Semisynthetic Analogues of Insulin

THE USE OF *N*-SUBSTITUTED DERIVATIVES OF METHIONINE AS ACID-STABLE PROTECTING GROUPS

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1. We describe the use of benzyloxycarbonylmethionine and ethoxycarbonylmethionine for the selective protection of the amino groups of glycine-A1 and lysine-B29 of pig insulin. We have used the Edman method to remove residues from the *N*-terminal end of the B-chain of the $N^{A1}N^{B29}$ -di-protected derivatives. The benzyloxycarbonyl group shows slight but noticeable lability in the acid-cleavage step, but the ethoxycarbonyl group remained intact even after five cycles of degradation. 2. We have prepared the following truncated forms of insulin via the di(ethoxycarbonylmethionyl) derivative: des-Phe^{B1}-insulin; des-(Phe^{B1}-Val^{B2})-insulin; des-(Phe^{B1}-Val^{B2}-Asn^{B3})-insulin; des-(Phe^{B1}-Val^{B2}-Asn^{B3}-Gln^{B4})-insulin; des-(Phe^{B1}-Val^{B2}-Asn^{B3}-Gln^{B4}-His^{B5})-insulin. 3. Insulin was re-synthesized from the di-protected des-Phe^{B1}-insulin by reaction with an active ester of t-butoxycarbonyl-L-phenylalanine. The product after deprotection crystallized, and the immunoreactivity of the crystalline material was identical with that of the native protein. 4. We have prepared the following analogues of insulin in a similar manner: [L-Ala^{B1}]insulin; [L-Val^{B1}]insulin; [L-Tyr^{B1}]insulin; [*m*-F-L-Phe^{B1}]insulin; [*o*-F-L-Phe^{B1}]insulin; [*o*-F-L-Phe^{B2}]des-Phe^{B1}-insulin. All had between 34 and 62% of the activity of insulin in the fat-cell test. 5. We have also investigated the use of the benzoyl, toluene-*p*-sulphonyl, *p*-nitrobenzyloxycarbonyl and 2,4-dinitrophenyl groups for the *N*-protection of the methionine active esters. Each should have had some particular advantage over the benzyloxycarbonyl and ethoxycarbonyl groups, but all proved in practice to have disadvantages that more than outweighed anything in their favour.

Several workers have carried out *N*-terminal modifications on insulin by stepwise semisynthesis. Usually residues are first removed by the Edman method and then replaced by active-ester coupling, although occasionally the degradation step has been omitted. The operations have been carried out on the A and B chains simultaneously (Brandenburg & Ooms, 1968) or on separated chains (Weinert *et al.*, 1969; Shimonishi, 1970; Weinert *et al.*, 1971; Brandenburg *et al.*, 1975; Krail *et al.*, 1975a), but it is probably best if at all possible to leave the disulphide bridges intact and exploit the selective reactivity of one or other of the α -amino groups in order to produce modifications in one chain only (Borras & Offord, 1970a,b; Krail *et al.*, 1971; Saunders & Offord, 1972; Krail *et al.*, 1975a,b; Geiger *et al.*, 1975a; Halban & Offord, 1975). All of the semisyntheses in this last group, whether they were of A-chain or B-chain analogues, involved a stage in which the amino groups of glycine-A1 and lysine-B29 were reversibly protected, leaving only the amino group of phenylalanine-B1 available for further reaction. Several of the papers cited use the method of Geiger *et al.* (1971) to achieve this protection. Geiger *et al.* (1971) found conditions in which the

Boc* group could be applied to residues A1 and B29 with only a little reaction on residue B1. Although it was necessary to separate the [N^{α} -Boc-Gly^{A1}, N^{α} -Boc-Lys^{B29}]insulin from the small quantities of mono- and tri-Boc-insulins also formed, the di-derivative was found to be substantially free of the other two possible isomers. Phenylalanine-B1 can be removed from this derivative by the Edman method, but, since the Boc group is labile to the acid used in the cyclization step, the amino-group protection is lost. Fortunately, the Boc groups can be reapplied to residues A1 and B29 of the des-Phe^{B1}-insulin with similar selectivity and, after purification, the diBoc derivative is ready to have residues replaced at position B1 (Krail *et al.*, 1971; Halban & Offord, 1975). Alternatively, as Geiger & Langner (1973) found, the diBoc-des-Phe^{B1}-insulin can be degraded further by successive applications of the Edman procedure followed each time by selective re-protection and isolation of the [N^{α} -Boc-Gly^{A1}, N^{α} -Boc-Lys^{B29}] derivative. They prepared by this means des-

* Abbreviations: Boc, t-butoxycarbonyl; Z, benzyloxycarbonyl; Dns, 5-dimethylaminonaphthalene-1-sulphonyl; Eoc, ethoxycarbonyl.

