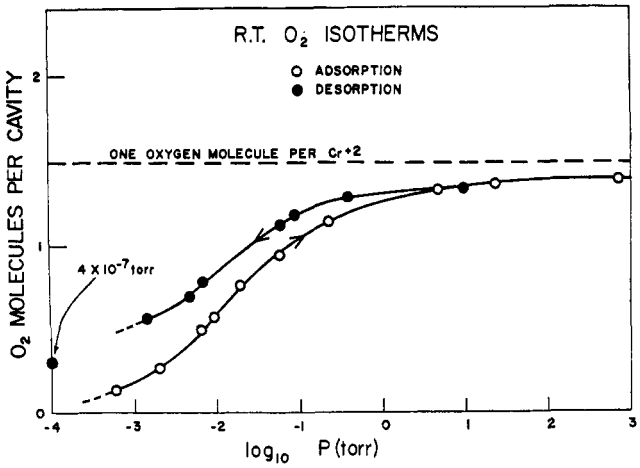

Figure 1. The oxygen chromium(II) exchanged anhydrous zeolite A room temperature isotherm. The adsorption is approximately 80% reversible at room temperature. The oxygen uptake also produces reversible color changes in the sample (see text).

5) with a magnetic moment at room temperature of 5.0 BM. A calculation of the energy levels of a quintet d^4 ion under a D_{3h} ligand field potential has been performed and will be published. Two spin allowed transitions are predicted and the 12,300 and 17,000 cm^{-1} peaks of the anhydrous $(Na_{0.75}Cr(II)_{0.125})$-A are assigned to the $^5E' \rightarrow {}^5E''$ and $^5E' \rightarrow {}^5A_1'$ transitions where the states $^5E'$, $^5E''$, and $^5A_1'$ originate by the splitting of the atomic term ^5D under the D_{3h} potential. On the basis of the agreement between the experimental and theoretical spectra, the divalent chromium ion is coordinated by the zeolitic oxygen six ring, as are the Ni(II)[6,7] and Co(II) ions.[8]

X-Ray diffraction patterns of the hydrated and anhydrous chromium(II) exchanged zeolites were compared to the hydrated nonexchanged zeolite pattern and showed that neither the ion exchange nor the subsequent dehydration degraded the zeolitic structure.

Exposure of the pale blue-lilac anhydrous zeolite to dry oxygen at 760 Torr instantly induces a gray color. The diffuse reflectance electronic spectrum of the oxygenated sieve is quite different from that of the nonoxygenated sieve (Table I) indicating a specific interaction between the chromium and the oxygen. This interaction is confirmed by the oxygen adsorption isotherm obtained using a quartz spring microbalance. Figure 1 shows that the uptake of oxygen starts at *ca.* 5×10^{-4} Torr and that at a pressure of 1 Torr the oxygen molecule–chromium ratio is close to unity. Desorption of the oxygen is affected by reduction of the oxygen pressure above the sample and is accompanied by a return to the blue-lilac color of the nonoxygenated sample.

In accord with the proposed binding schemes for other reversibly oxygenated transition metal complexes,[11-13] we suggest that the oxygen molecule accepts an electron from the rather easily oxidized Cr(II) ion and that the resulting superoxide anion is bound in π geometry to the now formally trivalent chromium ion.

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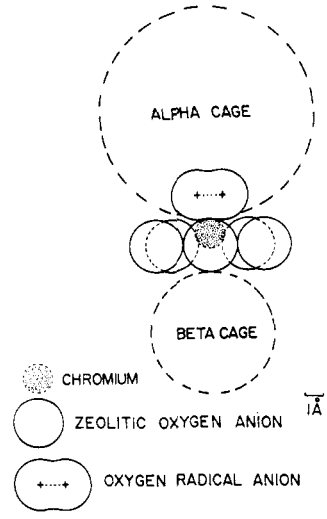

