(obtained with 70-v ionizing electrons) are shown in Figure 2. Significant differences between the two sets of mass spectra support the conclusion that while extensive dissociation of parent molecule ions takes place in both experiments, fundamentally different energy-transfer and dissociation mechanisms must operate. The role of kinetic to internal energy transfer is established by the fact that the recombination energy in ArD^+ is insufficient to bring about some of the decomposition processes observed if one accepts the validity of electron-impact, appearance-potential thresholds as a measure of actual minimum energy requirements. The most striking differences in the spectra are found in the two carbon fragment ion region where $C_2H_4^+$ or CH_3CH^+ ions are detected only from propylene while $C_2H_2^+$ is seen only from cyclopropane.

Studies with isomeric butenes show significant differences in spectra between butene-1 and *cis*- and *trans*-butene-2. Spectra obtained with Ar^+, where the recombination energy is approximately 5 ev greater than target ionization energies, tend to obscure differences between isomeric species. The use of ArD^+ reduces the recombination energy from that of the ionization potential of Ar (15.7 ev) to that of D (13.6 ev) minus the binding energy of $Ar–D^+$ (approximately 3 ev). No evidence in any of these experiments is found for D exchange or the incorporation of D in the target molecule ions. There is the possibility that HD and neutral CH_3D products may be formed in the ion-impact collision. If this were a very probable process, we would expect a relatively smaller yield of parent molecule ion.

Details of mechanisms operative in producing these spectra are so far not clearly understood. The potential of this technique in studies which attempt to correlate molecular structure with mass spectra is evident. Low-velocity ion-impact techniques provide a very sensitive method of energy deposition in target molecules and hence are capable of reflecting in mass spectra relatively subtle differences in molecular structure.

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The Synthesis of Bovine Insulin by the Solid Phase Method[1]

Sir:

We wish to report the synthesis of bovine insulin by the solid phase method.[2] This polypeptide hormone has already been synthesized independently by three other laboratories,[3] using the classical methods of peptide chemistry. With these methods, many months are required for the synthesis of each of the protected component peptide chains, and the over-all yields are low. Using the solid phase method we have, in a matter of days and in high yields, synthesized both protected chains of insulin. The chains were then combined to form the active hormone.

The B chain was synthesized in a stepwise manner beginning with 1.9 mmoles of *t*-butyloxycarbonyl-L-alanine esterified to 8 g of the supporting cross-linked polystyrene resin.[2,4] Twenty-nine cycles of deprotection, neutralization, and coupling were carried out with appropriate Boc-amino acids[5] according to previously developed procedures,[4] producing the fully protected triacontapeptide esterified to the resin. Boc-amino acids with protected side chains were O-Bzl-Glu, S-Bzl-Cys, O-Bzl-Ser, O-Bzl-Tyr, N^{im}-Bzl-His, N^{ϵ}-Z-Lys, and N^G-Tos-Arg. All coupling reactions to form peptide bonds were mediated by dicyclohexylcarbodiimide[6] except those involving the carboxyl groups of Asn and Gln which were used as the nitrophenyl esters.[7] Cleavage of the peptide from the resin by hydrogen bromide was done as described[4] except that the HBr was first bubbled through a solution of resorcinol[8] in TFA in order to prevent bromination of tyrosine residues and the peptide resin was suspended in TFA containing methionine in order to prevent benzylation of S-Bzl-Cys. The peptide was precipitated from water to remove the methionine. The yield of partially protected peptide was 64%, based on the amount of alanine originally esterified to the resin. Amino acid analysis gave:[9] Asp, 0.78; Thr, 0.99; Ser, 0.83; Glu, 3.16; Pro, 0.97; Gly, 3.26; Ala, 2.08; Val, 2.81; Leu, 4.06; Tyr, 1.76; Phe, 3.22; Lys, 0.90; Arg, 1.15; Bzl-His, 1.67; Bzl-Cys, 2.37 (Cys, 0; His, 0). The total time required for synthesis and cleavage was 11 days. The partially protected peptide was thoroughly dried and then treated with sodium in liquid ammonia as described by Niu, *et al.*,[10] except that the stable light blue end point was limited to exactly 15 sec in order to prevent excessive cleavage of the Thr-Pro bond (B_{27-28}).[11] Under these conditions this cleavage was only 20–25%,[12] whereas 80% was lost during a 60-sec treatment. The shorter time was adequate for complete debenzylation of His and Cys and complete detosylation of Arg (as determined by direct amino acid analyses for the protected amino acids). The deprotected triacontapeptide was converted to the S-sulfonate.[13] On electrophoresis,[13] there was a major Pauly-positive spot with the same mobility as that of the B-chain S-sulfonate ($BSSO_3$) obtained by sulfitolysis of natural bovine insulin, and a

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minor contaminant probably due to B_{1-27}. The over-all yield from the first alanine residue was 21%. Amino acid analysis gave: Asp, 0.82; Thr, 0.72; Ser, 0.90; Glu, 3.15; Pro, 0.75; Gly, 3.35; Ala, 2.07; Cys, 1.80; Val, 2.89; Leu, 4.35; Tyr, 1.78; Phe, 3.05; Lys, 0.76; His, 1.51; Arg, 1.18 (Bzl-Cys, 0; Bzl-His, 0; Tos-Arg, 0).