(Phe^{B1}-Val^{B2})-insulin and des-(Phe^{B1}-Val^{B2}-Asn^{B29})-insulin. The *N*-terminal residue of the B-chain of the latter derivative, glutamine-B4, was found to be partially cyclized to pyroglutamic acid. Geiger & Langner (1973) do not report whether or not the parent compound of the des-(Phe^{B1}-Val^{B2}-Asn^{B3})-insulin {the [*N*^ε-Boc-Gly^{A1}, *N*^ε-Boc-Lys]des-(Phe^{B1}-Val^{B2})-insulin} was free of its other two possible isomers, in which the Boc groups are on residues A1 and B3 or on B3 and B29.

It is clearly desirable to avoid the need to re-protect and purify the derivative each time by the use of an acid-stable group for the amino-group protection. Geiger & Langner (1975) found that the phthaloyl group (which is stable to acid) was as easily directed to residues A1 and B29 as was the Boc group, but that although satisfactory for less extensively truncated molecules, its removal from the des-(Phe^{B1}-Val^{B2}-Asp^{B3}-Gln^{B4}-His^{B5})-insulin by hydrazine led to some degradation of the protein molecule. Geiger *et al.* (1975b) have reported the selective protection of residues A1 and B29 by the methylsulphonylethoxycarbonyl group, but although insulin itself appears to withstand the vigorously alkaline conditions needed for deprotection, there is as yet no report of the effect of these conditions on insulin analogues, some of which might be less stable. Busse & Carpenter (1974, 1976) and Busse *et al.* (1974) have reported that methionine derivatives can be cleaved from the amino groups of insulin by CNBr without apparent damage to the rest of the protein. We report here the use of active esters of amino-protected methionine for the selective protection of residues A1 and B29. The resulting di-derivatives have been used for the preparation of a number of truncated forms of insulin, and, by replacement with other amino acids, some analogues modified at positions B1 and B2. Part of this work has been described by Saunders (1974).

Materials

Pig insulin (3x crystallized, lot no. S311069 MS) was from Novo Terapeutisk Laboratorium A/S, Copenhagen, Denmark. DL-Methionine and benzyl chloroformate were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Ethyl chloroformate (laboratory reagent) was from BDH Chemicals, Poole, Dorset, U.K. Formic acid (98–100%, BDH, analytical grade), methanol (BDH, analytical grade) and CNBr (Koch-Light) were all used without further purification. L-Phenylalanine, L-valine and Boc-L-tyrosine were from Sigma (London) Chemical Co., London S.W.6, U.K. Boc-L-alanine was a gift from Dr. J. Warnke of the Dyson Perrins Laboratory, Oxford. Dicyclohexylcarbodi-imide (Koch-Light, puriss grade) was used without purification. Urea (BDH analytical grade) was prepared as an 8M

solution and filtered through macerated Whatman no. 3 paper. It was deionized before use by passage through a column of Amberlite MB3 monobed resin (BDH). DEAE-Sephadex A25 and Sephadex LH-20 were from Pharmacia (G.B.), London W.5, U.K. Tris was from Sigma (Trizma base, reagent grade). Polyacrylamide thin-layer plates for identification of Dns-amino acids were from BDH, and used as described by Gonzalez & Offord (1971). All other materials were obtained and purified as described by Halban & Offord (1975).

