SYNTHESIS OF AN INSULIN-LIKE COMPOUND CONSISTING OF THE B CHAIN OF INSULIN AND AN A CHAIN CORRESPONDING TO THE A AND D DOMAINS OF HUMAN INSULIN-LIKE GROWTH FACTOR I*

Gerald P. SCHWARTZ, G. Thompson BURKE, Maqsood SHEIKH, Lin ZONG and Panayotis G. KATSOYANNIS

Department of Biochemistry, Mount Sinai School of Medicine of the City University of New York, New York, New York 10029, U.S.A.

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This paper is dedicated to the memory of Dr Karel Bláha, a distinguished colleague and a fine human being.

We report the synthesis and biological evaluation of a two-chain, disulfide-linked, insulin-like compound in which the A chain amino acid sequence corresponds to that of the A- and D-domains of human insulin-like growth factor I (IGF-I), and the B chain is that of bovine insulin. The compound displays reduced insulin-like activity, but considerably increased growth-promoting activity relative to insulin, and is not recognized by IGF carrier proteins. These data confirm some of our earlier conclusions regarding the role of the A-, B- and D-domains in the expression of the biological profile of IGF-I: The A-domain, but not the B- or D-domain is associated with the growth-promoting activity of IGF-I; the B-domain, but not the A- or D-domain, contains determinants for the recognition of IGF carrier proteins; and the D-domain acts to supress insulin-like activity in IGF-I.

The insulin-like growth factors I and II (IGF-I and IGF-II) are polypeptides in human plasma that show considerable homology with proinsulin[2,3]. The homologous sequences are confined to an "A-domain" and a "B-domain" which show c. 40% homology to the A and B chains of insulin, respedtively. Interestingly, part of the putative receptor-binding region of insulin is conserved in the A- and B-domains of the IGFs. The primary structure** of human IGF-I is shown in Figure 1.

* A preliminary discussion of this work was presented[1] (PGK) to the 19th European Peptide Symposium, Porto Carras, Greece, 1986.

** All the chiral amino acids mentioned in this work are of the L-configuration; abbreviations: Ac, Acetyl; Bzl, benzyl; Boc, tert-butoxycarbonyl; Cl_2Bzl, 2,6-dichlorobenzyl; DCC, N,N'-dicyclohexylcarbodiimide; DMF, dimethylformamide; DPM, diphenylmethyl; EEDQ, 2-ethyl-1-ethoxycarbonyl-1,2-dihydroquinoline; HOBT, 1-hydroxybenzotriazole; NMM, N-methylmorpholine; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; Tos (tosyl), *p*-toluenesulfonyl; Tris, tris(hydroxymethyl)aminomethane; Z, benzyloxycarbonyl.

As in proinsulin the N-terminal B-domain (sequence 1 – 30) is connected via a C-domain (sequence 31 – 41) to the A-domain (sequence 42 – 62). Unlike proinsulin, the IGFs contain an extension peptide at the C-terminus, the D-domain, an octapeptide in IGF-I (sequence 63 – 70) and a hexapeptide in IGF-II. The A- and B-domains of IGF-I are c. 60% homologous to the corresponding domains of IGF-II whereas their C-domains, 11 and 8 amino acid residues long respectively, show little if any homology. Computer-graphic studies indicate that the IGFs can assume conformations identical with that of insulin as far as the A- and B-domains and the hydrophobic core are concerned[4,5].

Not surprisingly, in view of the substantial structural homology and conformational similarity, IGFs and insulin display a similar spectrum of biological activities, although the relative potency of the IGFs and insulin vary considerably. Thus insulin is more potent than the IGFs in insulin-like effects (e.g. the stimulation of glucose transport and lipogenesis), whereas the IGFs are more potent than insulin in growth-promoting effects (e.g. the stimulation of DNA synthesis in fibroblasts)[6,7]. A unique property of the IGFs, not shared by insulin, is that they circulate in plasma bound to specific carrier proteins which appear to prolong their half-life and to modulate their delivery to target tissues[8].

Studies are under way in our laboratory designed to identify those regions in the IGF molecules that contribute to their particular biological behavior[1]. We are pursuing this goal through the synthesis and biological evaluation of disulfide-linked two-chain insulin-like molecules embodying structural features of insulin and the IGFs. We have already reported the synthesis and biological evaluation of four such hybrid molecules: one in which the hexapeptide D-domain of IGF-II was attached as a C-terminal extension to the A chain of insulin ($A_{INS} - D_{IGF-II}/B_{INS}$)[9,10];

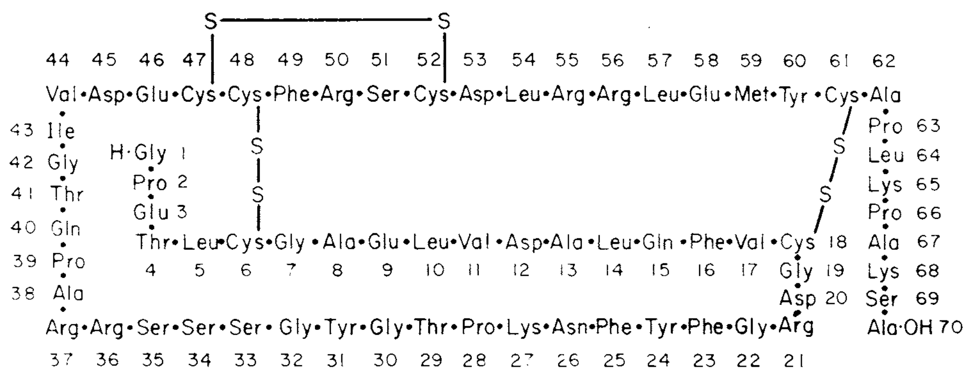

FIG. 1
Primary structure of human IGF-I

another in which the B chain of insulin was substituted by a B chain corresponding to the B-domain of IGF-I (A_{INS}/B_{IGF-I})[10,11]; a third in which the A chain of insulin was extended at its C-terminus with the D-domain of IGF-II and the insulin B chain was replaced by the B-domain of IGF-I ($A_{INS}-D_{IGF-II}/B_{IGF-I}$)[12]; and a fourth hybrid in which the A chain of insulin was replaced by an A chain corresponding to the A-domain of IGF-I (A_{IGF-I}/B_{INS})[13]. Biological evaluation of these hybrid molecules (Table I) led to the following conclusions[1,10–15]: The B-domain of IGF-I is important for recognition of IGF carrier proteins and antibodies against IGF-I but does not, in itself, play an important role in the growth-promoting activity of IGF-I[1,10,11,14]; the A-domain of IGF-I is importantly involved in its growth-promoting activity but not in its binding to carrier proteins; the D-domain of IGF-II does not contribute directly to the growth-promoting activity of that factor or to recognition of carrier proteins, but it diminishes insulin-like activity, both in the hybrid and in IGF-II itself[10,12,14]. Interestingly, the computer-graphics studies suggest that the D-domain of the IGFs partially covers the putative "insulin receptor-binding region", a portion of which is conserved in these factors, and this may explain the low insulin-like activity which they possess. A final observation arising from the behavior of these hybrids is that the B-domain of IGF-I, independent of the presence of the D-domain, interferes with the interaction of the molecule with the insulin receptor, and may play a role in modulating specificity in receptor recognition[1].

