thr$_{1.80}$glu$_{2.30}$pro$_{0.92}$gly$_{2.0}$ala$_{1.2}$val$_{2.08}$leu$_{2.92}$tyr$_{1.71}$phe$_{1.75}$S-benzylcysteine$_{0.75}$ (*im*-benzylhistidine not determined).

N-Carbobenzoxy-L-phenylalanyl-L-valyl-L-asparaginyl-L-glutaminyl-*im*-benzyl-L-histidyl-L-leucyl-S-benzyl-L-cysteinylglycyl-L-serine hydrazide[3] was converted to the respective azide, and this in turn was coupled with XV to give the protected sulfhydryl form of the B chain. The blocking groups were removed by treatment with sodium in liquid ammonia[20] and the deblocked product was converted to the S-sulfonate form and purified by continuous-flow electrophoresis. The S-sulfonate of the B chain thus obtained exhibited on high-voltage paper electrophoresis a single Pauli-positive spot. Amino acid analysis of the synthetic material after acid hydrolysis gave a composition, in molar ratios, which is consistent with the theoretically expected values for the human B chain: lys$_{0.8}$his$_{2.7}$arg$_{1.0}$thr$_{1.0}$[21]ser$_{1.0}$glu$_{3.5}$pro$_{0.8}$gly$_{3.2}$ala$_{1.5}$cys$_{1.8}$val$_{3.5}$leu$_{4.5}$tyr$_{1.8}$phe$_{3.0}$.

Combination experiments between the synthetic human B chain and the natural A chain of bovine insulin[22] generated considerable insulin activity. As judged by the mouse convulsion method, the over-all yield of the hybrid insulin produced was 4 to 8% of theory. The yield is based on the amount of the S-sulfonates of the A and B chains used originally before their conversion to the sulfhydryl form and their oxidation and not on the protein content of the final product. Since crystalline insulin, generated by the combination of synthetic B chain of sheep insulin with the natural bovine A chain, was recently obtained in our laboratory,[1] work is now in progress for the isolation of crystalline insulin generated by combination of synthetic human B chain with the natural bovine A chain.

(20) R. H. Sifferd and V. du Vigneaud, *J. Biol. Chem.*, **108**, 753 (1935).
(21) Uncorrected.
(22) Prepared from crystalline zinc insulin by a new method: P. G. Katsoyannis and A. Tometsko, unpublished data.
(23) We wish to express our appreciation to Eli Lilly and Co. for carrying out part of the mouse convulsion assays, to Miss Roberta Klimaski for carrying out mouse convulsion assays in our own laboratories, and to Miss Karen Scheibe for the amino acid analyses.
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### Insulin Peptides. XII. Human Insulin Generation by Combination of Synthetic A and B Chains[1]

*Sir:*

In the preceding communication[2] we reported the synthesis of the B chain of human insulin, its isolation in the S-sulfonate form, and its combination with the natural A chain of bovine insulin[3] to generate insulin activity. We wish now to report the synthesis and isolation in the S-sulfonate form of the A chain of human insulin and its combination, either with the natural bovine B chain[3] or with the synthetic human B chain, to generate insulin activity. This last observation appears to represent the first chemical synthesis of a human protein.

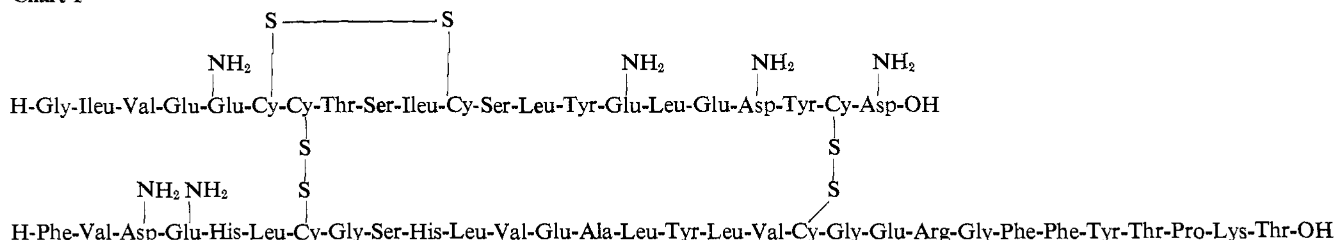
The structure of human insulin as proposed by Nicol and Smith[4] is shown in Chart I.