Methods

Preparation of protected methionyl N-hydroxy-succinimido esters

Z-DL-methionine was prepared by the method of Dekker & Fruton (1948). A strictly analogous method was used to prepare ethoxycarbonyl-DL-methionine, which could only be obtained as an oil. Yields of both products were generally about 60%. These materials were converted into their *N*-hydroxy-succinimido esters by the method of Anderson *et al.* (1964), but in ethyl acetate as the solvent. Recrystallization of the Z-derivative gave a compound melting at 100–101°C (literature 101–102°C). The ethoxycarbonyl derivative did not crystallize. Yields in both cases were about 80%.

Reaction of the methionyl N-hydroxysuccinimido esters with insulin

These conditions are similar to those of Geiger *et al.* (1971). Insulin (250mg, 125μmol of NH₂ groups) was suspended in 10ml of fresh 5% (w/v) NaHCO₃ and stirred at room temperature (20°C). Methanol (40ml) was added slowly. The insulin had dissolved by the time that the addition of the methanol was half complete. Any slight precipitate of bicarbonate was ignored. The Z or ethoxycarbonyl active ester (416μmol) was then added. The Z-derivative was added either as the solid or in solution in the methanol, but the ethoxycarbonyl derivative was always added in solution. The coupling reaction was left to continue for 16h at 20°C. A quantity of 6M-HCl equivalent to the NaHCO₃ was then carefully added. The methanol was removed under reduced pressure on a rotary evaporator. About 20ml of water was added to the sample half-way through this process. Evaporation was stopped when the volume of the sample had fallen to approx. 20ml. The resulting precipitate was collected by centrifugation, and the supernatant adjusted to pH4.8 with 1M-HCl. Any further precipitate was centrifuged down in a bench centrifuge and pooled with the original precipitate before being washed twice with 25ml of water, three times with acetone,

and twice with diethyl ether. The solids were air-dried. Little protein remained in the supernatants, as judged both by recovery of protein and by u.v. spectroscopy. Trial experiments suggested that the best yields of di(acylmethionyl)insulins were obtained with a 3.3-fold excess of active ester over amino groups. The approximate distribution of insulin derivatives was found by isoelectric focusing (see below) to contain no detectable unchanged insulin, about 10% mono-derivative, about 85% di-derivative, and about 5% tri-derivative. In contrast, the best yield of mono-derivative was obtained with a 1:1 molar ratio of active ester to amino groups.

We also investigated a completely volatile solvent system (methanol/water/*N*-ethylmorpholine, 32:8:1 by vol.) from which the protein derivative could be recovered simply by freeze-drying. When the same excess of the Z-form of the active ester as before was used, a shift in the balance of products occurred towards more acylated derivatives. Indeed, only di- and tri-derivatives were observed, in the approximate ratio 3:1. The addition of 1% (w/v) NaCl to the solvent system produced the spectrum of products characteristic of the non-volatile system.

Separation of (acylmethionyl)insulins

The ion-exchange system used by Brandenburg *et al.* (1972) for the separation of acetylinsulins was generally used for the purification of insulin with ethoxycarbonylmethionyl groups on residues A1 and B29. Derivatives were eluted from a DEAE-Sephadex A-25 column (2.4 cm × 13 cm) run at room temperature by applying a linear salt gradient (50–200 mM-NaCl in 7 M-urea/0.1 M-Tris, adjusted to pH 8.4 with conc. HCl) of total volume 800 ml. The di-derivative emerged at a salt concentration of about 133 mM. Peak tubes were pooled, adjusted carefully to pH 4.8 with conc. HCl, and dialysed against deionized water. The protein was recovered by freeze-drying. Up to 250 mg of insulin could be handled on a column of this size. The material was dissolved at a concentration of about 20 mg/ml in unadjusted starting eluant, and then adjusted to pH 8.4 with HCl before loading. Pure di-derivative, identified as such by isoelectric focusing, constituted 70–80% of the recovered material, and end-group determination indicated that it was substantially the homogeneous [*N*^α-acyl-Met-Gly^{A1},*N*^ε-acyl-Met-Lys^{B29}]insulin.