TABLE I

Biological evaluation of two-chain insulin-IGF hybrid molecules (values are expressed as percent of insulin except as otherwise indicated)

Compound	Insulin-like assays			Growth factor assays		
	Receptor binding	Lipogenesis	RIA	Receptor binding[a]	DNA synthesis[a]	Carrier protein binding[b]
A_{INS}/B_{INS}(insulin)	100	100	100	100	100	0
$A_{INS}-D_{IGF-II}/B_{INS}$	35	40	18	36	31	0
A_{INS}/B_{IGF-I}	2	2	0·3	9	3	16
$A_{INS}-D_{IGF-II}/B_{IGF-I}$	<0·3	0·3	0·05	17	20	16
A_{IGF-I}/B_{INS}	41	41	0·17	230	730	0
$A_{IGF-I}-D_{IGF-I}/B_{INS}$	[c]	21	0·16	570	440	0

[a] Assay carried out in chick embryo fibroblasts[10,12]; [b] values are expressed as percent of IGF (ref.[14]); [c] a wide range of potency values obtained in several assays does not permit the assignment of a definitive value (see text).

In the present communication, we report the synthesis and biological evaluation of a fifth hybrid molecule, in which the B chain of insulin is linked by disulfide bonds to a 29-amino acid residue A chain corresponding to the A- and D-domains of IGF-I (sequence 42–70 in Fig. 1) ($A_{IGF-I}-D_{IGF-I}/B_{INS}$). This compound displays reduced insulin-like activity when compared to insulin (21%) and also when compared with the previously synthesized hybrid[13], A_{IGF-I}/B_{INS}, which showed c. 40% of the activity of insulin (Table I). In contrast to this reduced insulin-like activity, $A_{IGF-I}-D_{IGF-I}/B_{INS}$ is a potent growth factor, 4–6 fold greater than insulin, as was the case with A_{IGF-I}/B_{INS} (ref.[13]). The present hybrid, similarly to A_{IGF-I}/B_{INS}, is not recognized by IGF carrier proteins. These data confirm conclusions drawn from our previous work, namely: the A-domain is associated with the growth-promoting activity of IGF-I but not with IGF carrier protein recognition; the D-domain of IGF-I, as was the case with the D-domain of IGF-II, is without obvious effect on growth-promoting activity or IGF carrier protein recognition; the D-domain of IGF-I, like that of IGF-II, suppresses insulin-like activity apparently, as the computer-graphics models suggest, by partially covering the putative "insulin receptor-binding region" which is substantially conserved in the hybrid and in IGF-I.

The low cross reactivity with antibodies against insulin displayed by the present compound (i.e. 0·16% relative to insulin) is readily explained. Important immunogenic determinants for anti-insulin antibodies are located in the C-terminal region and in the intra-chain loop of the A chain of insulin[16,17] (corresponding sequence 47–52 in IGF-I, in Fig. 1). Both these regions are considerably altered in the present hybrid, in which the A chain of insulin is replaced with the A- and D-domains of IGF-I.

It should be noted that although the growth-promoting activity of the present two-chain compound, like that of A_{IGF-I}/B_{INS} is considerably higher than that of insulin (c. 400–700% of insulin, Table I), it is still not equal to the growth-promoting activity of the single chain natural IGF-I (c. 15–26% relative to IGF-I in the stimulation of DNA synthesis[15]). Another two-chain insulin-like compound consisting of A and B chains corresponding to the A- and B-domains respectively of IGF-I (A_{IGF-I}/B_{IGF-I}) displayed growth-promoting activity comparable to A_{IGF-I}/B_{INS} and $A_{IGF-I}-D_{IGF-I}/B_{INS}$ (unpublished data). This could indicate that the C-domain, which joins the B- and A-domains in natural IGF-I (sequence 31–41 in Fig. 1), but which is absent from the two-chain molecules, may play a role in the expression of growth-promoting activity in IGF-I. The C-domain could act to orient the A- and B-domains in such a conformation as to enhance the activity of the single chain IGF-I relative to the two-chain synthetic compounds. It is also possible that the lower activity of the two-chain compounds relative to single chain natural IGFs results from the presence of additional charged groups (—COOH and —NH_2) at the ends of the B and A chains.

The synthesis of $A_{IGF-I}-D_{IGF-I}/B_{INS}$ was carried out by the procedure we have employed previously in the synthesis of the other hybrid molecules[11-13], namely the interaction of the S-sulfonated IGF-I [A – D]-chain *XXVI* with the S-sulfonated bovine insulin B chain in the presence of dithiothreitol at pH 10·5. The S-sulfonated B chain was obtained by sulfitolysis of bovine insulin as described previously[18]. The synthesis of the S-sulfonated IGF-I [A – D]-chain, patterned after that of the [$A_{INS}-D_{IGF-II}$]-chain[9], involved as the key intermediate the construction of the protected nonacosapeptide *XXV* containing the entire amino acid sequence of the human IGF-I [A – D]-domain (sequence 42 – 70 in Fig. 1). The synthesis of the latter polypeptide derivative was accomplished by the classical methods of peptide synthesis, namely a combination of stepwise elongation and fragment condensation approaches. The C-terminal tridecapeptide *XII* (sequence 58 – 70) was condensed with the adjacent tetrapeptide *XVI* (sequence 54 – 57) to yield the C-terminal heptadecaptide *XVII* (sequence 54 – 70). The latter intermediate was elongated by two stepwise additions to give the C-terminal nonadecapeptide *XIX* (sequence 52 – 70 Condensation of the latter nonadecapeptide *XIX* with the adjacent pentapeptide (sequence 47 – 51) afforded the C-terminal tetracosapeptide *XX* (sequence 47 – 70) which after five stepwise elongation steps was converted to the desired protected nonacosapeptide *XXV* (sequence 42 – 70). Removal of the protecting groups on exposure to liquid hydrogen fluoride in the presence of 2-mercaptopyridine and *p*-cresole, and sulfitolysis of the resulting reduced product afforded the S-sulfonated [A – D]-chain *XXVI*.

