The Reaction of Insulin with *N*-Acetyl-DL-homocysteine Thiolactone: Some Chemical and Biological Properties of the Products*

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Insulin has been thiolated by reaction with acetylhomocysteine thiolactone and the product formed has been analyzed by chromatography on DEAE-cellulose. The thiolated derivative has about 2 moles of acetylhomocysteine per mole of insulin. Analysis for free sulfhydryl or additional sulfhydryl plus disulfide groups shows that the product contains about 2 moles of extra sulfhydryl groups. The biological activity of thiolated insulin is much less than that of unmodified insulin. Carboxymethylation of the acetylhomocysteine residues in thiolated insulin does not restore the biological activity, whereas substitution of the amino groups of insulin with methionyl residues gives a product which retains most of the original activity. When *S*-carboxymethylhomocysteine derivatives of insulin are prepared under conditions which exclude disulfide interchange, the products are also biologically inactive.

In order to determine which groups are necessary for the expression of biological activity and also to study the effect of various modifications on the immunological and physical properties of the molecule, insulin has been treated in many ways. Some of the reagents used, such as the carbamino anhydride of leucine, react with amino groups (Fraenkel-Conrat, 1953). It has been found that the modification of the amino groups of insulin by acylation does not alter its biological activity and Fraenkel-Conrat *et al.* (1950) have concluded that the free amino groups are not necessary for its biological activity.

Benesch and Benesch (1958) have prepared acylated gelatin derivatives containing extra thiol groups by reacting the protein with *N*-acetyl-DL-homocysteine thiolactone (AHT).[1] Abadi and Wilcox (1960) have prepared monoacylated derivative of α-chymotrypsinogen using the same regent. The thiolation of ribonuclease with AHT and the enzymatic activity of the thiolated product have been investigated by White and Sandoval (1962). They also observed that disulfide interchange occurred as a secondary reaction to thiolation and an average interchange of one disulfide bond in the protein did not affect its enzymatic activity.

It appeared of interest to determine what effect thiolation has on the biological activity of insulin. The introduction of new sulfhydryl groups provides a means of anchoring other groups to the protein by carboxymethylation and other methods. We have also employed the carbamino anhydride of methionine for substituting the free amino groups of insulin.

MATERIALS

Crystalline insulin was kindly supplied by Eli Lilly and Co. Zinc was removed from the preparation by dialyzing it against several changes of 10^{-3} M EDTA adjusted to *p*H 7.6, followed by dialysis against water and lyophilization.

Other materials were obtained from the following sources: *N*-Acetyl-DL-homocysteine thiolactone from the California Foundation for Biochemical Research, Los Angeles; Dowex AC-2-X10 (200–400 mesh), a purified product from Bio-Rad Laboratories, Richmond, Calif.; DEAE-cellulose (Selectacel) (0.82 meq/g) from the Brown Co., Berlin, N. H.; the detergent BRIJ-35 from the Atlas Powder Co., Wilmington, Del.; *N*-ethylmaleimide from the Schwarz Laboratories, Inc., Mt. Vernon, N. Y.; crystalline iodoacetamide from the Sigma Chemical Co., St. Louis, Mo. Iodoacetic acid (Eastman Organic Chemicals, Rochester, N. Y.) was recrystallized from aqueous ethanol before use.

METHODS

Preparation of Insulin Derivatives.—REACTION OF INSULIN WITH THE CARBAMINO ANHYDRIDE OF METHIONINE.—[^{35}S]Methionine (23.5 mg) was suspended in 5 ml of dry dioxane and treated with phosgene gas at 80° for 15 minutes. All the amino acid went into solution. The solvent was distilled out of the reaction vessel, and more solvent was added and removed twice to eliminate the excess phosgene. The product was dissolved in 4 ml of dry dioxane. Insulin (200 mg) dissolved in 50 ml of 5% bicarbonate was treated with 1 ml of the dioxane solution of the carbamino anhydride with rapid stirring.