The hydrophobic nature of the Z-methionyl group suggested that insulins containing it should be separated well on the partition-chromatography system of Geiger & Langner (1973) and good separations of about 120 mg of mixed Z-methionyl-insulins were achieved with a short column (1.8 cm × 38 cm) of Sephadex LH-20 saturated with butan-1-ol and with flow rates between 40 and 80 ml/h. The

method is a simple and rapid one, particularly as pooled fractions could be recovered directly by freeze-drying, without the need for dialysis. Overall recoveries of pure di-derivative were 60–70%. Quantities larger than 120 mg could be handled by this column, but the tendency of the di-derivative to precipitate while being eluted made it difficult to monitor the effluent by u.v. absorption, and can lead to losses as a result of leakage caused by back-pressure.

*Edman degradation of the [*N*^α-acyl-Met-Gly^{A1},*N*^ε-acyl-Met-Lys^{B29}]insulins*

On reaction of the purified di(ethoxycarbonylmethionyl)insulin (145 mg) with phenyl isothiocyanate (40 μl) in 29 ml of pyridine/water solution (3:1, v/v) overnight at 8°C, a product was obtained which moved as tri-derivative on isoelectric focusing. It was recovered from the reaction mixture by removal of most of the pyridine under reduced pressure on the rotary evaporator (with particular care to avoid frothing towards the end of the operation), adjusting the mixture to pH 4.5 with dilute HCl, and centrifugation in the bench centrifuge. The pellet was washed twice each with water, acetone and diethyl ether, and then air-dried.

In contrast, the above conditions led to incomplete phenylthiocarbamoylation of di-(Z-methionyl)-insulin, even when larger excesses of phenyl isothiocyanate were used. Complete reaction was obtained as follows. The di-derivative was dissolved in degassed 8 M-urea at pH 8.75 at a concentration of 1–2 mg/ml, and the solution flushed with N₂. The pH was maintained by an autotitrator delivering 0.2 M-NaOH. Phenyl isothiocyanate (2 μl/mg of insulin derivative) was added and the inhomogeneous mixture stirred vigorously under a stream of N₂ at room temperature for 7–16 h. (The purpose of the N₂ was to prevent oxidation of the phenylthiocarbamoyl groups, which, as judged by the subsequent failure of the product to undergo acid cleavage, occurred to a serious extent if it was not used.) The reaction was stopped by adjusting to pH 4.5 with dilute HCl. A dense precipitate formed which, after recovery by centrifugation in the bench centrifuge, was washed once with water, twice with acetone and twice with diethyl ether and air-dried. Approx. 70–80% of the initial weight of di-derivative was recovered in this way. The remainder was recovered by dialysis of the supernatant against deionized water and freeze-drying, and pooled with the original precipitate.

Cyclization and removal of the phenylthiocarbamoyl residue was effected by treatment with redistilled anhydrous trifluoroacetic acid for 45 min at room temperature, with a protein concentration of about 10 mg/ml. Approx. 75% of the acid was then

removed under reduced pressure and the protein recovered by precipitation with cold diethyl ether. The precipitate was centrifuged down in the bench centrifuge and washed several times with ether before being allowed to dry in air.

The product of the acid treatment was found to behave mainly as a di-derivative on isoelectric focusing. A faint band of mono-derivative was seen, which was attributed to there having been some slight removal of the Z group (5–10%, as judged by the intensity of the band). For simple replacements at the B1-position this degree of removal might be tolerated, but for insulins that require further Edman degradation purification is necessary after each cycle. The Sephadex LH-20 partition column described above was used for this purpose.

It is probably preferable to use the ethoxycarbonyl protection when repeated degradations are planned, since this group was found, as expected, to be completely stable to acid treatment even after five cycles of degradation by the above conditions (see Table 2 below).

