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

Studies on Acid Hydrolysates of Vesicant-treated Insulin[1]

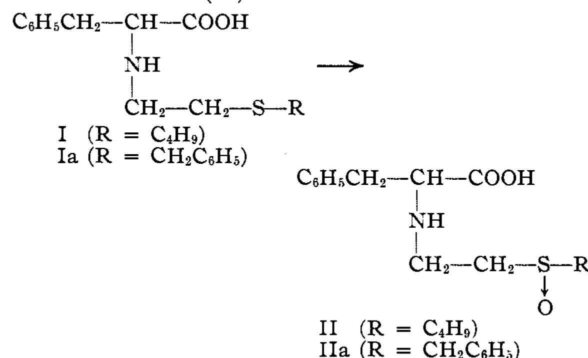
BY CARL M. STEVENS,[2] JOHN L. WOOD,[3] JULIAN R. RACHELE AND VINCENT DU VIGNEAUD

The treatment of insulin with benzyl β-chloroethyl sulfide (benzyl-H*) and *n*-butyl β-chloroethyl sulfide (butyl-H*) containing radiosulfur[4] has already been described.[5] Studies on these vesicant*-treated insulin preparations, containing an amount of vesicant* residues comparable to that found in tissues at the site of a burn, have shown that approximately 30% of the vesicant* residues was bound to the protein through linkages that were relatively stable to alkali.[6]

The experiments discussed in the present communication were designed to determine whether the vesicant* had reacted with the free amino groups in the insulin molecule. If such a reaction had occurred, the resultant linkages would be expected to be not only relatively stable to alkali but also stable to acid. The presence of such linkages in the protein might then be detected by the isolation of N-substituted vesicant* derivatives of amino acids from the acid hydrolysates of vesicant*-treated insulin.

A preparation of butyl-H*-treated insulin which contained approximately 5 vesicant* residues[7] per molecule of insulin was hydrolyzed with acid under various conditions. The "washing-out" technique was used to test for the presence of vesicant* derivatives of amino acids in the hydrolysates. The non-radioactive vesicant derivative of a given amino acid[8] was added to the hydrolysate to serve as a "carrier." The reference compound was then isolated from the mixture and examined for radiosulfur. The absence of radiosulfur in the isolated material indicated that the compound in question was not present in detectable amounts in the original hydrolysate. The presence of radiosulfur in the isolated material was not in itself regarded as sufficient evidence for the existence of the radioactive form of the reference compound in the hydrolysate. However, if the specific radioactivity of the isolated derivative reached a value which remained constant through successive recrystallizations and through chemical conversion of the compound to a suitable derivative, it was concluded that the hydrolysate contained the vesicant* derivative of the amino acid in question.

Since Jensen and Evans[9] had obtained evidence that the amino group of some of the phenylalanine moieties in insulin was chemically reactive,[10] we examined a sulfuric acid hydrolysate of butyl-H*-treated insulin for the presence of N-(β-butylmercapto)-ethyl-phenylalanine* (I). The non-radioactive form of I was dissolved in the hydrolysate and the N-(β-butylmercapto)-ethylphenylalanine was isolated from the mixture and recrystallized. It was found to contain radiosulfur; the content of radiosulfur did not change through several additional recrystallizations or through conversion to the sulfoxide (II).



The amount of radiosulfur in the amino acid derivative was equivalent to 5% of the total radiosulfur of the original butyl-H*-treated insulin. When the same preparation of butyl-H*-treated insulin was hydrolyzed with a mixture of hydrochloric and formic acids, the radiosulfur content of the isolated and purified compound I was equivalent to 3% of the total radiosulfur of the original protein preparation. This observed discrepancy in values from the two types of hydrolyses casts doubt upon the quantitative aspects of the results. Accordingly they are interpreted only in a qualitative manner to indicate the presence of N-(β-butylmercapto)-ethyl-phenylalanine* (I) in the acid hydrolysate of butyl-H*-treated insulin.