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-threonyl-L-serine methyl ester [mp 185–187°; $[\alpha]^{27}$D −16.4° (*c* 1, DMF[5a]) (*Anal.* Calcd for $C_{26}H_{33}N_3O_8S$: C, 57.1; H, 6.03; N, 7.7. Found: C, 57.3; H, 6.19; N, 8.0); after HBr in TFA[5b] treatment: $R_f$[6] 0.74, $R_f$[7] 4.1 × His, prepared by the reaction of N-carbobenzoxy-S-benzyl-L-cysteine *p*-nitrophenyl ester[8] with the product obtained by hydrogenolysis of N-carbobenzoxy-L-threonyl-L-serine methyl ester,[9] was deblocked on exposure to HBr in TFA and condensed with N-carbobenzoxy-S-benzyl-L-cysteine *p*-nitrophenyl ester to give N-carbobenzoxy-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-threonyl-L-serine methyl ester (I): mp 174–177°; $[\alpha]^{27}$D −26.0° (*c* 1, DMF) (*Anal.* Calcd for $C_{36}H_{44}N_4O_9S_2$: C, 58.4; H, 6.00; N, 7.6. Found: C, 57.9; H, 6.04; N, 7.3); after HBr in TFA treatment: $R_f$[6] 0.87, $R_f$[7] 4.45 × His. Exposure of I to HBr in TFA and reaction of the resulting product with N-carbobenzoxy-L-glutamine *p*-nitrophenyl ester[8] yielded N-carbobenzoxy-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-threonyl-L-serine methyl ester (II): mp 220–221°; $[\alpha]^{27}$D −26.9° (*c* 1, DMF) (*Anal.* Calcd for $C_{41}H_{52}N_6O_{11}S_2$: C, 56.7; H, 6.00; N, 9.7. Found: C, 56.4; H, 6.27; N, 9.2); for the hydrobromide: $R_f$[6] 0.75, $R_f$[7] 4.36 × His.

N-Carbobenzoxy-L-valyl-L-glutamic acid $\gamma$-*t*-butyl-$\alpha$-methyl ester [mp 91–93°; $[\alpha]^{27}$D −5.8° (*c* 1, DMF); lit[10] mp 90°; lit[10] $[\alpha]^{25}$D −29.5° (*c* 1, methanol); after hydrogenolysis: $R_f$[6] 0.65, $R_f$[7] 4.04 × His], prepared by the reaction of N-carbobenzoxy-L-valine *p*-nitrophenyl ester[11] and L-glutamic acid $\gamma$-*t*-butyl-$\alpha$-methyl ester, was hydrogenolyzed and condensed with N-carbobenzoxyglycyl-L-isoleucine azide[12] to give N-carbobenzoxyglycyl-L-isoleucyl-L-valyl-L-glutamic acid $\gamma$-*t*-butyl-$\alpha$-methyl ester (III): mp 171–174°; $[\alpha]^{27}$D −13.0° (*c* 1, DMF); lit[10] mp 202–203°; lit[10] $[\alpha]^{25}$D −13.2° (*c* 2, DMF) (*Anal.* Calcd for $C_{31}H_{48}N_4O_9$: C, 60.0; H, 7.74; N, 9.0. Found: C, 59.8; H, 7.86; N, 9.5); after hydrogenolysis: $R_f$[6] 0.86, $R_f$[7] 4.81 × His. Treatment of III with hydrazine afforded