Preparation of fluorophenylalanines for coupling

The racemic fluorophenylalanines were first converted into the *N*^α-Boc derivatives as described below and these were then resolved by a modification of the method developed by Bennett & Nieman (1950) for *N*^α-acetyl derivatives. The *N*^α-Boc derivatives were dissolved (3%, w/v) in 0.5M-sodium acetate/0.025M-cysteine hydrochloride, pH 6.0, and redistilled phenylhydrazine (1 equivalent) stirred into solution. A quantity of crude papain (Sigma, type III) was added, equal in weight to one-quarter of the amino acid substrates and the pH adjusted to 6.0 with dilute NaOH or acetic acid as necessary. After incubation at 37°C for 16h, the precipitated *N*^α-Boc-fluorophenylalanine phenylhydrazide was collected by filtration and washed with water. The vacuum-dried solid was dissolved in ethyl acetate (approx. 10mg/ml) and twice extracted with an equal volume of NaHCO₃ (5%, w/v). The organic layer was then dried over anhydrous Na₂SO₄, filtered and dried down by rotary evaporation. The residue was recrystallized twice from methanol with the addition of some water. The m.p. (uncorr.) of the *o*-isomer was 150–151°C. The *m*-isomer did not give a distinct m.p. (The *p*-isomer, which was also prepared, although not required for the present study, had a m.p. of 133–134°C.) Yields based on the original content of the L-form were 50–60%. The $[\alpha]_D^{20}$ value (1%, w/v, in methanol) was -17.3° for the *o*-isomer (and -17.1° for the *p*-isomer). Prolonged further incubation of the papain digests produced precipitates which after isolation and recrystallization had essentially the same m.p. The $[\alpha]_D^{20}$ values were of equal magnitude, but opposite sign. As is customary,

the early products were taken to be the L-forms and the late products the D-form.

The *N*^α-Boc-fluoro-L-phenylalanine phenylhydrazides were converted into the *N*^α-Boc-fluoro-L-phenylalanines by refluxing at a concentration of approx. 20mg/ml in water/methylcellosolve (approx. 1:1, v/v) for 2h with an approximately 10-fold molar excess of K₃Fe(CN)₆. We found in preliminary experiments that the Boc group was stable to these conditions. The mixtures were then cooled and adjusted to pH 3 with solid KHSO₄. They were then extracted with ethyl acetate and the organic layers dried and evaporated as above. Small samples of the product were deprotected with anhydrous trifluoroacetic acid and subjected to paper electrophoresis at pH 1.9 as described by Gonzalez & Offord (1971). Each isomer gave a major spot with ninhydrin of $m = 0.64$ (serine = 1.0) and a minor spot (roughly 10–20% of the total ninhydrin colour) of $m = 1.2$. The major spot was taken to be the amino acid (the racemic starting product had the same mobility). The minor spot probably resulted from di-(*N*^α-Boc-fluoro-L-phenylalanyl)phenylhydrazide, which the work of Milne *et al.* (1957) suggested would result from a side reaction during the oxidative step. The *N*^α-Boc-amino acids were easily freed from the by-product by extracting a solution of the crude material in ethyl acetate (approx. 20mg/ml) with aqueous NaHCO₃ (5%, w/v). The pure *N*^α-Boc-compounds could be recovered by re-acidification to pH 3 and re-extraction into ethyl acetate, as above, and their homogeneity was checked by electrophoresis. After recrystallization from diethyl ether with the addition of some hexane and cooling to -20°C , the yields were 80–85% based on the phenylhydrazide. Melting points were: *o*-, 64–67°C; *m*-, 72°C; (that of the *p*-isomer was 128–130°C). The hydroxysuccinimido esters of the *N*^α-Boc-fluoro-L-phenylalanines were prepared as described below for the other amino acids and recrystallized from propan-2-ol: melting points were: *o*-, 158–159°C; *m*-, 124–126°C (*p*-, 158–159°C).

Coupling of amino acid derivatives to [N^α-acyl-Met-Gly^{A1}, N^α-acyl-Met-Lys^{B29}]-des-Phe^{B1}-insulin

Boc-amino acyl-*N*-hydroxysuccinimido esters were used for most couplings (Table 1). They were prepared from the L-amino acids by the method of Anderson *et al.* (1964) via the *N*^α-Boc-amino acids [prepared and recrystallized by the methods of Schwyzer *et al.* (1959)]. 2,4,5-Trichlorophenyl esters were prepared in a similar manner.