EXPERIMENTAL

Details of materials and analytical procedures used in this investigation are given in previous publications[19-21]. The homogeneity of the intermediate peptide derivatives was ascertained by thin-layer chromatography on 6060 silica gel (Eastman chromagram sheet). The solvent systems used were 89 : 10 : 1 and 45 : 10 : 1 chloroform–methanol–water. A radioimmunoassay kit for insulin was purchased from Amersham. Cellulose acetate filters, 0·2 µm pore size, used in the receptor binding assays and in radioimmunoassay, were obtained from Sartorius. The insulin-like potency of the synthetic hybrid relative to bovine insulin was measured in three types of assay[20]: Insulin receptor binding in a rat liver membrane fraction, in which relative potency is calculated from the ratio of insulin to test compound required to displace 50% of specifically bound ^{125}I-insulin; lipogenesis in rat adipocytes, in which relative potency is calculated from the ratio of insulin to test compound required to achieve 50% of the maximum conversion of [3-^{3}H]glucose into organic-extractable material; and radioimmunoassay, in which insulin or the test compound was used to compete with ^{125}I-insulin binding to guinea pig antibodies raised against insulin. The assays for growth promoting potency of the synthetic hybrid are described in detail elsewhere[15].

Boc-Ser(Bzl)-Ala-OBzl (*I*)

To a solution of Boc-Ser(Bzl)-OH (5·9 g) in DMF (40 ml) containing NMM (2·2 ml) and cooled to –10°C, isobutyl chloroformate (2·6 ml) was added followed, 4 min later, by a solution of

H-Ala-OBzl.HCl (4·3 g) in DMF (20 ml) and NMM (2·2 ml). After 4 h at room temperature the reaction mixture was diluted with ethyl acetate (400 ml), washed (saturated $KHCO_3$, 0·2M-HCl and water), dried and concentrated to dryness. The product was obtained as an oil (8·5 g, 93%). This product was homogeneous on thin-layer chromatography and was used in the synthesis of the following compound without any further characterization.

Boc-Lys(Z)-Ser(Bzl)-Ala-OBzl (*II*)

A solution of compound *I* (10·0 g) in TFA (30 ml) was stored at room temperature for 30 min and then concentated, under reduced pressure, to dryness. The residue was dissolved in ethyl acetate (400 ml) and this solution was treated with cold 1M-Na_2CO_3 until the pH of the aqueous phase was c. 9. The organic layer was separated, washed with saturated NaCl, dried and concentrated to dryness. A solution of the residue in DMF (30 ml) was then mixed with a solution in DMF (30 ml) of the mixed anhydride prepared from Boc-Lys(Z)-OH (7·7 g), NMM (2·2 ml), and isobutyl chloroformate (2·6 ml) in the usual way. The reaction mixture was processed as described in the synthesis of compound *I* and the product was obtained as a low-melting amorphous solid (9·1 g, 58%). This product was homogeneous on thin-layer chromatography and was used in the synthesis of the following compound without any further characterization.

Boc-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*III*)

Compound *II* (8·0 g) was deblocked upon treatment with a mixture of TFA–AcOH (7 : 3, v/v) (30 ml) for 30 min and converted to the free base as described in the synthesis of compound *II*. To a solution of this product in DMF (30 ml) Boc-Ala-OH (3·8 g) was added followed by EEDQ (3·6 g). After 24 h the mixture was diluted with ethyl acetate (400 ml), washed in the usual way and concentrated to a small volume. Addition of ether caused the precipitation of the product which was collected and reprecipitated from methanol–ether; 7·0 g (80%); m.p. 152–154°C; $[\alpha]_D^{25}$ −20·4° (*c* 1, DMF). For $C_{42}H_{55}N_5O_{10}$ (789·9) calculated: 63·86% C, 7·02% H, 8·87% N; found: 64·2% C, 7·27% H, 8·9% N.

Boc-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*IV*)

A solution of compound *III* (7 g) in TFA–AcOH (7 : 3, v/v) (30 ml) was stored at room temperature for 30 min and then concentrated under reduced pressure to dryness. The residue was dried by the addition of toluene followed by evaporation under reduced pressure and solidified by trituration with ether. To a solution of this product in DMF (30 ml) containing NMM (0·9 ml) and cooled to 0°C was added the carboxyl component preactivated as follows. Boc-Pro-OH (3·2 g) was dissolved in DMF (20 ml) and to this solution cooled to 0°C were added HOBT (2·4 g) and DCC (3·0 g). After stirring at 0°C for 1 h and at room temperature 1 h the urea by-product was filtered off and the filtrate mixed with the free base of the deblocked peptide prepared as just described. After 24 h at room temperature the mixture was diluted with ethyl acetate (500 ml) and this solution washed as usual, dried and concentrated to a small volume. Addition of ether caused the precipitation of the product; 7·2 g (92%); m.p. 171–172°C; $[\alpha]_D^{25}$ −32·6° (*c* 1, DMF). For $C_{47}H_{62}N_6O_{11}$ (887·1) calculated: 63·64% C; 7·05% H, 9·47% N; found: 64·0% C, 7·46% H, 9·5% N.

Boc-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*V*)

Compound *IV* (7·0 g) was deblocked with TFA–AcOH (30 ml) as described above. To a solution of the deblocked product in DMF (25 ml) containing NMM (1 ml) was added Boc-Lys(Z)-OH

(5·8 g) preactivated in the presence of HOBT (2·5 g) and DCC (3·0 g) in DMF (40 ml) as described previously. The reaction mixture was processed as described in the synthesis of compound *IV*; 8·3 g (92%); m.p. 89–91°C; $[\alpha]_D^{25}$ −30·1° (*c* 1, DMF). For $C_{61}H_{80}N_8O_{14}$ (1 149) calculated: 63·75% C, 7·02% H, 9·75% N; found: 63·3% C, 7·30% H, 9·9% N.

Boc-Leu-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*VI*)

Compound *V* (8·8 g) was deblocked with TFA–AcOH (30 ml) and the free base of the resulting product was prepared as described in the synthesis of compound *II*. To a solution of this product in DMF (50 ml) were added Boc-Leu-OH (3·8 g), HOBT (2·4 g) and DCC (3·0 g). After 24 h the urea by-product was filtered off and the filtrate diluted with ethyl acetate (500 ml), washed in the usual way and concentrated to a small volume. Addition of ether caused the precipitation of the product; 6·7 g (69%); m.p. 113–115°C; $[\alpha]_D^{25}$ −34·8° (*c* 1, DMF). For $C_{67}H_{91}N_9O_{15}$ (1 263) calculated: 63·74% C, 7·27% H, 9·98% N; found: 63·4% C, 7·48% H, 9·6% N.

