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

Chemical Studies on Vesicant-treated Proteins[1]

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In a preceding paper[4] we have described the treatment of certain crystalline proteins (insulin, pepsin and tobacco mosaic virus (TMV)) with benzyl β-chloroethyl sulfide (benzyl-H*) and *n*-butyl β-chloroethyl sulfide (butyl-H*) containing radiosulfur.[5] The present report describes studies made on these vesicant*-treated proteins with the object of determining the nature of the attachment of some of the substituting groups.

The work of other investigators[6] on the nature of the linkages formed by the action *in vivo* of mustard gas on tissues had shown that a large percentage of the vesicant residues could be split from these tissues by alkali or heat. There was also evidence from the work of another Laboratory[7] that a large proportion of the vesicant residues bound to certain proteins by vesicant treatment *in vitro* could be removed by the action of alkali.

It was of interest to study in more detail the alkali lability of the linkages formed by the treatment of proteins with vesicant. For this purpose we used the vesicant*-treated protein preparations described previously,[4] which contained relatively minute amounts of vesicant* residues and were in this respect comparable to the tissue preparations.

A study was made of the effect of *p*H and temperature upon the rate of liberation of vesicant residues from two preparations of butyl-H*-treated insulin. These preparations contained 2.2 and 2.1 vesicant* residues[8] per mole of protein. The results are summarized in Table I and Fig. 1. Treatment with relatively strong alkali (Table I) liberated in a few minutes about 65% of the radiosulfur-containing residues in a form soluble in trichloroacetic acid; continued treatment up to fifty-two hours liberated very little more radiosulfur-containing material. Treatment with alkali under milder conditions (Fig. 1) demonstrated that the rate of liberation of radiosulfur-containing residues from butyl-H*-treated insulin was a function of both the *p*H and the temperature. At *p*H 9.5 (30°) and 11 (0°, 30°), the radioactive material was liberated initially at a rapid rate which decreased to a fairly constant value.

TABLE I

EFFECT OF ALKALI ON BUTYL-H*-TREATED INSULIN

Time, hr.	Radiosulfur liberated, % 1.2 *N* NaOH at 0°	0.1 *N* NaOH at 27°
0.25	65	57
0.75	64	67
3.00	67	71
5.00	68	71
52.00	75	75

The nature of the radiosulfur-containing residues split from vesicant*-treated insulin by alkali was also investigated. When the trichloroacetic acid solutions of the alkali-labile vesicant* residues from the above experiments were extracted with petroleum ether, the major portion of the radiosulfur-containing material was found to be in the organic solvent. These experiments were not carried further, but in similar experiments on benzyl-H*-treated insulin it was shown that, after treatment of the protein preparation with alkali, the portion of the radioactive material which could be extracted into ether consisted almost entirely of benzyl β-hydroxyethyl sulfide*. Identification of the compound* was accomplished through application of the "washing-out" technique. A known amount of non-radioactive benzyl β-hydroxyethyl sulfide was added to the ether extract as a carrier and the solution was treated with α-naphthyl isocyanate. The resulting urethan derivative was purified by successive recrystallizations to constant radioactivity. From the amount of radioactivity in this purified derivative, the amount of benzyl β-hydroxyethyl sulfide* present in the ether extract could be calculated.

A considerable proportion (30–50%) of the radiosulfur-containing residues could be split in an ether-extractable form from vesicant*-treated insulin by heating at 150° in neutral solution. Here again evidence was obtained that the residues consisted almost entirely of the hydroxy compounds ($RS^*CH_2CH_2OH$, R = C_4H_9— or $C_6H_5CH_2$—).

With benzyl-H*-treated pepsin (Preparation 10[4]), 95% of the radiosulfur-containing material could be extracted into ether after autoclaving the pepsin preparation at 150° for five hours, while

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(4) Wood, Rachele, Stevens, Carpenter and du Vigneaud, THIS JOURNAL, **70**, 2547 (1948).