A second portion of insulin (20 mg) in 25 ml of bicarbonate was treated with 1 ml of the reagent. The modified insulin preparations were subjected to exhaustive dialysis in the cold and lyophilized, and the methionine bound was calculated from the radioactivity of the preparations. One preparation had 0.89 mole of methionine per mole of insulin (mw 5740), the second had 2.5 moles per mole.

REACTION OF INSULIN WITH N-ACETYL-DL-HOMOCYSTEINE THIOLACTONE (AHT).—The reaction of AHT with insulin was carried out at room temperature (24–25°) at constant *p*H (7.8–8.0) in a *p*H-stat, with efficient stirring under an atmosphere of nitrogen. All aqueous solutions used were boiled and saturated with nitrogen. One g (0.75 mmole) of zinc-free insulin was suspended in 30 ml of distilled water in a covered vessel. The *p*H was adjusted to 8.0 with 1 N KOH, and 20 mg of EDTA was added, followed by 180 mg (1.1 mmoles) of AHT. The *p*H was maintained at the desired level by the addition of 0.1 N KOH.

After 24 hours the reaction mixture was added to 14 volumes of acetone; the precipitate was dissolved in oxygen-free water and dialyzed against a large volume of 10^{-4} M EDTA twice, then against water. The prod-

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[1] Abbreviations used in this work: AHT, *N*-acetyl-DL-homocysteine thiolactone; AHCySH, acetylhomocysteine; NEM, *N*-ethylmaleimide.

uct obtained after removal of the water by lyophilization will be designated I L24. When the extent of thiolation (reaction with AHT) was being studied, 1-ml samples were withdrawn from the reaction mixture at 3, 6, and 9-hour intervals. After dialysis as indicated above, the homocysteine content of each sample was determined.

Estimation of Homocysteine Residues in Thiolated Insulin Derivatives.—The number of moles of acetylhomocysteine residues (AHCySH) per mole of insulin was determined by a procedure similar to that described by Abadi and Wilcox (1960). Insulin (20–25 mg) was oxidized with 7 ml of performic acid for 4 hours at −4° as described by Hirs (1956). An equal volume of cold water was then added and the formic acid was removed on a rotary evaporator at room temperature. The oxidized protein was hydrolyzed with constant-boiling HCl for 18 hours, the acid was removed, and the hydrolysate was diluted to a known volume. Aliquots (1–2 ml) containing 1–2 μmoles of homocysteic acid were applied to a Dowex 2 column equilibrated with 0.01 M chloroacetic acid. The amino acid in 1-ml fractions from the column was determined by the colorimetric method of Moore and Stein (1954), comparing the colors against standard solutions of cysteic acid in 0.1 M chloroacetic acid. Under these conditions the separation of cysteic and homocysteic acids was complete.

In calculating the results of the analyses, loss of homocysteic acid equal to the loss of cysteic acid has been assumed (Abadi and Wilcox, 1960).

Fractionation of Thiolated Insulin Derivatives.—Column chromatography on DEAE-cellulose as described by O'Donnell and Thompson (1960) was used to fractionate the products obtained by the action of AHT on insulin. Jacketed columns were maintained at 3°. For analytical work using samples of 5–6 mg, columns 0.9 cm in diameter and 16 cm long were run at a flow rate of 4 ml/hour; for preparative work with samples of 90–100 mg, columns 3.0 cm in diameter and 21 cm long were run at a flow rate of 20 ml/hour. The columns were packed with purified DEAE-cellulose (O'Donnell and Thompson, 1960) and 0.005 M Tris–0.001 M EDTA–0.3 M KCl buffer at *p*H 7.8 under 10 psi nitrogen pressure. The buffer for elution was the same, except that it contained 0.25% detergent (BRIJ-35).

The protein was dissolved in aqueous solution at *p*H 7.8. After application of the sample to the column, buffer was run through at constant pressure and fractions were collected, 0.5 ml size when analysis was being performed or 5 ml when preparations were being made.

The protein concentration in the effluent was determined by the method of Moore and Stein (1954) or by the absorption of light at 276 mμ (Chrambach and Carpenter, 1960). Fractions from the columns were pooled as desired and were recovered by dialysis and lyophilization or precipitation with 18 volumes of acetone at *p*H 2 over a period of 14 hours. Fractions thus obtained were rechromatographed on small (analytical) columns.