Boc-Pro-Leu-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*VII*)

Compound *VI* (11·8 g) was deblocked in the usual way and the free base of the resulting product was prepared as described in the synthesis of compound *II*. To a solution of this product in DMF (50 ml) were added Boc-Pro-OH (6·0 g), HOBT (3·8 g) and DCC (3·85 g). After 24 h the urea by-product was filtered off and the filtrate diluted with ethyl acetate (500 ml). This solution was washed (2M-$KHCO_3$, 0·2M-HCl and water), dried, concentrated to a small volume and mixed with ether. The precipitated product was collected and reprecipitated from ethyl acetate–ether; 11·9 g (94%); m.p. 97–98°C; $[\alpha]_D^{25}$ −23·2° (*c* 1, DMF). For $C_{72}H_{98}N_{10}O_{16}$ (1 360) calculated: 63·61% C, 7·27% H, 10·30% N; found: 63·2% C, 7·26% H, 10·3% N.

Boc-Ala-Pro-Leu-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*VIII*)

Compound *VII* (7·9 g) was deblocked with TFA–AcOH and the free base was coupled in DMF (50 ml) with Boc-Ala-OH (2·9 g) in the presence of HOBT (2·4 g) and DCC (3·0 g) as described in the synthesis of compound *VII*. The product was reprecipitated from acetone–ether; 7·8 g (94%); m.p. 98–100°C; $[\alpha]_D^{25}$ −55·7° (*c* 1, DMF). For $C_{75}H_{103}N_{11}O_{17}.H_2O$ (1 449) calculated: 62·18% C, 7·31% H, 10·64% N; found: 62·0% C, 7·24% H, 10·7% N.

Boc-Cys(DPM)-Ala-Pro-Leu-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*IX*)

This decapeptide derivative was synthesized from compound *VIII* (7·6 g) and Boc-Cys(DPM)-OH (ref.[22]) (5·8 g), HOBT (2·7 g) and DCC (3·0 g) in DMF (40 ml) following the same procedure as described above for the synthesis of compound *VIII*; 7·5 g (84%); m.p. 131–133°C; $[\alpha]_D^{25}$ −20·9° (*c* 1, DMF). For $C_{91}H_{118}N_{12}O_{18}S$ (1 700) calculated: 64·29% C, 6·99% H, 9·89% N; found: 63·6% C, 6·90% H, 9·8% N.

Boc-Tyr(Cl_2Bzl)-Cys(DPM)-Ala-Pro-Leu-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*X*)

Compound *IX* (7·5 g) was deblocked as described in the synthesis of compound *IV*. To a solution of the resulting peptide trifluoroacetate in DMF (20 ml) containing NMM (0·45 ml) were added Boc-Tyr(Cl_2Bzl)-OH (4·4 g), HOBT (1·6 g) and DCC (2·0 g). After 24 h at room temperature the urea by-product was filtered off and the filtrate diluted with 2-propanol (100 ml) and ether (600 ml). The precipitated product was collected and reprecipitated from 95% ethanol; 6·6 g (74%); m.p. 198–200° C; $[\alpha]_D^{25}$ −50·1° (*c* 1, DMF). For $C_{107}H_{131}N_{13}O_{20}SCl_2$ (2 022) calculated: 63·55% C, 6·53% H, 9·00% N; found, 62·9% C, 6·77% H, 9·1% N.

Boc-Met(O)-Tyr(Cl_2Bzl)-Cys(DPM)-Ala-Pro-Leu-Lys(Z)-Pro-Ala-
-Lys(Z)-Ser(Bzl)-Ala-OBzl (*XI*)

Compound *X* (6·5 g) was deblocked as usual and the free base of the resulting product was isolated as described in the synthesis of compound *II*. To a solution of this product in DMF (30 ml) were added Boc-Met(O)-OH (2·7 g) and EEDQ (2·8 g). After 24 h the mixtuye was diluted with ethyl acetate (500 ml) and this solution was washed (1M-$KHCO_3$, 0·2M-HCl and water) and concentrated to a small volume. Addition of methanol (30 ml) and ether (200 ml) completed the precipitation of the product which was collected and reprecipitated from 95% ethanol; 6·0 g (86%); m.p. 198–200°C; $[\alpha]_D^{25}$ −50·1° (*c* 1, DMF). For $C_{112}H_{140}N_{14}O_{22}S_2Cl_2$ (2 169) calculated: 62·01% C, 6·50% H, 9·04% N; found: 62·00% C, 6·49% H, 9·0% N.

Boc-Glu(OBzl)-Met(O)-Tyr(Cl_2Bzl)-Cys(DPM)-Ala-Pro-Leu-
-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*XII*)

Compound *XI* (4·3 g) was deblocked with TFA–AcOH in the usual way and the resulting trifluoroacetate salt was dissolved in DMF (20 ml). To this solution, neutralized with NMM, was then added the carboxyl component preactivated as follows. Boc-Glu(OBzl)-OH (3·4 g) was dissolved in DMF (15 ml) and to this solution HOBT (1·6 g) and DCC (2·0 g) were added. After 1 h at room temperature the urea by-product was filtered off and the filtrate mixed with the free base prepared as just described. After 24 h the reaction mixture was filtered and the product precipitaed by dilution of the filtrate with 2-propanol and ether and reprecipitated from 95% ethanol; 3·6 g (76%); m.p. 182–183°C; $[\alpha]_D^{25}$ −32·1° (*c* 1, DMF). For $C_{124}H_{153}N_{15}O_{25}$-$S_2Cl_2$.2 H_2O (2 425) calculated: 61·42% C, 6·53% H, 8·66% N; found, 60·8% C, 6·65% H, 8·4% N. Amino acid analysis: Ser 1·0, Pro 2·0, Glu 1·0, Ala 3·0, Met 0·6, Leu 1·0, Tyr 0·9, Lys 2·1. Cys was not determined.

Boc-Arg(Tos)-Leu-OBzl (*XIII*)

p-Toluenesulfonate of H-Leu-OBzl (10·1 g) was partitioned between ethyl acetate (400 ml) and 1M-$KHCO_3$ (30 ml) and the organic layer was separated, washed with water, dried and concentrated to dryness. To a solution of the residue in THF (50 ml) and DMF (5 ml), Boc-Arg(Tos)-OH (10·0 g) and EEDQ (6·9 g) were added. After 24 h the reaction mixture was concentrated to a small volume and then diluted with ethyl acetate. This solution was washed (1M-$KHCO_3$, 0·2M-HCl and water), dried and concentrated to dryness. From a solution of the residue in ether the product was precipitated by the addition of light petroleum; 13·7 g (93%); m.p. 68–70°C; $[\alpha]_D^{25}$ −27·1° (*c* 1, DMF). For $C_{31}H_{45}N_5O_7S$ (631·8) calculated: 58·92% C, 7·18% H, 11·08% N; found: 58·7% C, 7·28% H, 10·8% N.