(5) An asterisk (*) is used to indicate the presence of radiosulfur (S^{35} of 87-day half-life) in a compound.

(6) Ball, *et al.*, Informal Progress Report to NDRC Section B4C, August 19, 1942; Moritz, Henriques, *et al.*, Informal Progress Report to Division 9:5:1, NDRC, November 10, 1943.

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

(8) 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.

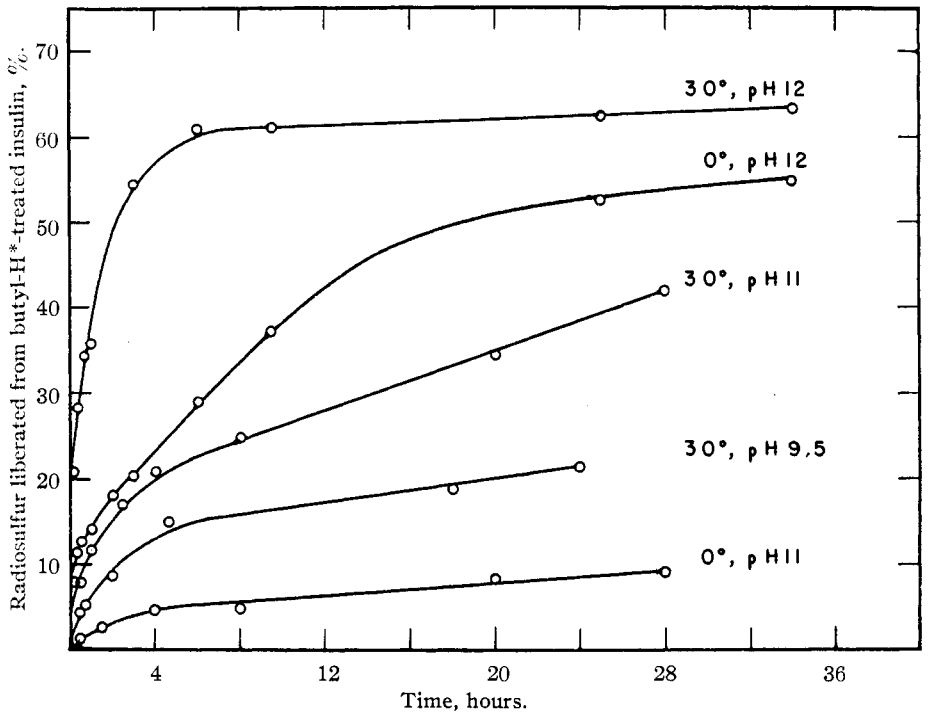

Fig. 1.—The liberation of radiosulfur-containing material, in a form not precipitated by trichloroacetic acid, from butyl-H*-treated insulin at various temperatures and *p*H levels.

70% of the vesicant* residues was liberated by treatment at *p*H 11 for three days.

We were interested in investigating vesicant*-treated TMV to determine what proportion, if any, of the vesicant residues was attached to the nucleic acid moiety. The nucleic acid which was separated from butyl-H*-treated TMV (Preparation 12[4]) was found to contain 5% of the radioactivity of the original TMV preparation. When it is recalled that TMV contains only 6% nucleic acid, it can be seen that the nucleic acid has "competed" quite effectively with the protein for the vesicant. In addition, it was noted that in the case of the nucleic acid moiety a smaller proportion of the vesicant* residues was attached through alkali-labile linkages than in the case of the protein moiety. Alkali treatment of the nucleic acid moiety liberated about 33% of the vesicant* residues, while similar treatment of the protein moiety resulted in the liberation of 86% of the vesicant* residues.

