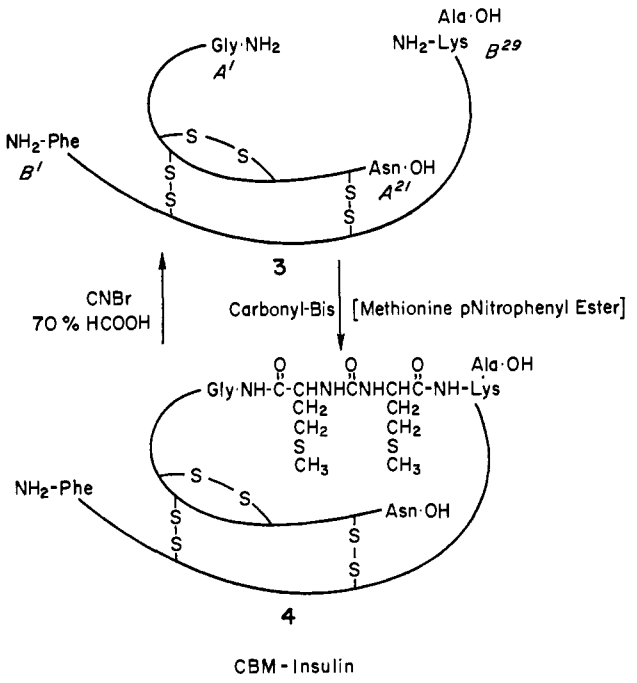

detected after oxidative sulfitolysis.[20] In immunoassays by the double antibody technique,[21] the CBM-insulin exhibited 90–92% of the activity of bovine insulin.

CBM-insulin **4** (10 mg/ml) was treated with CNBr (100 mg/ml) in 70% formic acid for 6 hr. The reaction mixture was diluted tenfold with water and lyophilized. Chromatography of the product on DEAE-cellulose (*vide supra*) followed by dialysis and lyophilization of the material in the main peak gave insulin in 70–75% yield. The product was shown to be identical with insulin by crystallization of the zinc complex, by cellulose acetate and sodium dodecyl sulfate–gel electrophoresis, and by amino acid composition, in circular dichroic spectrum and in immunoassay. The above results demonstrate that an insulin derivative which is cross-linked between the amino groups of glycine A1 and lysine B29 with the carbonylbis(methionyl) residue can be converted back to insulin in good yield by the cyanogen bromide cleavage reaction. The CBM-insulin **4** has proven to be a useful derivative in which the B chain of insulin can be degraded in a stepwise manner by the Edman method, and the degraded CBM-insulin used for the resynthesis of insulin or insulin analogs which are modified on the NH_2 terminus of the B chain. The carbonylbis(methionine *p*-nitrophenyl ester) should be applicable as a general reagent for both inter- and intramolecular cross-linking of proteins. In those proteins which are deficient in methionine or where the methionine is located in non-critical sites, the cross-linking residue can be removed by the CNBr reaction. This property could be useful in topography studies on oligomeric proteins such as ribosomes.[22]

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Carbonylbis(L-methionyl)insulin. A Proinsulin Analog Which Is Convertible to Insulin

Sir:

The previous communication[1] described the preparation of carbonylbis(methionyl)insulin (CBM-insulin), **1**, and its conversion back to insulin by the cyanogen bromide cleavage reaction. The present communication demonstrates that the disulfide bonds of the CBM-insulin may be opened by oxidative sulfitolysis, the *S*-sulfonates, **2**, reduced to sulfhydryls, **3**, and the latter allowed to reoxidize to yield the correct pairing of the disulfide bonds in good yield, Figure 1. Thus the CBM-insulin has the properties of a proinsulin analog[2] in that it promotes the correct pairing of the disulfide bonds between the two chains. This fact coupled with the facile conversion of CBM-insulin to insulin makes the carbonylbis(methionyl) residue potentially useful in a chemical synthesis of insulin in which the S-sulfonated A and B chains are first cross-linked[2b] from the amino group of glycine A1 to the epsilon amino group of lysine B29 before formation of the disulfide bonds.

CBM-insulin, **1** (25 mg in 0.5 ml 0.1 *M* Tris, 7 *M* urea at pH 7.6), was treated with Na_2SO_3 (29 mg in 1 ml of the Tris–urea buffer) and $Na_2S_4O_6$ (29 mg in 1 ml of the Tris–urea buffer)[3] for 4 hr at room temperature. The reaction mixture was chromatographed on a column of Sephadex G-50 (fine) (2.5 × 150 cm) equilibrated and developed with 0.05 *M* NH_4HCO_3. The main protein peak, which eluted at V_e of 314 ml as compared with a V_e of 390 ml for the starting CBM-insulin, was lyophilized to yield 22 mg (80%) of the S-sulfonated CBM-insulin **2**. In three parallel experiments the latter compound **2** (10 mg in 5 ml of 0.36 *M* Tris, 8 *M* urea, 0.25% EDTA at pH 8.6)[4] was treated under nitrogen with dithiothreitol (15 mg). After 1 hr at room temperature an aliquot (0.5 ml) was removed and subjected to carboxymethylation[4] to demonstrate the complete reduction to the sulfhydryl form **3**. The remaining solution was chromatographed on a Sephadex G25 (fine) column (2.5 × 40 cm) which was equilibrated and developed with 0.05 *M* phosphate at pH 7.8. The material in the main protein peak (V_e 85 ml) contained 5.5–5.8 sulfhydryls per mole of protein, as determined by the