(1) (a) Part of this work was reported (P. G. K.) in the National Academy of Sciences Autumn Meeting, Seattle, Wash., Oct 11–13, 1965; (b) presented (P. G. K.) at the Brookhaven National Laboratory Symposium on "Structure and Function of Polypeptide Hormones: Insulin," Upton, N. Y., Nov 8–11, 1965.
(2) P. G. Katsoyannis, A. M. Tometsko, J. Z. Ginos, and M. A. Tilak, *J. Am. Chem. Soc.*, **88**, 164 (1966).
(3) Prepared from crystalline zinc insulin by a new procedure: P. G. Katsoyannis and A. Tometsko, unpublished data.
(4) D. S. H. W. Nicol and L. F. Smith, *Nature*, **187**, 483 (1960).
(5) (a) DMF stands for N,N-dimethylformamide; (b) TFA stands for trifluoroacetic acid.
(6) The $R_f$ refers to the Partridge system: S. M. Partridge, *Biochem. J.*, **42**, 238 (1948).
(7) The $R_f$ refers to the system 1-butanol–pyridine–acetic acid–water, 30:20:6:24 (S. G. Waley and G. Watson, *Biochem. J.*, **55**, 328 (1953)), and is expressed as a multiple of the distance traveled by a histidine marker.
(8) M. Bodanszky and V. du Vigneaud, *J. Am. Chem. Soc.*, **81**, 5688 (1959).
(9) K. Hofmann, W. Haas, M. J. Smithers, and G. Zanetti, *ibid.*, **87**, 631 (1965).
(10) H. Zahn, H. Bremer, W. Sroka, and J. Meienhofer, *Z. Naturforsch.*, **20b**, 646 (1965).
(11) B. Iselin, W. Rittel, P. Sieber, and R. Schwyzer, *Helv. Chim. Acta*, **40**, 373 (1957).
(12) Y. Wang, *et al.*, *Sci. Sinica* (Peking), **13**, 2030 (1964).

Chart I




the respective hydrazide IV: mp 243°; $[\alpha]^{27}D$ −14.0° (*c* 1, DMF); lit[10] mp 243–244°; lit[10] $[\alpha]^{25}D$ −13.8 (*c* 1, DMF) (*Anal.* Calcd for $C_{30}H_{48}N_6O_8$: C, 58.1; H, 7.74; N, 13.6. Found: C, 57.7; H, 7.96; N, 14.0). Conversion of IV to the corresponding azide and coupling with the product obtained from II on exposure to HBr in TFA yielded the amino terminal nonapeptide derivative N-carbobenzoxyglycyl-L-isoleucyl-L-valyl-γ-*t*-butyl-L-glutamyl-L-glutaminyl-S-benzyl-L-cysteinyl-S-benzyl-L-cysteinyl-L-threonyl-L-serine methyl ester (V) [mp 257° dec; $[\alpha]^{27}D$ −30.85° (*c* 1, DMF) (*Anal.* Calcd for $C_{63}H_{90}N_{10}O_{17}S_2$: C, 57.2; H, 6.80; N, 10.6. Found: C, 57.2; H, 6.62; N, 10.1); after HBr in TFA treatment: $R_f^6$ 0.71, $R_f^7$ 4.39 × His; amino acid analysis after acid hydrolysis: $thr_{1.0}ser_{0.9}glu_{2.2}gly_{1.1}val_{0.8}ileu_{0.8}$S-benzylcysteine$_{1.9}$], which on treatment with hydrazine afforded the respective hydrazide (VI): mp 258° dec; $[\alpha]^{28}D$ −20.0° (*c* 1, hexamethylphosphoramide) (*Anal.* Calcd for $C_{62}H_{90}N_{12}O_{16}S_2 \cdot H_2O$: C, 55.5; H, 6.96; N, 12.6. Found: C, 55.3; H, 7.05; N, 13.2).