Experimental

Cleavage of Vesicant* Residues from Butyl-H*-treated Insulin Preparations by Treatment with Alkali.—In the general procedure employed, 1 volume of the appropriate buffer was mixed with 1 or in some cases 2 volumes of a dialyzed solution of a butyl-H*-treated insulin preparation.[4] The final concentration of insulin varied from 4 to 12 mg. per cc. The buffer and insulin solutions were brought to the same temperature before being mixed, and this temperature was maintained throughout the reaction. At noted time intervals after mixing, 1 cc. of the reaction mixture was withdrawn and delivered into 1 cc. of a 10% solution of trichloroacetic acid in 0.06 *N* hydrochloric acid. This served to stop the reaction and to precipitate the protein. (Control experiments had shown that treatment with trichloroacetic acid precipitated all the radiosulfur-containing material from the original vesicant*-treated insulin preparations.) The major portion of the protein precipitate was removed by centrifugation and the remainder by passing the supernatant liquid through a gravity micro filter. Analyses for radiosulfur[9] were carried out on the filtrate.

The rate of liberation of radiosulfur-containing material from Preparation 8[4] by treatment with alkali was determined under the following conditions: 1.2 *N* sodium hydroxide at 0° (Table I); 0.1 *N* sodium hydroxide at 27° (Table I); and 0.1 *M* borate buffer (*p*H 9.5) at 30° (Fig. 1). Similar studies were made on Preparation 9[4] in 0.1 *M* phosphate buffer (*p*H 12) at 0° and 30°, and in 0.1 *M* borate buffer (*p*H 11) at 0° and 30° (Fig. 1). The final *p*H of the reaction mixture was slightly lower than that of the buffer in some cases. The greatest decrease noted was that of 0.4 *p*H unit in the case of the *p*H 11 buffer.

Identification of Vesicant* Residues Liberated from Vesicant*-treated Insulin.—A solution of benzyl-H*-treated insulin (Preparation 4[4]; 18 mg.) in 1.8 cc. of 0.1 *N* sodium hydroxide was allowed to stand for seventy-two hours and was then extracted with ether. The ether layer contained 57% of the total radioactivity of the original benzyl-H*-treated insulin. To the ether extract were added 370 mg. of non-radioactive benzyl β-hydroxyethyl sulfide and 0.2 cc. of α-naphthyl isocyanate. The solution was evaporated to dryness and the residue was heated on a water-bath for five minutes. The resulting urethan derivative (β-(benzylmercapto)-ethyl 1-naphthalenecarbamate*) was recrystallized from hexane to constant radioactivity and melting point (86°).[10]

Anal. Calcd. for $C_{20}H_{19}NO_2S$: S, 9.50. Found: S, 9.96.

(9) The radioactivity estimations were carried out essentially by the method described by Henriques, Kistiakowsky, Margnetti and Schneider [*Ind. Eng. Chem., Anal. Ed.*, **18**, 349 (1946)].

(10) All melting points are corrected capillary melting points.

Radioactivity determinations on the purified derivative indicated that at least 90% of the ether-extractable radioactive material split from benzyl-H*-treated insulin by alkali was benzyl β-hydroxyethyl sulfide*.

Preparation 4[4] of benzyl-H*-treated insulin was treated with alkali under milder conditions (borate buffer; *p*H 9) for twenty-four hours. After extraction with ether, 17% of the initial radioactivity was found in the ether layer, and 95% of the radioactivity in the ether layer was shown to be due to the presence of benzyl β-hydroxyethyl sulfide*.

An aqueous solution of butyl-H*-treated insulin (Preparation 6[4]; 10 mg.) was heated at *p*H 6.5 in a sealed tube at 150° for five hours. The resulting solution was extracted with ether. The ether extract contained 30% of the radioactivity of the original protein solution. Nonradioactive *n*-butyl β-hydroxyethyl sulfide (92 mg.) and α-naphthyl isocyanate (0.1 cc.) were added to the ether solution. The mixture was evaporated to a sirup, heated on a steam-bath for a few minutes and extracted with hot hexane. When the hexane solution was cooled, the β-(*n*-butylmercapto)-ethyl 1-naphthalenecarbamate* crystallized. It was recrystallized to constant radioactivity. The product had a melting point of 74.5–75.5°; there was no depression in melting point upon admixture with a sample of the urethan prepared from authentic *n*-butyl β-hydroxyethyl sulfide.