Figure 2. The proposed structure of the anhydrous chromium(II) ion exchanged zeolitic molecular oxygen complex. The figure is drawn to scale. The dimensions of the oxygen radical anion are taken from ref 11. The chromium ion is pulled out of the six ring by the oxygen.

$$(Na_{0.75}Cr(II)_{0.125})\text{-A} \underset{\text{vacuum}}{\overset{O_2}{\rightleftharpoons}} (Na_{0.75}[Cr(III)\text{-}O_2^-]_{0.125})\text{-A}$$

The room temperature magnetic moment of the complex is 3.7 BM, indicating spin pairing between the chromium ion and the oxygen radical anion. This behavior is also typical of transition metal ion–oxygen complexes, many of which are diamagnetic.[13] On the assumption that the spin pairing arises from weak magnetic interaction and that the chromium in the oxygen complex can be treated as a d^3 ion within the framework of ligand field theory, the electronic spectrum Table I (right) is to be interpreted as that of a d^3 ion under C_{1v} ligand field. Preliminary calculations show reasonable agreement with the observed spectrum. The proposed structure of the complex is given in Figure 2. However, other geometries are not excluded and further experimentatal data are needed to resolve structural details.

In summary, divalent chromium on anhydrous zeolite A is a particularly simple reversible oxygen binder. The nonoxygenated chromium is coordinated by three zeolitic oxygens and to a good approximation behaves as an ionic complex. No simpler oxygen carrier appears to have been reported.

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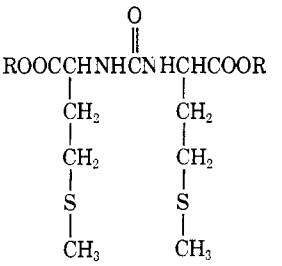
Carbonylbis(L-methionine *p*-nitrophenyl ester). A New Reagent for the Reversible Intramolecular Cross-Linking of Insulin

Sir:

The biological synthesis of insulin occurs through a single peptide chain (proinsulin) in which the COOH

terminus of the B chain is connected to the NH_2 terminus of the A chain through a peptide of about 33 amino acid residues.[1] Whereas the disulfide bonds of proinsulin can be reduced and then reoxidized to give the parent molecule in good yield (*ca.* 70%),[2] similar treatment of the two-chain insulin molecule results in a poor yield of reoxidized products containing the active insulin with correct pairing of disulfide bonds.[3] This fact has handicapped chemical syntheses of insulin involving, as a last step, the combination of the two separate chains through formation of the disulfide bonds.[4] The three-dimensional structure of insulin[5] has revealed that the NH_2-terminal glycine of the A chain is located quite close (*ca.* 10 Å) to the ϵ-amino of lysine B29. This fact had been indicated as early as 1958 when Zahn and Meienhofer found that the bifunctional reagent, difluorodinitrobenzene, formed an intramolecular cross-link between these two amino groups.[6] Recently several investigators[7] have prepared intramolecularly cross-linked insulins involving linkage of the amino groups of A1 to B29 through a series of dicarboxylic acids. Insulin derivatives which were cross-linked with the suberoyl[8] ($-OC(CH_2)_6CO-$) or adipoyl[9] ($-OC(CH_2)_4CO-$) residues could be reduced and reoxidized to give good yields of products with the correct pairing of the disulfide bridges as judged by physical and chemical properties of the reoxidized products. A disadvantage of these proinsulin analogs, in which the cross-linking residues could not be removed, has been overcome by the use of the di(BOC)-α,α'-diaminosuberoyl residue.[10] After removal of the BOC groups by trifluoroacetic acid, the diaminosuberoyl moiety is removed by an Edman degradation. The conditions used were similar to those developed in this laboratory for the removal of BOC groups and amino acids from insulin.[11] In the present report we describe a new cross-linking reagent for insulin, the carbonylbis(methionyl) residue (CBM), **1**, which can be removed to regenerate insulin in good yields in a simple one-step reaction. The removal step involves the cyanogen bromide cleavage reaction[12] for specific splitting of peptide chains at methionine residues. The absence of methionine in most species of insulin[13] coupled with the stability of insulin to the conditions of the cyanogen bromide cleavage[14] were key factors in the development of the reversible cross-linking reagent.