Boc-Arg(Tos)-Arg(Tos)-Leu-OBzl (*XIV*)

Compound *XIII* (7·2 g) was deblocked with TFA (30 ml) and the free base was prepared as described for the synthesis of compound *II*. To a solution of this product in THF (40 ml) and DMF (5 ml) Boc-Arg(Tos)-OH (5·37 g) and EEDQ (2·95 g) were added. The reaction mixture was processed as described in the synthesis of compound *XIII* and the product was isolated by precipitation from methanol–ether; 9·0 g (86%); m.p. 99–100°C; $[\alpha]_D^{25}$ −31·4° (*c* 1, DMF). For $C_{44}H_{63}N_9O_{10}S_2.H_2O$ (960·2) calculated: 55·04% C, 6·82% H, 13·13% N; found: 55·3% C, 7·09% H, 12·9% N.

Boc-Leu-Arg(Tos)-Arg(Tos)-Leu-OBzl (*XV*)

Compound *XIV* (7·0 g) was deblocked with TFA and then coupled with Boc-Leu-OH (3·7 g) in the presence of EEDQ (3·1 g) following the procedure described for the synthesis of compound *XIV*. The product precipitated during concentration of the ethyl acetate solution; 5·1 g (65%); m.p. 181–184°C; $[\alpha]_D^{25}$ −29·1° (*c* 1, DMF). For $C_{50}H_{74}N_{10}O_{11}S_2$ (1 055·3) calculated: 56·91% C, 7·07% H, 13·27% N; found: 56·4% C, 7·20% H, 13·1% N.

Boc-Leu-Arg(Tos)-Arg(Tos)-Leu-OH (*XVI*)

Compound *XV* (3·0 g), dissolved in a mixture of methanol (100 ml) and DMF (20 ml), was hydrogenated over 10% Pd/C catalyst (3·0 g) for 3 h. The catalyst was then filtered off and the filtrate concentrated to dryness. The product was isolated from the residue by precipitation from DMF–water; 2·6 g (95%); m.p. 150–152°C; $[\alpha]_D^{25}$ −13·5° (*c* 1, DMF). For $C_{43}H_{68}N_{10}O_{11}S_2$. .$H_2O$ (983·2) calculated: 52·5% C, 7·18% H, 14·25% N; found: 52·3% C, 7·10% H, 14·2% N.

Boc-Leu-Arg(Tos)-Arg(Tos)-Leu-Glu(OBzl)-Met(O)-Tyr(Cl_2Bzl)-Cys(DPM)-Ala-
-Pro-Leu-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*XVII*)

The protected tridecapeptide *XII* (3·4 g) was treated with TFA–AcOH (7 : 3, v/v, 20 ml) for 30 min and subsequently the reaction mixture concentrated and dried by the addiion of toluene followed by evaporation under reduced pressure. To a solution of the residue in 1-methylpyrrolidinone (30 ml), TEA (1·4 ml) was added followed by ether (350 ml). The precipitated free base was collected and dissolved in DMF (30 ml). To this solution was then added the tetrapeptide derivative *XVI* (3·06 g) followed by DCC (0·6 g) and N-hydroxy-5-norbornene-2,3-dicarboxylic acid imide (0·55 g). After 72 h at 4°C the reaction mixture was poured into cold water (1 liter) containing saturated $KHCO_3$ (10 ml). The precipitated heptadecapeptide derivative was filtered off, washed with water, dried and reprecipitated from 95% ethanol; 3·2 g (70%); m.p. 174–178°C; $[\alpha]_D^{25}$ −34·4° (*c* 1, DMF). Amino acid analysis: Ser 1·2, Pro 2·0, Glu 1·0, Ala 3·2, Met 0·4, Leu 3·0, Tyr 1·0, Lys 1·9, Arg 2·0. Cys was not determined.

Boc-Asp(OBzl)-Leu-Arg(Tos)-Arg(Tos)-Leu-Glu(OBzl)-Met(O)-Tyr(Cl_2Bzl)-
-Cys(DPM)-Ala-Pro-Leu-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*XVIII*)

Compound *XVII* (3·2 g) was deblocked with TFA–AcOH and converted to the free base as described above. To a solution of this product in DMF (10 ml) was added the carboxyl component which was preactivated as follows. Boc-Asp(OBzl)-OH (1·57 g) was dissolved in DMF (10 ml) and to this solution HOBT (0·7 g) and DCC (1·0 g) were added. After 30 min at 0°C and 40 min at room temperature the mixture was filtered and the filtrate mixed with the solution of the free base prepared as just described. The mixture was stirred for 24 h, filtered, and the filtrate diluted with 2-propanol (30 ml) and ether (350 ml). The precipitated product was collected and reprecipitated from ethanol; 2·5 g (73%); m.p. 195–198°C; $[\alpha]_D^{25}$ −35·5° (*c* 1, DMF). Amino acid analysis: Asp 1·0, Ser 1·1, Pro 2·0, Glu 1·0, Ala 3·1, Met 0·6, Leu 3·0, Tyr 1·0, Lys 2·1, Arg 1·9. Cys was not determined.

Boc-Cys(DPM)-Asp(OBzl)-Leu-Arg(Tos)-Arg(Tos)-Leu-Glu(OBzl)-Met(O)-Tyr(Cl_2Bzl)-
-Cys(DPM)-Ala-Pro-Leu-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*XIX*)

Compound *XVIII* (2·5 g) was deprotected and converted to the free base as described above. A solution of this product in DMF (10 ml) was mixed with a solution of Boc-Cys(DPM)-OH

(2·0 g) in DMF (15 ml) preactivated in the presence of HOBT (0·8 g) and DCC (1·0 g) in the usual way. After 24 h the mixture was filtered and the filtrate was poured into dilute $KHCO_3$. The precipitated product was filtered off, washed, dried, triturated with warm ethyl acetate and reprecipitated from ethanol; 2 g (74%); m.p. 213—215°C; $[\alpha]_D^{25}$ −30·1° (*c* 1, DMF). Amino acid analysis: Asp 0·9, Ser 1·0, Pro 1·9, Glu 1·0, Ala 3·0, Met 0·6, Leu 3·0, Tyr 1·0, Lys 2·1, Arg 1·9. Cys was not determined.