It is conceivable that the phenylalanine derivative I was formed during the hydrolysis procedure and was not actually present in the original vesicant*-treated insulin. If this were the case, one would expect to be able to detect in these insulin hydrolysates vesicant* derivatives of other amino acids known to be present in insulin. Accordingly,

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(4) An asterisk (*) indicates the presence of radiosulfur (S^{35} of 87-day half-life) in a compound.

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

(6) Carpenter, Wood, Stevens and du Vigneaud, *ibid.*, **70**, 2551 (1948).

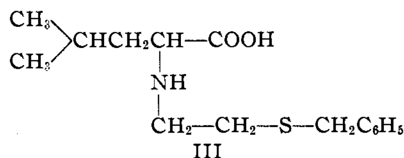
(7) This value represents a statistical average of the number of vesicant residues per molecule in the particular preparation, and is not intended to indicate that every molecule contains this number of residues.

(8) du Vigneaud, Stevens, McDuffie, Wood and McKennis, *ibid.*, **70**, 1620 (1948).

(9) Jensen and Evans, *J. Biol. Chem.*, **108**, 1 (1935).

(10) Sanger [*Biochem. J.*, **39**, 507 (1945)] has since reported data indicating that there are free amino groups of both phenylalanine and glycine in the insulin molecule.

a hydrolysate of benzyl-H*-treated insulin was tested for the presence of N-(β-benzylmercapto)-ethyl-leucine* (III) by the "washing-out" tech-



nique. Despite the fact that insulin contains approximately 30% leucine, none of the leucine derivative III could be detected in the hydrolysate.

The possibility of the formation of new vesicant* molecules and their reaction with released amino acids or protein fragments during the hydrolysis of the insulin preparation was also investigated. When mixtures of benzyl-H* and phenylalanine or N-benzoyl-phenylalanine were treated with acid under conditions used for protein hydrolysis, no detectable amount of N-(β-benzylmercapto)-ethyl-phenylalanine* (Ia) was formed. Moreover, after a mixture of insulin and butyl-H* had been hydrolyzed together, the radioactive phenylalanine derivative I could not be detected in the hydrolysate.

It was also conceivable that there might be some transfer of the radiosulfur onto the "carrier" compound from vesicant* decomposition products in the hydrolysate of vesicant*-treated insulin. A mixture of non-radioactive N-(β-benzylmercapto)-ethyl-DL-phenylalanine (Ia) and benzyl-H* was treated with acid under the conditions of protein hydrolysis. The phenylalanine derivative Ia was isolated from the mixture and was found to contain radiosulfur. However, the radiosulfur was lost upon conversion to the sulfoxide (IIa).

Because of the interruption of this work by more pressing wartime studies, the investigations were not carried further. However, the data obtained strongly indicate that when a vesicant* derivative of an amino acid is found in a hydrolysate of vesicant*-treated insulin, the derivative was originally present in the protein preparation before hydrolysis and was not formed by subsequent reactions during the hydrolysis procedure. On this basis it may be concluded that when insulin was treated with a minute amount of vesicant at *p*H 7.4, a fraction of the vesicant became attached to the amino group of phenylalanine.

Experimental

Detection of N-(β-Butylmercapto)-ethyl-phenylalanine* (I) in Hydrolysates of Butyl-H*-treated Insulin.—One hundred milligrams of crystalline insulin was dissolved in 5 cc. of *p*H 7.4 borate buffer and the solution was stirred gently for twenty-four hours with approximately 4 mg. of butyl-H*.[5] The solution was extracted three times with ether, and the protein was precipitated by the addition of 2 volumes of 0.3 *N* trichloroacetic acid, ground with several portions of dry ether and dried *in vacuo*. A determination of radiosulfur[11] on this material showed it to contain 5.3 vesicant* residues per molecule of insulin (on the basis of a molecular weight of 40,000 for insulin).

