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(19) (CA) **CANADIAN PATENT** (12)

(54) Hypoglycaemic Peptides

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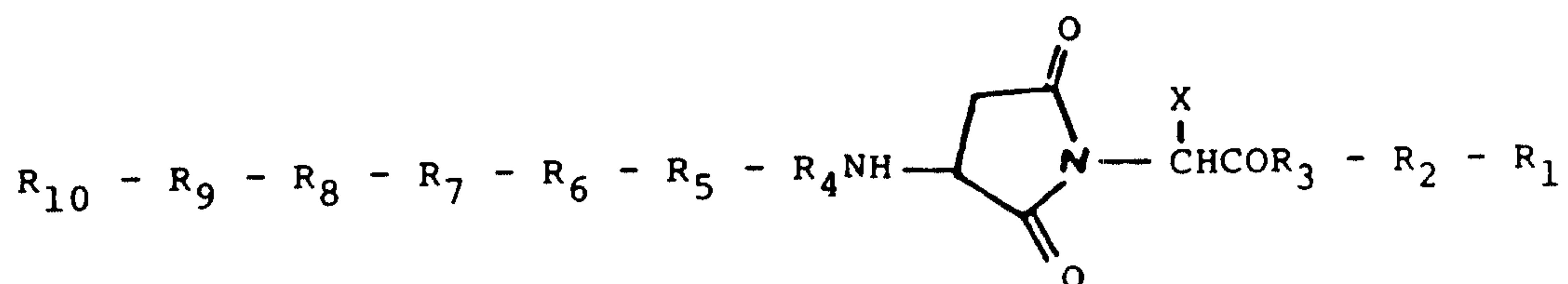
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ABSTRACT OF THE DISCLOSURE

A compound of general formula I:



wherein X is hydrogen, $-\text{CHCONH}_2$ or $-\text{CH}_2\text{CH}_2\text{CONH}_2$; each of R_1 , R_2 and R_3 is an L- α -amino acid, a δ -amino acid or an ϵ -amino acid; R_4 is an L or D α -amino acid, a δ -amino acid or an ϵ -amino acid; and each of R_5 to R_{11} is hydrogen or an L or D α -amino acid, a δ -amino acid or an ϵ -amino acid, or a pharmaceutically acceptable salt thereof. Methods of synthesis of peptides according to general Formula I, compositions containing the peptides, and uses of the peptides are also disclosed and claimed.

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PEPTIDES

5 This invention relates to methods of controlling blood glucose levels, and in particular to novel compounds with hypoglycaemic activity and to processes for producing these compounds.

Background of the Invention

10 The principal factor controlling blood glucose levels is the hormone insulin. Diabetes may result either from insufficient production of insulin or from resistance to the action of circulating insulin.

It has been suggested that a peptide fragment of human growth hormone (hGH), derived from enzymic digestion of pituitary, can counter the effects of natural inhibitors of insulin (Australian patent application no. 87961/75 published July 7, 5 1977. This α -peptide chain, having sequence leu-ser-arg-leu-phe-asp-asn-ala, is comprised of amino acids all of which have the L configuration.

This peptide, equivalent to hGH 6-13, was stated to be able to counteract the inhibition of insulin by somantin, a 10 degradation product of growth hormone, and was thought to potentiate the effects of insulin, to induce synthesis of insulin receptor sites on cells, and to sensitize β cells in the islets of Langerhans to glucose.

However, it has subsequently been found that the 15 peptide disclosed does not have these activities. In fact, inconsistency in demonstrating the claimed biological activities, due to the absence of detailed knowledge of the precise conformational structure of the active substance, prevented further development and validation of the original 20 work by Bornsten and co-workers, inventors in respect of 879661/75, and this application lapsed.

We have now prepared a family of novel β -imide linked L-aspartyl-L-asparaginyl peptides wherein the general structure of the peptide may additionally involve between 2 25 and 6 other α -amino acid residues of either the D- or L-stereochemical form, or involve other non α -amino acid substitutions at positions 1 or 6 to 12.