Boc-Cys(DPM)-Cys(DPM)-Phe-Arg(Tos)-Ser(Bzl)-Cys(DPM)-Asp(OBzl)-Leu-Arg(Tos)--Arg(Tos)-Leu-Glu(OBzl)-Met(O)-Tyr(Cl_2Bzl)-Cys(DPM)-Ala-Pro-Leu-Lys(Z)-Pro-Ala--Lys(Z)-Ser(Bzl)-Ala-OBzl (*XX*)

A solution of compound *XIX* (1·4 g) in TFA–AcOH (7 : 3) (10 ml) was stored at room temperature for 45 min and then diluted with ether. The precipitated nonadecapeptide trifluoroacetate was dissolved in DMF (15 ml) and this solution was diluted with 1M-Na_2CO_3 (5 ml) and poured into cold water saurated with NaCl. The precipitated free base of the partially protected nonadecapeptide was collected and dried over P_2O_5 in vacuo. To a solution of this product in DMF (10 ml) was added the azide prepared from Boc-Cys(DPM)-Cys(DPM)-Phe-Arg(Tos)-Ser(Bzl)--$NHNH_2$ (ref.[13]) (1·93 g) in the usual way[9]. The reagents used in the conversion of this pentapeptide hydrazide to the corresponding azide are as follows. DMF (15 ml), 4·66M-HCl in DMF (0·96 ml), tert-butyl nitrite (0·27 ml) and TEA (0·64 ml). After 48 h at 4°C, the mixture was diluted with AcOH (1 ml) and 95% ethanol (30 ml) The precipitated product was filtered off and washed with water A suspension of the wet product in methanol (c. 200 ml) was heated to c 55°C and cooled at room temperature The solid product was then filtered off and washed with methanol; 1·4 g (43%); m.p. 170—172°C; $[\alpha]_D^{25}$ −30·1° (*c* 1, DMF). Amino acid analysis: Asp 1·0, Ser 1·9, Pro 1·9, Glu 1·0, Ala 2·9, Met 0·6, Leu 3·0, Tyr 0·9, Phe 1·2, Lys 2·0, Arg 3·1. Cys was not determined.

Boc-Glu(OBzl)-Cys(DPM)-Cys(DPM)-Phe-Arg(Tos)-Ser(Bzl)-Cys(DPM)-Asp(OBzl)--Leu-Arg(Tos)-Arg(Tos)-Leu-Glu(OBzl)-Met(O)-Tyr(Cl_2Bzl)-Cys(DPM)-Ala-Pro-Leu--Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*XXI*)

Compound *XX* (2·3 g) was deblocked in the usual way and the trifluoroacetate of the resulting deblocked product isolated by precipitation with ether. To a solution of this product in DMF (10 ml), pentachlorophenol (0·266 g) and TEA (0·2 ml) were added just before the addition of the carboxyl component which was preactivated as follows. Boc-Glu(OBzl)-OH (0·8 g) was dissolved in DMF (10 ml) and to this solution, cooled to 0°C, were added HOBT (0·5 g) and DCC (0·6 g). After 30 min at room temperature the urea by-product was filtered off and the filtrate mixed with the solution of the free base prepared as just described. After 24 h the reaction mixture was filtered and the filtrate poured into dilute $KHCO_3$. The precipitated product was collected and reprecipitated from DMF–ether; 2·3 g (97·8%); m.p. 180—181°C; $[\alpha]_D^{25}$ −23·4° (*c* 1, DMF). Amino acid analysis: Asp 0·9, Ser 2·3, Pro 1·9, Glu 2·0, Ala 3·0, Met 0·6, Leu 3·4, Tyr 1·0, Phe 1·2, Lys 2·0, Arg 3·2. Cys was not determined.

Boc-Asp(OBzl)-Glu(OBzl)-Cys(DPM)-Cys(DPM)-Phe-Arg(Tos)-Ser(Bzl)-Cys(DPM)--Asp(OBzl)-Leu-Arg(Tos)-Arg(Tos)-Leu-Glu(OBzl)-Met(O)-Tyr(Cl_2Bzl)-Cys(DPM)-Ala--Pro-Leu-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*XXII*)

Compound *XXI* (2·2 g) was deblocked and the trifluoroacetate salt of the deprotected peptide was isolated in the usual way. To a solution of the product in DMF (6 ml), pentachlorophenol (0·27 g) and TEA (0·2 ml) were added just before the addition of the carboxyl component pre-

activated as usual. Reagents used are as follows: Boc-Asp(OBzl)-OH (1·37 g), DMF (6 ml), HOBT (0·57 g) and DCC (0·88 g). The reaction mixture was processed as in the synthesis of compound *XXI*; 2·3 g (95·8%); $[\alpha]_D^{25}$ −27·4° (*c* 1, DMF); m.p. 235−237°C (dec.). Amino acid analysis: Asp 2·1, Ser 2·1, Pro 1·8, Glu 2·2, Ala 2·9, Met 0·6, Leu 2·9, Phe 1·1, Tyr 0·9, Lys 1·6, Arg 2·9. Cys was not determined.

Boc-Val-Asp(OBzl)-Glu(OBzl)-Cys(DPM)-Cys(DPM)-Phe-Arg(Tos)-Ser(Bzl)-Cys(DPM)-
-Asp(OBzl)-Leu-Arg(Tos)-Arg(Tos)-Leu-Glu(OBzl)-Met(O)-Tyr(Cl_2Bzl)-Cys(DPM)-Ala-
-Pro-Leu-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*XXIII*)

This compound was synthesized by the same procedure used in the synthesis of compound *XXI*. The materials used are as follows. Compound *XXII* (2·2 g), TEA (0·2 ml), pentachlorophenol (0·27 g), Boc-Val-OH (0·71 g), HOBT (0·45 g) and DCC (0·67 g). A suspension of the final product in a mixture of ethyl acetate (75 ml) and ethanol (25 ml) was warmed to 60°C, cooled to room temperature and diluted with ether (350 ml); 2·2 g (98%); m.p. >250°C; $[\alpha]_D^{25}$ −25·7° (*c* 1, DMF). Amino acid analysis: Asp 2·1, Ser 1·9, Pro 2·0, Glu 2·0, Ala 2·8, Val 1·3, Met 0·6, Leu 3·0, Phe 1·1, Tyr 0·9, Lys 1·8, Arg 2·9. Cys was not determined.

Boc-Ile-Val-Asp(OBzl)-Glu(OBzl)-Cys(DPM)-Cys(DPM)-Phe-Arg(Tos)-Ser(Bzl)-
-Cys(DPM)-Asp(OBzl)-Leu-Arg(Tos)-Arg(Tos)-Leu-Glu(OBzl)-Met(O)-Tyr(Cl_2Bzl)-
-Cys(DPM)-Ala-Pro-Leu-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*XXIV*)

This compound was synthesized by the same procedure described above. The materials used are as follows. Compound *XXIII* (2·2 g), pentachlorophenol (0·27 g), TEA (0·2 ml), Boc-Ile-OH (1·3 g) HOBT (0·91 g) and DCC (1·3 g); 2·1 g (93%); m.p. >250°C. Amino acid analysis: Asp 2·0, Ser 1·7, Pro 2·1, Glu 2·1, Ala 2·8, Val 0·8, Met 0·6, Ile 0·7, Leu 2·8, Phe 1·1, Tyr 0·9, Lys 2·2, Arg 2·9. Cys was not determined.