Assay for Sulfhydryl Groups Using N-Ethylmaleimide (NEM).—The free SH groups in the thiolated insulin derivatives were estimated by the method of Alexander (1958) with slight modifications using 2 μmoles of protein derivatives and 10 μmoles of NEM at room temperature and *p*H 7. The reaction was carried out in phosphate buffer 8 M in urea in order to increase the solubility of the derivatives.

Assay for Sulfhydryl Disulfide by the Disulfide-Interchange Reaction.—The total SH + (–S–S–/2) in the thiolated insulin derivatives was measured as described by Glazer and Smith (1961), using reaction times of 98 and 180 hours, although after 98 hours the mono-DNP-cystine concentration in the reaction mixture did not change by more than 2%.

Carboxymethylation of Thiolated Insulin.—Ten μmoles of insulin derivative was reacted with 100 μmoles of iodoacetic acid at constant *p*H (8.5 ± 0.1) in a *p*H-stat. After 20 minutes at 20°, the reaction mixture was added to 14 volumes of acetone and 14 hours were allowed for the precipitation of insulin derivative. The precipitate was dissolved in water, dialyzed against water to remove acetone, and lyophilized.

Thiolation of Insulin in Presence of Iodoacetate or Iodoacetamide.—Four hundred mg (80 μmoles) of insulin was reacted with either 40 mg (250 μmoles) of AHT and 60 mg iodoacetic acid (320 μmoles) or 60 mg AHT (380 μmoles) and 80 mg iodoacetamide (420 μmoles) at *p*H 8, in a *p*H-stat for 24 hours. Twenty-five mg of sodium EDTA was also present in the reaction mixture. After the reaction, the protein was precipitated with acetone and dissolved in water. The product was recovered by dialysis and lyophilization and the *S*-carboxymethylhomocysteine content of the protein was estimated.

Estimation of S-Carboxymethylhomocysteine in Carboxymethylated-thiolated Insulin.—Carboxymethylated-thiolated insulin, 6 mg, was hydrolyzed with 6 N HCl for 20 hours in a sealed tube. The HCl was removed in a vacuum desiccator over anhydrous $CaCl_2$ and NaOH. Residual HCl was removed by repeated evaporation after additions of small volumes of water. The amino acid mixture was dissolved in 0.5 M acetic acid and applied to a column of Dowex 1 equilibrated with 0.5 M acetic acid (Hirs, *et al.*, 1954). Two-ml fractions were collected, and after 180 ml of 0.5 M acetic acid had passed through the column development was continued with 1.0 M acetic acid. The amino acid in 1 ml of each fraction was determined by the ninhydrin analysis of Rosen (1957). The elution pattern is shown in Figure 4.

In recovery experiments, 29 mg of crystalline insulin and 10 μmoles each of *S*-carboxymethylhomocysteine and *S*-carboxymethylcysteine were hydrolyzed as described. The hydrolysate was diluted up to 5 ml in 0.5 M acetic acid and 1-ml aliquots were applied to Dowex 1 columns for amino acid analysis.

Biological Assay.—An approximate assay of the insulin derivatives as compared with that of crystalline bovine insulin was carried out by the mouse-convulsion method (British Pharmacopoeia, 1948). Additional assays were made by Eli Lilly and Co., using the same method or the rabbit method (blood sugar).

Thiolated insulin and the carboxymethylated derivatives of thiolated insulin were also tested by the immunological method involving the displacement of [^{131}I]insulin bound to guinea pig antiserum (Grodsky *et al.*, 1959).

Results and Discussion

Reaction of AHT with Insulin.—The reaction was carried out using a ratio of protein to AHT of 1:6 on a molar basis. Under these conditions it was found that thiolation gradually increased with time (Table I) in agreement with the results of Abadi and Wilcox (1960), who used α-chymotrypsinogen. When the reaction was carried out in the absence of EDTA and in 0.1 M KCl, considerable precipitation occurred. This was probably due to intermolecular disulfide bonding (Benesch and Benesch, 1958).