In one experiment, 9.5 mg. of the butyl-H*-treated insulin was hydrolyzed by heating it in a sealed tube with 1 cc. of concentrated sulfuric acid and 4 cc. of water at 95–105° for four days. The hydrolysate was diluted to 5.3 cc. To 2 cc. of the hydrolysate was added 54 mg. of N-(β-butylmercapto)-ethyl-DL-phenylalanine[8] (I) and enough acetic acid to give a clear solution. Addition of 50 cc. of water caused the derivative to separate from the solution. The material was recrystallized and then analysed for radiosulfur. On successive recrystallizations of the material the radiosulfur content remained constant and was equivalent to 5% of the total radiosulfur originally attached to the insulin. Repetition of the experiment using 3 cc. of the hydrolysate and 96 mg. of the phenylalanine derivative I gave a similar result. A sample of the isolated radiosulfur-containing derivative I was then converted to the sulfoxide II by the procedure described below. The radiosulfur content of the sulfoxide was equivalent to that of the starting derivative I.

A second sample of the same butyl-H-treated insulin preparation (5 mg.) was heated with 1 cc. of a 1:1 mixture of hydrochloric and formic acids at 100° for six days. The solution was evaporated to dryness to remove the acids. The residue was dissolved in 5 cc. of water and 100 mg. of the phenylalanine derivative I was added. The solution was made 30% in acetic acid and was heated to boiling. After centrifugation to remove a small amount of insoluble material, the supernatant liquid was diluted with water. The derivative was recovered and recrystallized from dilute acetic acid. The radiosulfur content of the derivative was equivalent to 3% of the original radiosulfur in the protein preparation. The value did not change after four recrystallizations.

Test for N-(β-Benzylmercapto)-ethyl-leucine* (III) in a Hydrolysate of Benzyl-H*-treated Insulin.—One hundred milligrams of crystalline insulin was treated with 4 mg. of benzyl-H*[5] and then precipitated with trichloroacetic acid under conditions similar to those described above for the preparation of butyl-H*-treated insulin. Analysis for radiosulfur showed that 4.1 vesicant* molecules had combined with 1 molecule of insulin.

A portion of this preparation (20.4 mg.) was allowed to stand in 2 cc. of 0.1 *N* sodium hydroxide for thirty-six hours. The vesicant* residues that had been cleaved by the alkali were extracted into ether. Analyses for radiosulfur on the aqueous layer indicated that 1.4 vesicant* residues were attached to 1 molecule of insulin through linkages stable to this alkali treatment. Ten cubic centimeters of a 1:1 mixture of hydrochloric and formic acids was added to the aqueous layer and the resulting solution was heated at 100–110° for fifty hours. Aliquots of this hydrolysate were used for the experiments below.

To 1 cc. of the hydrolysate were added 79.5 mg. of N-(β-benzylmercapto)-ethyl-DL-leucine[8] (III), 1 cc. of glacial acetic acid, 1 cc. of hydrochloric acid and 3 cc. of water. The resulting solution was heated on the steam-bath for one and one-half hours, and then concentrated to dryness. The residue was recrystallized twice from 33% aqueous acetic acid. The resulting product did not contain a detectable amount of radiosulfur. From the sensitivity of the measurement, it was calculated that the hydrolysate contained less than 0.5% of its radiosulfur in the form of the leucine derivative III.

On the other hand, when 82 mg. of the N-(β-benzylmercapto)-ethyl-DL-phenylalanine[8] (Ia) was added in place of the leucine derivative III and the mixture was treated in exactly the same manner as that described above for the leucine derivative III, the purified phenylalanine derivative Ia contained an amount of radiosulfur equivalent to approximately 5% of that originally present in the hydrolysate.

Preparation of Sulfoxides.—N-(β-Butylmercapto)-ethyl-DL-phenylalanine (I) (100 mg.) was dissolved in 5 cc. of glacial acetic acid. Then 0.4 cc. of a 3% solution of hydrogen peroxide in acetic acid was added and the flask was warmed on the water-bath for one hour. The

(11) The analyses for radiosulfur were carried out essentially by the method of Henriques, Kistiakowsky, Margnetti and Schneider [*Ind. Eng. Chem., Anal. Ed.*, **18**, 349 (1946)].

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.

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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]

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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).