Z-Gly-Ile-Val-Asp(OBzl)-Glu(OBzl)-Cys(DPM)-Cys(DPM)-Phe-Arg(Tos)-Ser(Bzl)-
-Cys(DPM)-Asp(OBzl)-Leu-Arg(Tos)-Arg(Tos)-Leu-Glu(OBzl)-Met(O)-Tyr(Cl_2Bzl)-
-Cys(DPM)-Ala-Pro-Leu-Lys(Z)-Pro-Ala-Lys(Z)-Ser(Bzl)-Ala-OBzl (*XXV*)

This nonacosapeptide was synthesized by the procedure described in the preparation of compound *XXIII*. The following materials were used. Compound *XXIV* (2·1 g), pentachlorophenol (0·27 g), TEA (0·2 ml), Z-Gly-OH (1·23 g), HOBT (0·8 g) and DCC (1·2 g). The final product was triturated with absolute ethanol; 2·0 g (94%). Amino acid analysis: Asp 2·1, Ser 1·6, Pro 1·9, Glu 2·3, Gly 1·4, Ala 3·0, Val 0·8, Met 0·6, Ile 0·8, Leu 2·7, Tyr 0·9, Phe 0·9, Lys 2·2, Arg 2·8. Cys was not determined.

Gly-Ile-Val-Asp-Glu-Cys(SO_3^-)-Cys(SO_3^-)-Phe-Arg-Ser-Cys(SO_3^-)-Asp-Leu-Arg-Arg-
Leu-Glu-Met-Tyr-Cys(SO_3^-)-Ala-Pro-Leu-Lys-Pro-Ala-Lys-Ser-Ala-OH
(Human IGF-I [A-D]-Chain S-Sulfonate) (*XXVI*)

The deblocking of the nonacosapeptide derivative *XXV* with liquid hydrogen fluoride in the presence of 2-mercaptopyridine and *p*-cresole and the sulfitolysis of the resulting reduced polypeptide chain was carried out essentially by the procedure used in the synthesis of human insulin B chain S-sulfonate[23]. The protected nonacosapeptide *XXV* (200 mg) was treated with anhydrous liquid hydrogen fluoride (10 ml) in the presence of 2-mercaptopyridine (200 mg) and *p*-cresole (1·0 g) for 80 min at 0°C. After removal of the liquid hydrogen fluoride, by the use of a vacuum

pump, the residue was triturated with ethyl acetate (3×; 50 ml each) and light petroleum (2× 50 ml each) and dried in high vacuum over KOH. This product was subsequently dissolved in 8M-guanidine hydrochloride (20 ml) and to this solution adjusted to pH 8·9 with dilute NH_4OH, were added sodium sulfite (800 mg) and freshly prepared sodium tetrathionate (600 mg). The mixture was stirred for 4 h at room temperature and then placed in a Spectrapor membrane tubing no. 3, and dialyzed against four changes of distilled water (4 liters each) at 4° C for 24 h. Lyophilization of the dialysate afforded the crude S-sulfonated human IGF-I [A—D]-chain as a white powder (130 mg).

For purification this product (56 mg) was dissolved in 5 ml, 0·02M-Tris-HCl buffer (pH 7·4) and applied to a Whatman DE-52 cellulose column (1·2 × 24 cm) equilibrated with the same buffer. Elution of the column was carried out with a linear NaCl gradient formed by adding to the above buffer (200 ml) 0·4M-NaCl in the same buffer (200 ml). The effluent of the column was monitored by an ISCO spectrophotometer at 278 nm and a conductivity meter (Radiometer, Copenhagen). The elution pattern is shown in Figure 2. The eluate under the major peak (200 to 240 ml) was collected, dialyzed (Spectrapor membrane tubing no. 3) against four changes of distilled water (4 liters each) and lyophilized to a white fluffy material (22 mg).

Amino acid analysis of the purified material after acid hydrolysis gave a composition expressed in molar ratios in good agreement with the theoretically expected values (Table II). Digestion of the synthetic material with aminopeptidase M and amino acid analysis of the digest gave the molar ratios shown in Table II. It is apparent that the synthetic polypeptide was completely digested by the enzyme indicating that the stereochemical homogeneity of the constituent amino acids was preserved during the synthetic processes. On electrophoresis on cellulose acetate membranes in Tris (0·024 mol l^{-1})–barbital (0·006 mol l^{-1})–sodium barbital (0·024 mol l^{-1}) buffer, pH 8·8 and 250 V, the synthetic chain exhibited a single band (data not shown).

S-Sulfonated B Chain of Bovine Insulin

The B chain S-sulfonate was prepared by oxidative sulfitolysis of bovine insulin followed by separation of the resulting A and B chain S-sulfonates by carboxymethyl cellulose chromatography as we have reported previously[18] with the only difference that the sulfitolysis was carried out for 3 instead of 24 h.

Synthesis and Isolation of the Insulin-like Compound Consisting of the [A—D]-Chain of IGF-I and the B chain of Insulin

The synthesis of this hybrid molecule was accomplished by the interaction of the S-sulfonated derivative of the [A—D]-chain of IGF-I with the S-sulfonated B chain of insulin in the presence of dithiothreitol at pH 10·5 as described previously[24]. To a solution of human IGF-I [A—D]-chain S-sulfonate (*XXVI*) (30 mg) and insulin B chain S-sulfonate (25 mg) in 0·1M-glycine buffer (pH 10·5; 9 ml) cooled to 4°C, dithiothreitol (7 mg) was added. After 24 h at 4°C the mixture was diluted with acetic acid (1 ml) and chromatographed on a Sephadex G-50 column (2·5 × 48 cm), equilibrated and eluted with 1M-acetic acid. The effluent from the peak representing the monomer fraction (c. 120—160 ml; using insulin as standard), which contains the biologically active material (insulin assays) was collected and lyophilized; c. 5 mg. This material was subjected to reversed phase HPLC using a Vydac 218 TP column (0·45 × 25 cm) connected to a Laboratory Data Control liquid chromatography system. Elution of the column was carried out with a 10 to 50% linear gradient of 2-propanol in 0·1% trifluoroacetic acid over 60 min at a flow rate of 0·5 ml/min. The chromatographic profile monitored at 278 nm is shown in Figure 3. The fraction containing the active material (insulin assays) eluting at c. 39 min was collected, concentrated

to a small volume and rechromatographed under the same conditions. The chromatogram exhibited a sharp single peak (data not shown). The effluent under this peak was collected and used for amino acid analysis and biological evaluation; 200 μg. Amino acid analysis of the purified material after acid hydrolysis gave the following composition, expressed in molar ratios, in good agreement with the theoretically expected values (shown in parenthesis): Asp 3·4 (3), Thr 0·8 (1), Ser 3·0 (3), Pro 2·9 (3), Glu 5·3 (5), Gly 3·9 (4), Lys 3·0 (3), His 1·9 (2), Met 0·7 (1), Ile 0·5 (1), Leu 6·7 (7), Tyr 2·5 (3), Pre 3·6 (4), Lys 3·0 (3), His 1·9 (2), Arg 4·2 (4). Cys was not determined.