Furthermore, the in vitro and in vivo properties of these β -imido-L-aspartyl-L-asparaginyl peptides as potent 30 hypoglycaemic agents with functional capabilities of potentiating insulin action are demonstrated. In this application we describe (i) the synthesis of these peptides via solid and solution phase peptide synthetic protocols; (ii) the purification and structural characterization of 35 these peptides; (iii) the in vitro evaluation of these

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peptides in various biological assay systems; (iv) the evaluation of these peptides in lowering blood glucose levels in vivo.

Brief Description of the Figures

- 5 Figure 1 represents a stereochemical view of a representative peptide according to the invention;
- Figure 2 represents far ultraviolet circular dichroic spectra for synthetic peptide Pep (6-13) β -aspartimido form I() and synthetic peptide Pep (6-13) α -form (-----). Spectra recorded in water at 21°C using 1 mg/ml solutions in 0.1 mm cells;
- 10 Figure 3 represents infra-red spectrum for active synthetic peptide (6-13) β -aspartimido form I. The spectrum was measured between 2000-1500 cm^{-1} after the peptide sample 15 (ca. 5 mg) was pressed into a KBr disc;
- Figure 4 represents infra-red spectrum for synthetic peptide Pep (6-13) α -form, inactive. The spectra were measured between 200-1500 cm^{-1} . (a) the sample (ca. 5 mg) of peptide was pressed into a KBr disc. (b) the sample (ca. 5 mg) 20 of peptide was saturated with Nujol to form a mull;
- Figure 5 represents fast atom bombardment (FAB) mass spectrometric spectra of the synthetic peptides active β -aspartimido form I Pep (6-13) and inactive α -form Pep (6-13);
- 25 Figure 6 represents ^1H N.M.R. spectrum of active β -aspartimido form I synthetic peptide Pep (6-13);
- Figure 7 represents paper electrophoresis comparing synthetic peptide β -aspartimido form I Pep (6-13) active and synthetic peptide α -form dissolved in water and dilute (1M) 30 NaOH.
- (a) Electrophoresis was performed at pH 6.5 for 1 h at 1.5 kV.
- (b) Electrophoresis was performed at pH 3.0 for 2.5 h at 1.5 kV.
- 35 Peptides were detected by spraying with ninhydrin;

Figure 8 represents HPLC chromatograms of synthetic Pep (6-13) peptides using a shallow gradient (0% - 20% Solvent B over 20 min).

(a) Injection of purified synthetic Pep (6-13)
5 β -aspartimido form I, active.

(b) Injection of partly hydrolysed synthetic Pep (6-13) β -aspartimido form I.

(c) Simultaneous injection of inactive synthetic Pep (6-13) α -form peptide and active β -aspartimido form I
10 Pep (6-13) peptide α -form;

Figure 9 represents the change in blood glucose level in rats following injection of various synthetic inactive α form and active β -aspartimido forms of peptides according to the invention;

15 Figure 10 represents the effect of various doses of Pep (6-13) β imide during intravenous insulin sensitivity tests on normal rats;

Figure 11 represents the effect of Pep (6-13) on glucose tolerance tests in normal rats;

20 Figure 12 represents in vivo testing of Pep (6-13) (Asn¹¹Asp¹²) (Figure 11a) and Pep 6-13 (Asp¹²) (Figure 11b) for insulin-potentiating activity on "untrained" female mice. The dotted lines represent control animals (injected with 0.2 ml of saline) in each case. The unbroken lines represent test
25 animals (injected with 2.4 mg/kg of peptide in 0.2 ml of saline). Insulin was injected at t_0 ;

Figure 13 represents in vivo testing of peptides for insulin-potentiating activity on "untrained" and "trained" male mice. The dotted line represents the mean fall in blood
30 glucose for control animals (injected with 0.2 ml of saline). The unbroken line represents the mean fall in blood glucose for test animals, injected with 2.3 mg/kg of peptide in 0.2 ml of saline, except for (a), in which the dose was 2.4 mg/kg.