Fractionation of Thiolated Insulin (I L24).—Analysis

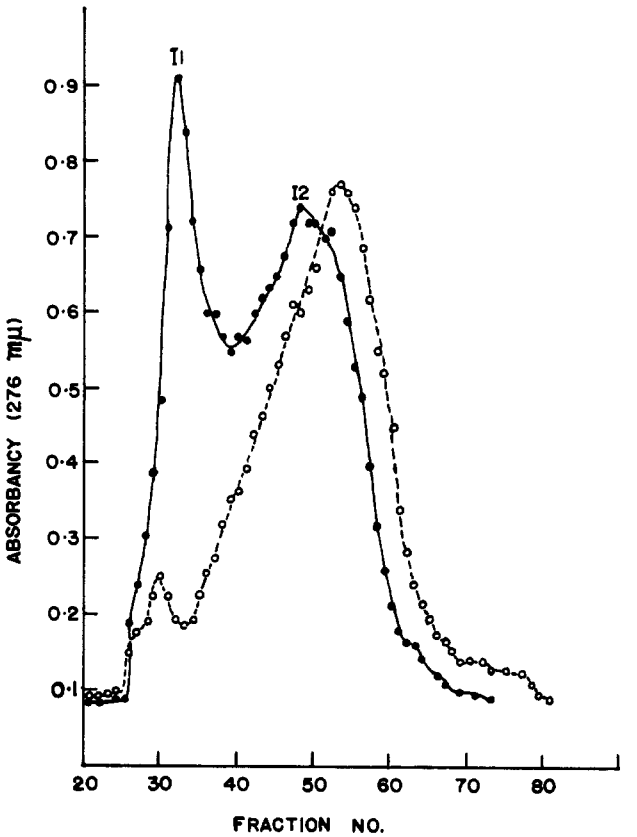

FIG. 1.—Chromatography of I L24 on a preparative DEAE-cellulose column at 3°. The eluting buffer was 0.3 M KCl–0.005 M Tris–0.001 M EDTA, *p*H 7.8. Other characteristics of the column are described in the text. The fractions were assayed by measuring the absorbancy at 276 mμ. O, native insulin; ●, I L24.

TABLE I

EXTENT OF THIOLATION OF INSULIN[a]

Time (hr)	Moles of AHCySH Residue per Mole Protein	Time (hr)	Moles of AHCySH Residue per Mole Protein
3	Trace	9	0.8
6	0.5	24	1.7

[a] Conditions: *p*H 8.0; insulin concn, 1.75×10^{-4} M; AHT concn, 1.1×10^{-3} M.

of the labeled insulin in DEAE-cellulose columns using a single buffer (KCl-Tris at *p*H 7.8) for development showed the presence of two peaks designated I 1 and I 2 (Fig. 1). It is seen that fraction I 1, which represents approximately 40% of the total protein, has an R_F value less than native insulin, whereas fraction I 2 has an R_F about the same as native insulin. The same elution pattern and R_F values was observed with another batch of I L24.

When the *p*H of the buffer was increased, the protein was held on the column longer and eventually emerged as a broad peak. Similar results were obtained when the ionic strength of the buffer was decreased or the temperature of the column was increased. These effects were also observed by O'Donnell and Thompson (1960).

When fraction I 1 was rechromatographed on an analytical column, the protein again emerged as a single peak with the same R_F as before; but when fraction I 2 was rechromatographed, two peaks appeared (Figs. 2 and 3). The minor fraction, 10%, had an R_F value