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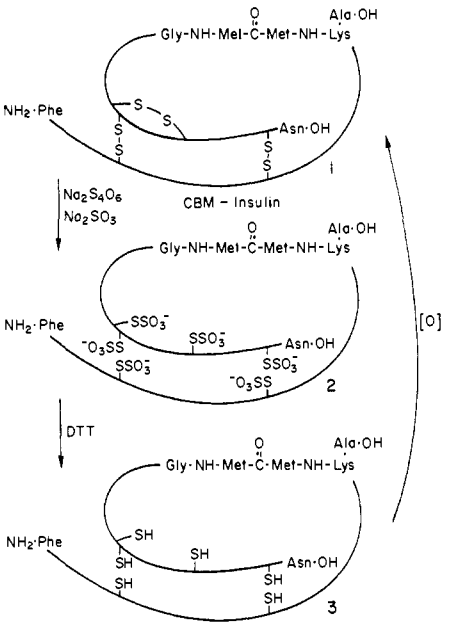

Figure 1. Diagram of the oxidative sulfitolysis of carbonylbis-(methionyl)insulin (CBM-insulin), **1**, followed by the reduction of the *S*-sulfonate **2** with dithiothreitol (DTT) to yield the sulfhydryl form **3** which in turn is oxidized in air to yield the CBM-insulin.

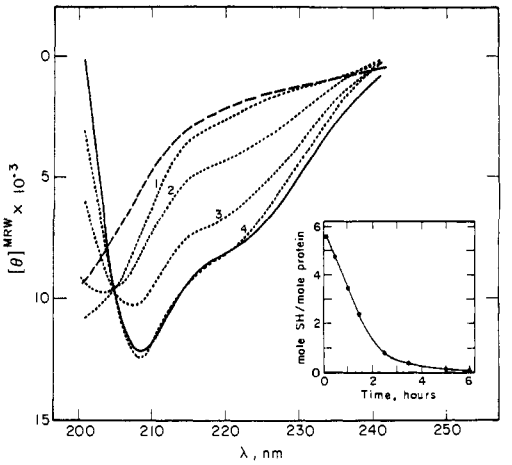

Figure 2. Mean residue weight ellipticity ($[\theta]^{MRW}$) as a function of wavelength at a concentration of 0.1 mg/ml in 0.05 *M* phosphate at pH 9.5 at 27° for the *S*-sulfonate of CBM-insulin **2** (---), for CBM-insulin **1** (—), and for reoxidation mixtures of the reduced CBM-insulin **3** (·····) at various time intervals in which the reoxidation mixture contained moles of sulfhydryls per mole of protein of (1) 5.25 at 15 min, (2) 3.5 at 60 min, (3) 2.0 at 105 min, and (4) 0.1 at 360 min. Insert. Rate of disappearance of sulfhydryls *vs.* time in the reoxidation of reduced CBM-insulin **3** at 0.1 mg/ml in 0.05 *M* phosphate at pH 9.5 at room temperature.

Ellman reagent.[5] The eluate was diluted to give 0.1 mg of reduced protein per milliliter, the pH was adjusted to 9.5 and the solution contained in an erlenmeyer flask coated with bovine serum albumin[6] was stirred in air at room temperature for 10 hr. The reoxidation mixture was lyophilized and the residue chromatographed on a Sephadex G50 (fine) column (2.5 × 150 cm) which was equilibrated and developed with 0.05 *M* NH_4HCO_3. The material in the main peak (V_e of 390 ml) was lyophilized to yield 6.8–7.8 mg (75–86%) of CBM-insulin **1** which was shown to be identical with the starting material by cellulose acetate and sodium dodecyl sulfate–gel electrophoresis, circular dichroic (CD) spectrum, and immunoassay.[7] The rate of reoxidation of the sulfhydryls of the reduced CBM-insulin as well as the CD spectra at various stages of reoxidation are shown in Figure 2. The CD spectra indicate the presence of at least one isosbestic point located at 204 nm (Figure 2) which suggests that only two conformations, the fully reduced and the completely oxidized, contribute to the CD spectra. However, more extended studies are necessary to confirm this hypothesis.

CBM-insulin which had been subjected to the above reduction and reoxidation (12.4 mg) was treated with CNBr (344 mg) in 3 ml of 70% formic acid at room temperature for 18 hr. Insulin was isolated from the reaction mixture as previously described[1] to give 8.4 mg (68%) of product which was identical with the native hormone in amino acid analysis, amino end groups, and CD spectrum. The product yielded a crystalline zinc complex and gave the same fragmentation pattern as insulin on chymotrypsin digestion.[8] This conversion to insulin is an unambiguous demonstration that the correct pairing of disulfide bonds occurred during reoxidation of the reduced CBM-insulin.

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Evidence for the Much Greater Rate of β-Scission Involving the *tert*-Butoxy Group Compared to Permutational Isomerization in a Cyclic Tetraalkoxyphosphoranyl Radical

Sir:

Esr[1] and chemical studies[2] have provided evidence that the oxidation reactions (eq 1) of alkoxy radicals

$$R'O\cdot + (RO)_3P \xrightarrow{a} \underset{\mathbf{1}}{R'OP(OR)_3} \xrightarrow{b} R'\cdot + OP(OR)_3 \quad (1)$$

with trialkyl phosphites proceed *via* discrete phosphoranyl radical intermediates, **1**. The product forming step in reaction 1 is termed β-scission. Near-trigonal bipyramidal structures, **2**, are usually proposed[1] for **1**. Chemical evidence[2a] has been presented which shows that R'O· in reaction 1 does not enter the trigonal bipyramidal intermediate **2** in a configurationally random way and by analogy to pentacovalent phos-

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