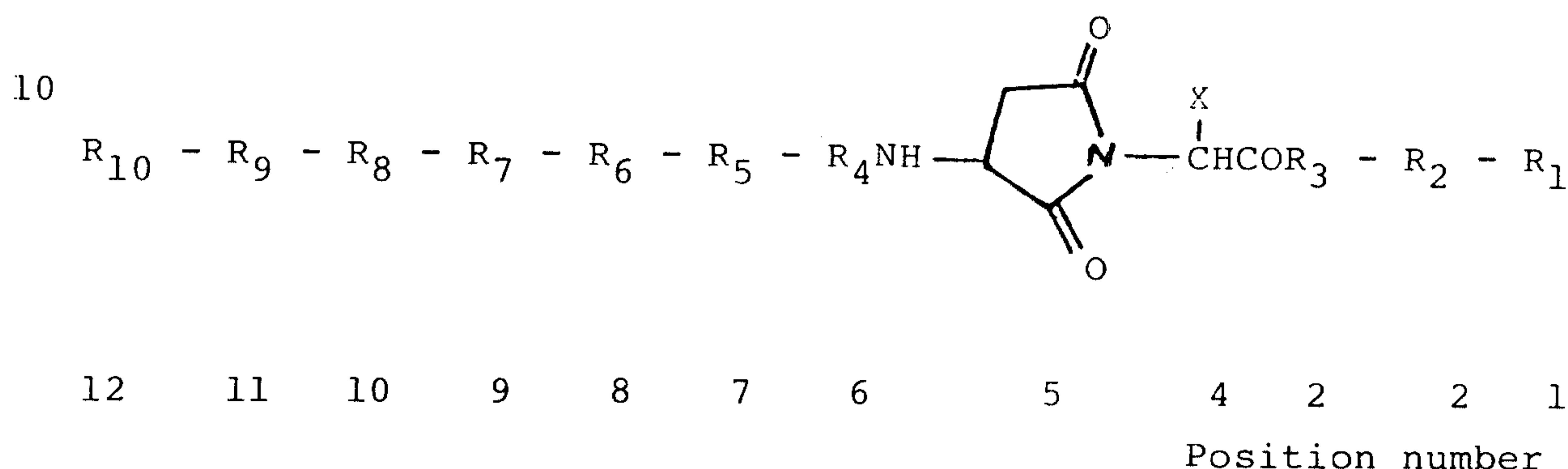
(a) Pep 6-13 (Gln¹¹Asp¹²), "untrained"
35 (b) Pep 6-13 (Gln¹¹Asp¹²), "trained"
(c) Pep 6-13 (Asn¹¹Asp¹²), "trained"
(d) Pep 6-13 (Asp¹²), "trained"

Insulin was injected at t_0 ;

Figure 14 represents the in vitro effect of Pep (6-13) β imide on glucose oxidation in isolated rat adipocytes in the absence and in the presence of various levels of 5 insulin.

Summary of the Invention

According to one aspect of the present invention there is provided a compound of general formula I:



15 wherein X is hydrogen, $-\text{CHCONH}_2$ or $-\text{CH}_2\text{CH}_2\text{CONH}_2$;

each of R_1 , R_2 and R_3 is an L- α -amino acid, a δ -amino acid or an ϵ -amino acid;

R_4 is an L or D α -amino acid, a δ -amino acid or an ϵ -amino acid; and

20 each of R_5 to R_{11} is hydrogen or an L or D α -amino acid, a δ -amino acid or an ϵ -amino acid,

or a pharmaceutically acceptable salt thereof.

Preferably R_5 is a L or D α -amino acid, a δ -amino acid or an ϵ -amino acid.

25 Preferably each of R_1 , R_2 and R_3 is selected from the group consisting of alanine, glycine and phenylalanine.

Preferably R_4 is a hydrophobic amino acid; more preferably R_4 is selected from the group consisting of L-phenylalanine, L-tyrosine, L-tryptophan and L-histidine.

30 Preferably R_5 is a hydrophobic amino acid; more preferably R_5 is selected from the group consisting of L- or D- leucine, isoleucine and histidine.