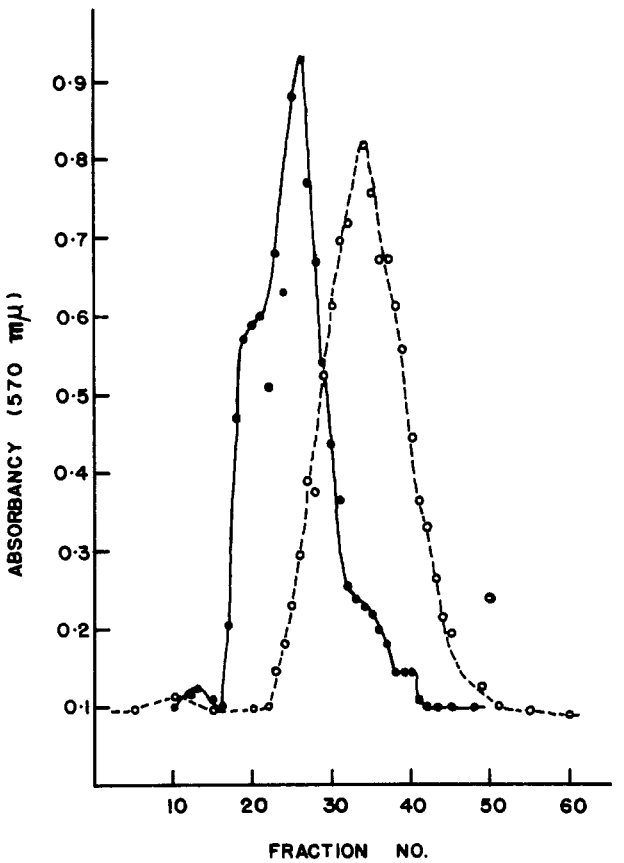

FIG. 2.—Chromatography of I 1 on an analytical DEAE-cellulose column at 3°. The eluting buffer was 0.3 M KCl–0.005 Tris–0.001 M EDTA, *p*H 7.8. Other characteristics of the column are described in the text. Fractions were assayed by the colorimetric ninhydrin procedure. O, native insulin; ●, I 1.

almost the same as I 1; the major fraction had an R_F value the same as that of unreacted insulin. Therefore fraction I 2 is probably unreacted insulin, slightly contaminated with I 1.

Sulfhydryl and Disulfide Groups Found in Thiolated Insulin.—The results of these determinations, given in Table II, show that the unfractionated thiolated insulin

TABLE II

ANALYSIS ON INSULIN AND DERIVATIVES

Insulin Preparation	Sulfhydryl Group by NEM (moles SH per mole insulin)	Total Sulfhydryl and Disulfide by Exchange (moles per mole insulin)	Change in Sulfhydryl (Δ)[a]
Insulin	0.00	6.10	+0.10
I L24	0.70	6.85	+0.85
I 1	1.65	7.95	+1.95
I 2	0.00	5.90	−0.10

[a] Moles total [SH + (—S—S—/2)] − 6 (6 is subtracted to correct for the moles of half-cystine in native insulin).

has about 0.7 mole of SH per mole of insulin derivative by NEM method (Alexander, 1958). This result agrees reasonably well with that of the extra sulfhydryl plus disulfide groups (Δ), found in the same preparation by the method of disulfide exchange (Glazer and Smith, 1961), namely, 0.85. It is of interest to note that no