BIOLOGICAL EVALUATION

The hybrid compound was compared with bovine insulin with regard to competition with ^{125}I-insulin in binding to insulin receptors in rat liver plasma membranes. In five separate assays, we were unable to obtain consistent values of relative potency

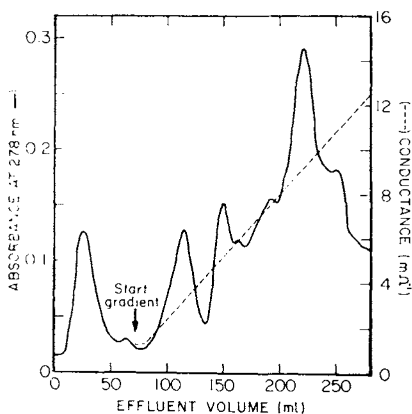

FIG. 2

Chromatography of crude IGF-I [A—D]--chain S-sulfonate on a 1·2 × 24 cm Whatman DE 52 column with 0·02M-Tris-HCl buffer (pH 7·4) and a linear NaCl gradient. The column effluent was monitored by an ISCO recording spectrophotometer and a conductivity meter. The S-sulfonated chain was recovered by dialysis and lyophilization of the effluent (200—240 ml)

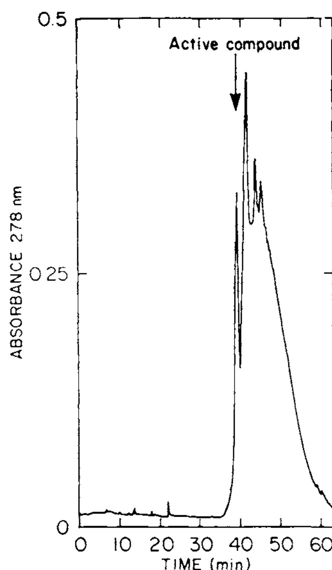

FIG. 3

Reversed-phase HPLC of the combination mixture of the S-sulfonated human IGF-I [A—D]-chain and S-sulfonated bovine insulin B chain on a 0·45 × 25 cm Vydac 218 TP column at 0·5 ml/min with a 10—50% linear gradient of 2-propanol in 0·1% TFA over 60 min. The active material A_{IGF-I} — D_{IGF-I}/B_{INS}) was eluted at c. 39 min

TABLE II

Amino acid composition of an acid hydrolysate and an enzymatic digest (Aminopeptidase M) of the S-sulfonated human IGF-I [A—D]-chain

Amino acid	Acid hydrolysis		Enzymatic hydrolysis	
	theory	found	theory	found
Lys	2	1·9	2	2·0
Arg	3	3·0	3	2·6
Asp	2	2·0	2	1·8
Sér	2	1·7	2	2·2
Pro	2	2·0	2	2·1
Glu	2	2·0	2	1·7
Gly	1	1·0	1	1·6
Ala	3	3·0	3	3·5
Cys	4	[a]	0	0
Val	1	0·6	1	1·1
Met	1	0·8	1	1·0
Ile	1	0·6	1	1·3
Leu	3	3·0	3	2·9
Tyr	1	0·9	1	1·0
Phe	1	0·9	1	0·9
S-Sulfo-Cys	0	0	4	4·5

[a] Not determined.

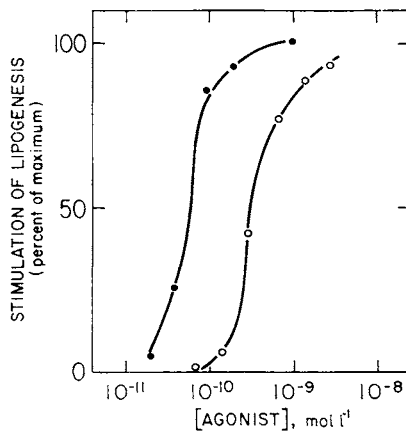

FIG. 4

Effect of bovine insulin (●) and hybrid compound (○) upon the stimulation of lipogenesis in isolated rat adipocytes. The stimulation, expressed as percent of maximum, is presented as a function of the molar concentration of test compound. The data points represent the means of triplicate determinations in a representative experiment which was performed three times. In this experiment, zero and 100% stimulation refer respectively to 6·4 and 94·0 nmol glucose converted into organic-extractable material per mg cells per hour

for the hybrid compound, and no dose-response curve is presented. We obtained a range of 2·8–41·7% relative to insulin, with no trend toward higher or lower values over time, using three different preparations of plasma membranes. We have observed ranges of this magnitude in receptor-binding assays only once before[25]. At that time, we ascribed the variation in potency to limited solubility of the test compound, resulting in unpredictable precipitation of the test compound during the assay. A similar situation is possible with the present compound, although there was no evidence of gross precipitation of the hybrid compound in stock solutions.

We were, however, able to obtain consistent values for the potency of the hybrid compound in lipogenesis assays, perhaps because the inherently greater sensitivity of this assay permitted the use of lower concentrations of test compound. The ability of bovine insulin and the hybrid compound to stimulate lipogenesis in isolated rat adipocytes is shown in Figure 4. The hybrid compound is a full agonist, reaching the same maximum stimulation as that seen with insulin, and the dose-response curves are essentially parallel. The calculated potency of the hybrid compound, based upon three experiments, is 21·2 ± 3% relative to bovine insulin.

In insulin radioimmunoassays (data not shown), the hybrid compound showed little cross-reactivity with the anti-insulin antibody, giving a calculated potency of 0·16% relative to bovine insulin.

The hybrid compound was compared with IGF-I and with insulin in growth factor assays[15]. The synthetic compound displayed c. 440% the potency of insulin and c. 15·4% the potency of IGF-I in stimulating the incorporation of ^{3}H-thymidine in chick embryo fibroblasts; in assays measuring the ability to displace ^{125}I–IGF-I from IGF receptors in the same cells the hybrid showed 570% the potency of insulin and c. 21·9% the potency of IGF-I. The synthetic compound, however, showed no reactivity toward IGF carrier proteins derived from rat serum.

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