Typically, couplings were performed by treating the truncated di-derivative with an 8–15-fold molar excess of active ester at a protein concentration of 3–10mg/ml in freshly redistilled dimethylformamide (Table 1). A few microlitres of *N*-ethylmorpholine

Table 1. *Conditions for coupling*

Active ester	Molar excess of active ester over amino groups	Molar excess of 1-hydroxybenztriazole over amino groups	Time for coupling at 20°C (h)
Boc-L-phenylalanine hydroxysuccinimido ester	11	20	17
Boc-L-alanine hydroxysuccinimido ester	10	20	16
Boc-L-valine hydroxysuccinimido ester	20	20	22
Boc-L-tyrosine hydroxysuccinimido ester	15	20	18
Boc- <i>m</i> -fluoro-L-phenylalanine hydroxysuccinimido ester	16	16	18
Boc- <i>o</i> -fluoro-L-phenylalanine hydroxysuccinimido ester	16	16	18

were used to adjust the apparent pH [externally indicated on damp Whatman BDH paper, pH range 6–8, by the method described by Rees & Offord (1976)] to 8.5–9. After 16–24 h at room temperature the protein was precipitated with ether, centrifuged down, washed three times with ether, and air-dried. Generally, isoelectric focusing showed complete conversion of the protein from a di-derivative into a tri-derivative. Amino acid analysis showed the incorporation of the appropriate extra residue (Table 2, below).

Certain active esters, notably that of *o*-fluoro-L-phenylalanine, coupled rather slowly. Following the report of König & Geiger (1972) on the acceleration of sterically hindered couplings in certain solvents by the addition of *N*-hydroxybenztriazole, we found that the inclusion of a quantity of *N*-hydroxybenztriazole equimolar to the active ester decreased the time for complete coupling from 18 to 1 h. A similar result was obtained for the *m*-fluoro-L-phenylalanine ester. Therefore, many of the coupling times shown in the Table could probably be shortened, but it was found convenient to use the longer times and no harm ensued.

Deprotection of acylmethionylinsulins with CNBr

The derivatives were dissolved at a concentration of 5 mg/ml in 70% (v/v) formic acid and treated with a 150-fold molar excess of CNBr over methionine content (i.e. 5 mg of CNBr/mg of protein) for 16 h at 20°C. These conditions are also sufficient to remove the Boc group quantitatively. The protein was most commonly recovered by freeze-drying after dilution with water to about 4% formic acid. Occasionally, when small quantities were used, the protein was recovered from the deprotection mixture by precipitation with cold ether, but in view of the hazardous nature of CNBr this is not recommended as a general procedure. The extent of cleavage, as judged by isoelectric focusing and the percentage of residual methionine in the insulin sample, ranged from 80–90% for materials that had been subjected to rather little handling, to 70–80% for materials

that had been through several stages of modification. It is possible that the failure to cleave is due to the partial conversion of the methionine into the sulphoxide. (The sulphoxide reverts to methionine on hydrolysis of the derivative for amino acid analysis and would not have been detected as such.) Wide variations of solvent, time, and the excesses of CNBr were investigated, but did not lead to improved efficiency of cleavage.

In general the small contaminating quantities of acylinsulin remaining after cleavage could be removed as follows. The sample was set up for crystallization in the rhombohedral system (with two atoms of Zn per protein hexamer) of Schlichtkrull (1958), except that the final mixture was adjusted to pH 8 with 1 M-NaOH rather than to pH 6.1–6.3 as is usual. The mixture was left for 1–2 h at room temperature. The pH was then adjusted to pH 6.1–6.3 to allow the recrystallization to begin. Any precipitate that appeared immediately after the pH was adjusted could be shown by isoelectric focusing to be mainly the uncleaved acylinsulins, and was discarded. Crystals formed over the course of a few hours to a few days. (If the pH 8 step was omitted almost all of the sample precipitated at pH 6.1–6.3: the effect of the alkaline conditions may be to reverse any N → O acyl shifts that might have occurred, or to remove formyl groups.)

Characterization of insulin derivatives

End-group and amino acid analyses were carried out as described by Gonzalez & Offord (1971). Isoelectric focusing in polyacrylamide gels was carried out as described by Borras & Offord (1970b). Two ranges of Ampholines were used. Mixtures of all derivatives, or samples expected to contain predominantly insulin or monoacylinsulin, were examined with the pH 5–7 range; samples expected to have changed from diacylinsulin to triacyl-derivative, or vice versa, were examined with the pH 4–6 range, as the wider separation of the bands of di- and tri-derivatives more than compensated for the poorer resolution of less substituted derivatives.