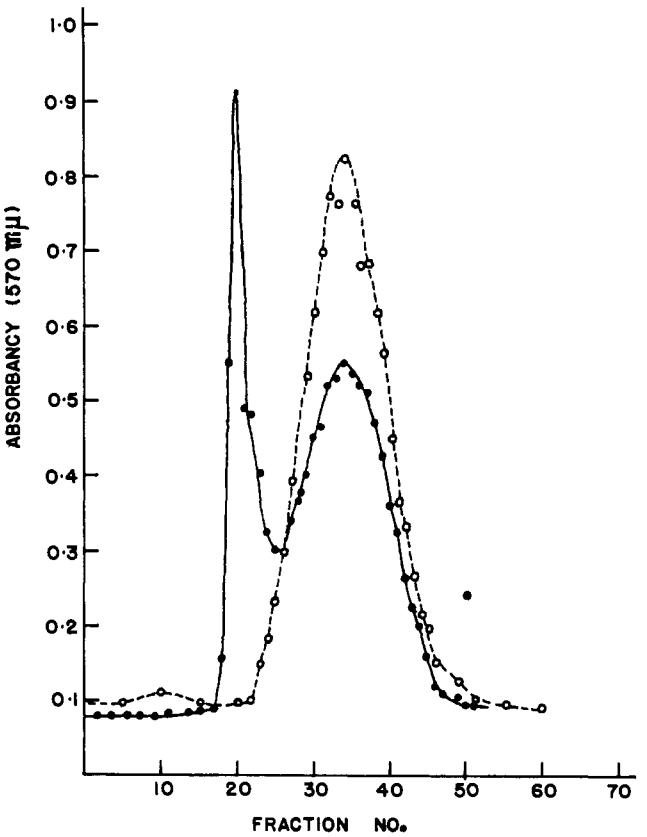

FIG. 3.—Chromatography of I 2 on an analytical DEAE-cellulose column at 3°. The eluting buffer was 0.3 M KCl–0.005 M Tris–0.001 M EDTA, *p*H 7.8. Other characteristics of the column are described in the text. The fractions were assayed by the colorimetric ninhydrin procedure. O, native insulin; ●, I 2.

extra sulfhydryl was found by either method in the preparation I 2 used in these experiments, supporting the hypothesis that this peak represents unreacted insulin. On the other hand, preparation I 1 contained 1.65 (NEM) or 1.95 (exchange) moles of extra sulfhydryl per mole of thiolated insulin.

Carboxymethylation of Thiolated Insulin.—The conditions chosen for the carboxymethylation of thiolated insulin derivative (I 1) did in fact lead to complete carboxymethylation of the sulfhydryl groups of AHCySH residues in fraction I 1. When the carboxymethylated derivative was subjected to the disulfide-exchange reaction of Glazer and Smith (1961), no extra sulfhydryl or disulfide groups, other than those found in native insulin, could be detected. This is to be expected if the AHCySH residues in fraction I 1 have been completely carboxymethylated. It has been found by Gundlach *et al.* (1959) that iodoacetic acid is not completely specific for the sulfhydryl groups and reacts with other groups in proteins. However, it is assumed that, under the conditions of *p*H and concentration of iodoacetic acid employed here for the carboxymethylation of sulfhydryl groups in the insulin derivative, side reactions are minimal (Sela *et al.*, 1959).

Estimation of S-Carboxymethylhomocysteine in Carboxymethylated-thiolated Insulin.—Recovery experiments showed that there was considerable loss of *S*-carboxymethyl derivatives of cysteine and homocysteine during hydrolysis. Recoveries of *S*-carboxymethylhomocysteine were only about 50% and those of *S*-carboxymethylcysteine about 63% (Crestfield *et al.*, 1963).

Carboxymethylated-thiolated insulin was found to

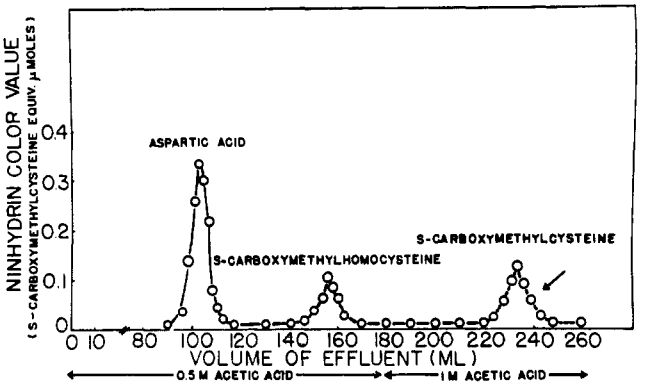

FIG. 4.—Estimation of *S*-carboxymethyl derivatives of homocysteine and cysteine in carboxymethylated-thiolated insulin, by column chromatography on Dowex 1-X8, 200–400 mesh, 1 × 30 cm, acetate form. Flow rate, approximately 10 ml/hour.

contain 1.6 moles of *S*-carboxymethylhomocysteine per mole of insulin (corrected for losses during hydrolysis). This result agrees well with the values found for free sulfhydryl groups (Table II) in the thiolated insulin derivative, I 1. No *S*-carboxymethylcysteine could be detected in the hydrolysates of carboxymethylated-thiolated insulin.