N-Carbobenzoxy-S-benzyl-L-cysteinyl-L-serine methyl ester[13] was decarbobenzoxylated on treatment with HBr in TFA and coupled with N-carbobenzoxy-L-isoleucine *p*-nitrophenyl ester[8] to give the tripeptide N-carbobenzoxy-L-isoleucyl-S-benzyl-L-cysteinyl-L-serine methyl ester [mp 196–198°; $[\alpha]^{27}D$ −31.0° (*c* 1, DMF) (*Anal.* Calcd for $C_{28}H_{37}N_3O_7S$: C, 60.1; H, 6.62; N, 7.5. Found: C, 60.7; H, 6.91; N, 8.1); for the hydrobromide: $R_f^6$ 0.84, $R_f^7$ 5.24 × His], which on exposure to hydrazine yielded the corresponding hydrazide VII: mp 219–220°; $[\alpha]^{27}D$ −22.0° (*c* 1, DMF) (*Anal.* Calcd for $C_{27}H_{37}N_5O_6S$: C, 58.0; H, 6.62; N, 12.5. Found: C, 58.6; H, 6.79; N, 12.0).

L-Leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester, produced from the respective protected derivative[14] by treatment with HBr in TFA, was allowed to react with the azide which was prepared from VII in the usual way to give the carboxyl terminal dodecapeptide derivative N-carbobenzoxy-L-isoleucyl-S-benzyl-L-cysteinyl-L-seryl-L-leucyl-L-tyrosyl-L-glutaminyl-L-leucyl-L-glutamyl-L-asparaginyl-L-tyrosyl-S-benzyl-L-cysteinyl-L-asparagine *p*-nitrobenzyl ester (VIII): mp 272° dec; $[\alpha]^{27}D$ −28.1° (*c* 1, dimethyl sulfoxide) (*Anal.* Calcd for $C_{92}H_{118}N_{16}O_{25}S_2$: C, 57.8; H, 6.18; N, 11.7. Found: C, 57.5; H, 6.61; N, 11.3); after HBr in TFA treatment: $R_f^6$ 0.83, $R_f^7$ 4.76 × His; amino acid analysis after acid hydrolysis: $asp_{2.0}ser_{0.80}glu_{2.0}val_{0.90}leu_{2.0}tyr_{1.6}$S-benzylcysteine$_{1.6}$.

In the final synthetic step the nonapeptide hydrazide VI was converted to the respective azide and this in turn was condensed with the product obtained by HBr in TFA treatment of the dodecapeptide derivative VIII to yield the partially protected sulfhydryl form of the human A chain.

The protecting groups were removed by successive treatment with TFA and sodium in liquid ammonia,[15] and the ensuing product was converted to the S-sulfonate form upon reaction with sodium sulfite and sodium tetrathionate. The S-sulfonate form of the human A chain thus obtained was purified by chromatography on Sephadex. Amino acid analysis of the synthetic material after acid hydrolysis gave a composition, in molar ratios, in good agreement with the theoretically expected values for the human A chain: $asp_{1.5}thr_{1.0}ser_{1.9}glu_{4.2}gly_{1.2}cys_{4.3}val_{0.9}ileu_{1.7}leu_{2.0}tyr_{1.8}$. On high-voltage paper electrophoresis, the synthetic human A chain exhibited a sharp, single Pauli-positive spot and had a mobility very similar to that of the natural bovine A chain.

Combination experiments carried out between the synthetic human A chain and the synthetic human B chain[2] or the natural bovine B chain[3] led to generation of considerable insulin activity. As judged by biological assays using the mouse convulsion method (over 120 mice for each assay), the over-all yield of the all-synthetic human insulin produced was approximately 2% of theory, and the over-all yield of the hybrid insulin produced (synthetic human A–natural bovine B) was 8% of theory. The over-all yields reported are based on the amounts of the S-sulfonates of the A and B chains used and include three distinctive steps: (1) conversion of the S-sulfonates to the sulfhydryl form, (2) isolation of the chains in the sulfhydryl form, and (3) the oxidation step.

We have recently developed[16] in our laboratory a procedure by which natural bovine A and B chains can be recombined to produce insulin in yields ranging from 45 to 55%. We anticipate that application of this new recombination procedure to the synthetic chains will lead to considerably higher recombination yields than the ones we presently report, which should permit the isolation of crystalline synthetic and hybrid insulins.

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(17) We wish to express our appreciation to Miss Karen Scheibe for the amino acid analyses and to Miss Roberta Klimaski for the biological assays.
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