Table 2. *Amino acid compositions of the products*
 All hydrolyses were with 6M-HCl for 24h at 108°C. The results are not corrected for destruction or incomplete release of some residues. The dashes indicate peaks lost through failure of the amino acid analyser.

A, Pig insulin	J, [<i>m</i> -F-L-Phe ^{B1}]-insulin																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																														
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* Control runs with the authentic amino acids (see the Materials section) showed that *m*-fluoro-L-phenylalanine was eluted from the analyser with phenylalanine and that *o*-fluoro-L-phenylalanine was eluted with tyrosine.

Other amino-protecting groups

The slight but significant removal of the Z group during the acid cyclization step of the Edman degradation makes the ethoxycarbonylmethionyl group preferable for preparations involving the deletion of several residues. However, in contrast with the Z group, the ethoxycarbonyl group imparts insufficient hydrophobicity to its derivatives to make it possible to take advantage of the very rapid and simple partition-chromatography system of Geiger & Langner (1973). We have investigated other groups which are both acid-stable and more hydrophobic, but have found none entirely satisfactory. The benzoyl group seemed a likely alternative, as it is very stable to acid, and benzoylmethionylinsulins separated as well as Z derivatives in the partition system. However, the insulin derivatives were only poorly deprotected by CNBr treatment. We ascribe this failure to homoserine formation without cleavage via a δ -iminolactone intermediate (Saunders, 1974). A similar mechanism was proposed to explain the poor cleavage undergone by simple acetylmethionylpeptides (Carpenter & Shiigi, 1974). The toluene-*p*-sulphonyl derivative was removed even more poorly than was the benzoyl derivative. Although the *p*-nitrobenzyloxycarbonyl group is more acid resistant than the Z group the protected methionine residue and its active ester tended to decompose (cf. Carpenter & Gish, 1952). The use of the 2,4-dinitrophenyl derivative of methionine seemed attractive, as it allows simple determination of the extent of insulin protection by measurement of the characteristic absorption at 350nm. Unfortunately the extent of deprotection seemed to fall as the age of the samples increased. This may have been a result of a light-dependent oxidation of the methionine's sulphur atom, promoted by the dinitrophenyl group.

Results and Discussion

The amino acid compositions and end-groups of a number of insulin analogues produced by these methods are given in Table 2.

In a control experiment the native sequence was re-synthesized by the replacement of L-phenylalanine in position B-1 (see Table 1). This product crystallized well in the rhombohedral form with two atoms of Zn per protein hexamer under the conditions of Schlichtkrull (1958). The competition curve against ^{125}I -labelled insulin in the radioimmunoassay of this crystalline material was superimposable on that of a sample of Novo insulin (Fig. 1). The [Ala^{B1}]- and [Val^{B1}]-insulin analogues also crystallize well in the rhombohedral form. The [Tyr^{B1}]- and the three [F-L-Phe^{B1}]insulins do not crystallize in the rhombohedral form, but the analogue containing tyrosine produces monoclinic crystals in the phenol-containing buffer described by Schlichtkrull (1958).

Fig. 1. Immunological comparison between crystalline native insulin (○) and crystalline resynthesized insulin (▲; product F, Table 2)

The samples were assayed by Dr. P. A. Halban, University of Geneva, by competition with ^{125}I -labelled insulin for guinea-pig antiserum as described by Herbert *et al.* (1965).

The ring of phenylalanine-B1 is, in the hexameric unit of the rhombohedral crystals, close to a hydrogen-bond formed between the side chain of two tyrosine-A14 residues in adjacent monomers (Blundell *et al.*, 1972). It is possible that the introduction of a hydrogen-bond donor or acceptor into this region disrupts the balance of forces that stabilize the rhombohedral crystal, and that this accounts for the failure of the analogues containing tyrosine and fluorophenylalanine to crystallize in this form. The packing of the subunits in this region of the monoclinic form may be different, and this constraint might not apply.

All the analogues that have substitutions at positions B-1 or B-2 have biological activities of between 34 and 61% in the isolated rat adipocyte assay of Glieman & Gammeltoft (1974).

We conclude that the ethoxycarbonyl and Z derivatives are both suitable for semisynthetic work with insulin, and possibly in other proteins that lack methionine.

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