The A chain was synthesized by the automated solid phase procedure.[14] Three grams of Boc-L-Asn resin (esterified in ethyl acetate) was carried through 20 reaction cycles as described for the B chain except that the reagents used for all deprotection and neutralization steps were those described by Stewart and Woolley.[15] The peptide was cleaved from the resin as described above (yield 69%). The total time required was 8 days. The S-benzyl protecting groups were removed by treatment with sodium in liquid ammonia,[16] and the four cysteine residues were converted to the S-sulfonates ($ASSO_3$) (over-all yield 37%). Amino acid analysis of a 24-hr HCl hydrolysate gave: Asp, 1.80; Ser, 2.06; Glu, 4.41; Gly,[17] 1.00; Ala, 1.19; Cys, 3.40; Val,[17] 2.01; Ile,[17] 1.07; Leu, 2.41; Tyr, 1.70 (Bzl-Cys, 0). This heneicosapeptide was further purified by chromatography with Sephadex G-15 and by countercurrent distribution.[18] On electrophoresis[13] it had a major Pauly-positive spot with exactly the same mobility as that of the bovine A-chain S-sulfonate obtained from insulin, as well as a small amount of contaminating material.

The synthetic A- and B-chain sulfonates were combined with each other and with the complementary natural chains.[19] The mixtures were first reduced to the thiol derivatives with thioglycolic acid at 25° by a modification of the method of Du, *et al.*[10,13] The reduced chains were precipitated together at pH 3.8, washed, and then oxidized[13] in air at pH 10.0 to form insulin. The molar ratio of $ASSO_3$:$BSSO_3$ at the beginning of reductions was 4:1. The oxidation mixtures were extracted once by the method of Du, *et al.*,[13] and the extracts were lyophilized. On electrophoresis, these insulin preparations appeared as Pauly-positive spots with the same mobility as standard bovine insulin. The preparations were assayed by a mouse convulsion method[20] (Table I). It is clear from these data that both synthetic chains could participate in the formation of biologically active insulin when they were combined with the natural chains or with each other.

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Table I. Insulin Activities[a] of Recombined Chains

Sample[b]	Recovery of B chain[c] after extraction, %	Specific activity Units/mg[d]	Specific activity %[e] (av)
A_NB_N[f]	3.4	20–24	95
A_NB_S	8.4	12–16	60
A_SB_N	10	1–2	6
A_SB_S	2	1.5–2.5	8
A_N	g	0[h]	0
B_N	2	0[h]	0

[a] By mouse convulsion assay.[20] [b] All the samples were carried through the reduction, oxidation, and extraction steps described in the text. Subscript N refers to natural chains, S to synthetic. [c] Based on B-chain content which was determined from quantitative amino acid analyses (Thr, Phe, Lys, His, and Arg occur only in the B chain). [d] The observed range of activity from several groups of animals, based on the maximum possible insulin present, as determined from B-chain content. [e] Compared with crystalline insulin of 24 units/mg. [f] Crystals were obtained from this preparation.[13] [g] 12% of the A chain was recovered in the extract. No B chain could be detected. [h] None of the mice convulsed on doses of 80–460 μg.

Comparison of our biological activities with those published by other laboratories[10] shows that the peptide chains made by the solid phase method produce insulin of approximately the same specific activity as do chains synthesized by classical techniques. We think that the remarkable simplicity of operation, the short time required for the synthesis, and the high yields of these large polypeptides emphasize the advantages of the solid phase method.[21]

Acknowledgment. This work is dedicated to the memory of Dr. D. W. Woolley. Without his encouragement and wise guidance the principles of solid phase peptide synthesis could not have been developed and this synthesis of insulin could not have been undertaken.

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(21) We thank Professor Helmut Zahn and Dr. Bernd Gutte of the Deutsches Wollforschungs-institut, Aachen, for their generous advice and instruction in the recombination methods given to A. M. when he was a guest in their laboratories. We also thank Miss Angela Corigliano for her indispensable assistance in these studies.

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