L-Methionine in 3 *N* NaOH was treated with 0.5 equiv of phosgene in toluene at 0° with vigorous stirring. Recrystallization from 15% acetic acid of the product obtained upon acidification of the reaction mixture gave carbonylbis(L-methionine) (**1**) in 30–40% yield, mp 167–168°, $[\alpha]^{25}D$ +4.8° (*c*, 1, methanol). Reaction of **1** in dimethylformamide with 2 equiv of dicyclohexylcarbodiimide and *p*-nitrophenol[15] yielded the active ester **2** in 80% yield (after recrystallization



1, R = H
2, R = $C_6H_4NO_2$-*p*

from tetrahydrofuran–ether), mp 183–184°, $[\alpha]^{25}D$ −41° (*c*, 0.5, dimethylformamide).

Bovine insulin, **3**, was treated with 1 equiv of the active ester **2** in dimethyl sulfoxide in the presence of excess triethylamine at room temperature for 18 hr.[7b] The product was precipitated by methanol–ether and separated according to size by gel chromatography on Sephadex G-50 (fine) in 10% acetic acid to give a 70% yield of monomers which, after dialysis and lyophilization, were separated according to cationic charge on CM-cellulose in a solvent which was 7 *M* in urea, 0.1 *M* in acetic acid, and 0.075 *M* in NaCl. The fraction in the main peak, after dialysis and lyophilization, was separated according to anionic charge by chromatography on DEAE-cellulose at pH 7.2 in 0.01 *M* Tris, 0.09 *M* NaCl, and 7 *M* urea. Dialysis followed by lyophilization of the main fraction gave CBM-insulin **4** in overall yield of 30–40% from the starting insulin. The product **4** exhibited homogeneity of charge in cellulose acetate electrophoresis at pH 4.8[16] and of size in sodium dodecyl sulfate–gel electrophoresis.[17] It had the correct amino acid composition including two methionines. The only free amino group as determined by the Edman[18] or the dansyl[19] method was present on phenylalanine. Only one peptide chain could be

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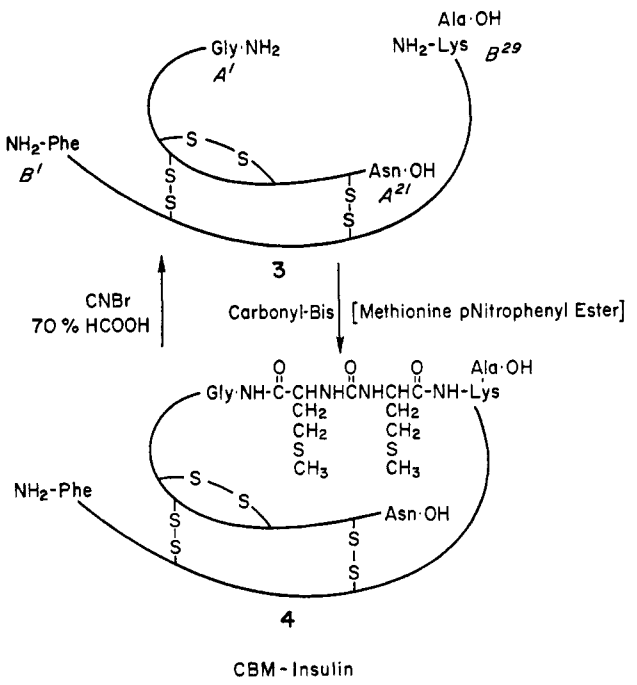

detected after oxidative sulfitolysis.[20] In immunoassays by the double antibody technique,[21] the CBM-insulin exhibited 90–92% of the activity of bovine insulin.

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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