It may be concluded that no appreciable reduction of the disulfide bonds of insulin occurred during the reaction of insulin with AHT, under the conditions of our experiments. High concentrations of thiol which are required for the reduction of the disulfide bonds of insulin were not present during the reaction. Also, the free sulfhydryl groups in thiolated insulin could be accounted for as *S*-carboxymethylhomocysteine in carboxymethylated-thiolated insulin.

However, it is possible that "scrambling" of disulfide bonds in the protein could have occurred due to disulfide interchange. This could have been initiated by the sulfhydryl group of free AHCySH in the reaction mixture, as well as by the AHCySH in the thiolated protein during chromatography on DEAE-cellulose (White and Sandoval, 1962). No attempt was made to detect mixed disulfide groups in the thiolated insulin.

Biological Activity of Insulin Derivatives.—The results of biological assay carried out on the various derivatives of insulin are shown in Table III. The unfractionated-thiolated insulin (I L24) with 0.85 mole of sulfhydryl group per mole of insulin had one-half the activity of beef insulin. This high activity may be attributed to the presence of unreacted insulin in the preparation. The fractionated-thiolated insulin, I 1, which contains 1.95 moles of sulfhydryl groups per mole of insulin, showed very little activity when assayed either by the mouse-convulsion test or the immunological assay.

If the free amino groups of insulin are not necessary for its biological activity, as indicated by the acetylation experiments of Fraenkel-Conrat and Fraenkel-Conrat (1950), acylation with AHCySH residues should not lead to the inactivation of insulin. If loss of biological activity is due to the introduction of new sulfhydryl groups, carboxymethylation of the AHCySH residues in the thiolated insulin might be expected to restore the biological activity. However carboxymethylation of the thiolated derivative (I 1) did not restore the activity.

Disulfide interchange may have occurred during the thiolation of insulin or after the AHCySH residues had been introduced and the effect of this process on the biological activity of insulin must also be considered

TABLE III
BIOLOGICAL ACTIVITY OF INSULIN DERIVATIVES

Insulin Preparation	Degree of Substitution of Amino Groups (moles residue/mole insulin)	Biological Activity Mouse-Convulsion Assay (% of beef insulin)	Immunological Assay (% of beef insulin)
1. Thiolated, unfractionated,[a] I L24	0.85	50.0	
2. Thiolated, fractionated,[a] I 1	1.95	12.0	10.0
3. Thiolated, fractionated, I 1, carboxymethylated	1.65		12.0
4. Thiolated, carboxymethylated[b]	0.85		55.0
5. Thiolated, carboxymethylated[c]	2.10	0.1	8.0
6. Methionyl insulin[d]	0.89	62.0[e]	
7. Methionyl insulin[d]	2.50	54.0[e]	

[a] Insulin reacted with AHT. [b] Insulin reacted with AHT in presence of iodoacetic acid. [c] Insulin reacted with AHT in presence of iodoacetamide. [d] Insulin reacted with the carbamino anhydride of methionine. [e] Blood sugar depression in fasted rabbits.

(White and Sandoval, 1962). When insulin was thiolated in the presence of iodoacetate or iodoacetamide to exclude disulfide interchange, in one case a product was obtained which had 0.85 mole of carboxymethylhomocysteinyl residue per mole of insulin. This preparation had about one-half the activity of beef insulin in the immunological assay. This high activity is probably owing to the presence of some native insulin in the preparation. Another preparation which contained 2.1 moles of the amide of *S*-carboxymethylhomocysteine per mole of insulin had very little biological activity both in the mouse-convulsion and immunological assay.

In contrast, when methionyl groups rather than acetylhomocysteinyl residue or *S*-carboxymethylhomocysteinyl residues were substituted on the free-amino groups of insulin, the derivatives retained more than 50% of the original activity, even though in one preparation 2.5 moles of methionyl residues were present in the derivative. Little impairment of biological activity was also observed when leucine residues were substituted on the free-amino groups of insulin (Fraenkel-Conrat, 1953). Therefore it appears that biological activity of insulin not only depends on the group modified, but also on the modification introduced.

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