after dialysis against running water for twenty-four hours, was transferred to a 25-cc. volumetric flask; 1 cc. of 0.1 *N* hydrochloric acid was added and the volume was adjusted to 25 cc. by addition of water. A 6-cc. aliquot of this solution (containing 24 mg. of vesicant*-treated insulin) was added to a solution consisting of 10.5 cc. of 0.67 *M* phosphate buffer (*p*H 7.15), 5 cc. of water and 0.84 cc. of 1 *N* hydrochloric acid. One cubic centimeter of zinc acetate solution (2.5 mg. of zinc), 2.1 cc. of acetone and 0.6 cc. of 1 *N* ammonium hydroxide were added. The *p*H of the solution was then adjusted to 6.2 with 0.1 *N* hydrochloric acid, and it was allowed to stand at 5° for twelve hours and at room temperature for twenty-four hours. The yield of crystalline material was 12 mg.; from a control experiment with untreated insulin, 16 mg. of crystals was obtained. The recoveries were thus 50 and 66%, respectively, of the total protein. The radioactivity of the vesicant*-treated protein indicated that it contained 1.5 benzyl-H* residues per molecule of insulin.

For recrystallization, 9 mg. of the crystals was dissolved in 1 cc. of 0.05 *N* hydrochloric acid. A small residue was removed by centrifugation. To the solution was added a slightly warmed mixture of 7.5 cc. of water, 7.5 cc. of phosphate buffer (*p*H 7.15) and 1.5 cc. of acetone. The mixture was centrifuged and the supernatant liquid was decanted. The *p*H of the supernatant was lowered from 7.1 to 6.1 by addition of 1 *N* hydrochloric acid and the solution was allowed to stand overnight at room temperature. A small amount of crystalline precipitate formed. This was collected, washed with water and then with absolute alcohol, and dried. The yield was approximately 2 mg. The radioactivity of this recrystallized sample indicated the presence of 1.1 benzyl-H* residues per molecule of insulin. The specific activity was thus 73% of that of the once-recrystallized material.

Treatment of Tobacco Mosaic Virus (TMV) with Vesicants*.—Preparations 11, 13 and 14 (Table I) were obtained by treatment of solutions of TMV (20 mg./cc.) in 0.05 *M* borate buffer (*p*H 7.5) with benzyl-H* (0.4–1.2 mg./cc. of protein solution) under conditions similar to Procedure A for insulin.

For Preparation 12, 1 g. of TMV was dissolved in 25 cc. of 0.25 *M* borate buffer (*p*H 7.5). To this solution, 1 cc. of an ethanolic solution of butyl-H* (2.5 mg.) was added in ten portions over a period of six hours with continuous stirring. The solution was then dialyzed against running water for twenty-four hours.

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Summary

A method is described for the synthesis of the mustard-type vesicants, benzyl β-chloroethyl sulfide (benzyl-H) and *n*-butyl β-chloroethyl sulfide (butyl-H), containing radiosulfur. Studies were made of the reaction of these radioactive vesicants with three highly purified proteins: insulin, pepsin and tobacco mosaic virus.

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