Anal. Calcd. for $C_{17}H_{21}NO_2S$: S, 10.57. Found: S, 11.05.

The results of the radioactivity determinations indicated that 85% of the ether-extractable radioactive material split from butyl-H*-treated insulin by heat was *n*-butyl β-hydroxyethyl sulfide*.

Heating of benzyl-H*-treated insulin (Preparation 4[4]) under the same conditions resulted in the liberation of 53% of the radiosulfur-containing material in an ether-extractable form. In this case, 70% of the radioactivity in the ether extract could be accounted for as benzyl β-hydroxyethyl sulfide*.

Separation of Protein and Nucleic Acid Moieties from Vesicant*-treated Tobacco Mosaic Virus.—The procedure of Cohen and Stanley[11] was used to cleave the virus into nucleic acid and protein. An aliquot of a solution of benzyl-H*-treated TMV (Preparation 11[4]), which contained 1.25 mg. of virus per cc., was adjusted to *p*H 5.5 and made 0.1 *M* with respect to sodium chloride by the addition of salt. The solution was boiled for two minutes and cooled overnight. The precipitate of protein was collected, washed and analyzed for radiosulfur. It contained 84% of the radioactivity of the original benzyl-H*-treated TMV preparation. Control experiments indicated that protein separated in this manner contained less than 0.25% nucleic acid.

A cleavage experiment was also carried out using butyl-H*-treated TMV (Preparation 12[4]). The protein moiety was separated as a precipitate by the heat treatment described above. The supernatant liquid was made acid to congo red paper and the nucleic acid was precipitated by the addition of an equal volume of ethanol. The protein precipitate contained approximately 75% of the radioactivity originally present in the butyl-H*-treated TMV preparation, while the nucleic acid contained approximately 5% of the radioactivity of the original TMV preparation. The nucleic acid was dissolved as the ammonium salt, the solution was acidified, and the nucleic acid was reprecipitated with ethanol. The specific radioactivity of the nucleic acid was unchanged by this process.

The reprecipitated nucleic acid was treated with 5% sodium hydroxide for two hours at 0°, the solution was acidified and the nucleic acid was again precipitated with ethanol. The alkali-treated nucleic acid had a specific radioactivity equivalent to two-thirds that of the original nucleic acid, thus indicating that 33% of the vesicant* residues was attached to the nucleic acid moiety through alkali-labile linkages. The protein moiety was treated with 5% sodium hydroxide for two hours at 0° and then precipitated with trichloroacetic acid. Analyses for radiosulfur on the precipitate and the supernatant liquid indicated that 86% of the vesicant* residues attached to the protein moiety had been liberated by the action of alkali.

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Summary

A study was made of the cleavage by alkali of the linkages between protein and vesicant residues in preparations obtained by the treatment of insulin, pepsin and tobacco mosaic virus with benzyl β-chloroethyl sulfide (benzyl-H) or *n*-butyl β-chloroethyl sulfide (butyl-H) containing radiosulfur. The extent of liberation of vesicant residues varied with the different proteins; in all cases, a certain fraction of the vesicant residues was not cleaved by the alkali under the conditions studied.

A more detailed investigation of the action of alkali on vesicant-treated insulin showed that the rate of liberation of vesicant residues was dependent upon the temperature and *p*H of treatment. Heating of vesicant-treated insulin at 150° in neutral solution also resulted in the liberation of vesicant residues.

By application of the "washing-out" technique to the radiosulfur-containing material cleaved from butyl-H- or benzyl-H-treated insulin, the cleavage product was demonstrated to consist mainly of the corresponding alkyl β-hydroxyethyl sulfide.

Studies on vesicant-treated tobacco mosaic virus indicated that vesicant residues were attached to both the nucleic acid and protein moieties of the virus.

(11) Cohen and Stanley, *J. Biol. Chem.*, **144**, 589 (1942).

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