R_6 is a basic amino acid, and is preferably selected from the group consisting of L- or D- arginine, lysine, and histidine.

R_7 is preferably a substantially hydrophobic amino acid, and is preferably selected from the group consisting of L- or D- serine, leucine and isoleucine.

R_8 is preferably selected from the group consisting of L- or D- leucine, phenylalanine, proline or isoleucine, an ϵ -amino acid such as 6-aminohexanoic acid,
10 4-aminocyclohexane-1-carboxylic acid or similar amino acids.

Each of R_9 and R_{10} is preferably a substantially hydrophobic amino acid, and is most preferably selected from the group consisting of L- or D- leucine, phenylalanine, proline, isoleucine or tyrosine.

15 R_8 , R_9 or R_{10} may optionally be coupled to R_1 via a bifunctional agent such as 6-aminohexanoic acid.

The amino acids may optionally have side chain substituents.

R_1 , R_2 , R_8 , R_9 , or R_{10} may be absent from the
20 peptide, allowing coupling between C-terminal and N-terminal amino acids via a bifunctional agent such as 6-aminohexanoic acid.

R_6 is equivalent in terms of sequence alignment to residue 8 of human growth hormone.

25 The cyclic imide structure as shown in general Formula I is essential for activity. Activity increases with chain length up to 8 residues. A stereochemical view of a representative peptide is shown in Figure 1.

The peptide according to the invention forms an
30 amphipathic helix structure having a fluid hydrophobic face and a second face carrying an exposed imide group.

It will be apparent to persons skilled in the art that pharmacologically active analogues and derivatives of the compound of general formula I are within the scope of the
35 invention, e.g. o-, m-, or p-aminobenzoic acid or α - or δ -lactams in place of the aspartimide ring.

According to a second aspect of the invention there is provided a method of solid phase synthesis of a peptide of general formula I comprising protection of the α -amino functionality of added amino acids using t-BOC or f-MOC and
5 treating the resin-bound protected α /straight chain L-aspartyl-L-asparaginyl peptide with a base to produce the stable β -imido form of the peptide.

The base is a dialkylamine such as piperidine, or a trialkylamine, preferably triethylamine.

10 According to a third aspect of the invention there is provided a method of lowering the level of blood glucose in a mammal in need of such treatment, comprising administering to that animal a hypoglycaemically effective dose of a compound of general formula I. Said compound may optionally
15 be administered together with or in conjunction with a second hypoglycaemic agent.

Detailed Description of the Invention

The invention will now be described in detail by way of example only, with reference to the accompanying drawings.

20 1. PEPTIDE SYNTHESIS

The various peptide analogues of the general formula I above were synthesised either by standard solution phase synthetic procedures or by using modifications of the Merrifield solid phase peptide synthesis procedure (R.B.
25 Merrifield, J. Amer. Chem. Soc., 85, 2149-2145, 1963), of which the following protocols are representative. Peptides were initially synthesised in the alpha/straight chain form with all amino acid side chain groups protected. For the t-BOC-synthetic approach, the α -amino functionality of the
30 incoming amino acid was protected with the tert-butyloxycarbonyl (t-BOC) group with deprotection, following coupling, with trifluoroacetic acid in CH_2Cl_2 . Side chain protecting groups such as O-benzyl, tosylbenzylester and chlorobenzylloxycarbonyl were used for the trifunctional amino
35 acids. The initial coupling to the benzhydrylamine resin

employed a 3-fold excess of the designated side chain protected amino acid R_1 , dissolved in N,N' -dimethylformamide (DMF). Subsequent couplings of the second, third, eighth amino acid (counting asp and asn separately) employed a 5 2 fold excess of side chain protected amino acid in DMF. The amino acids were sequentially activated using tert-butanol and dicyclohexylcarbodiimide in approximately 5ml DMF and allowing reactants to stir at room temperature for 5-10 min. The acylurea was removed by filtration and the asymmetrical 10 anhydride added to the resin. The suspended resin was stirred at room temperature for reaction times generally between 60 and 120 min. Each coupling was monitored for unreacted amino groups using 2,4,6-trinitrobenzenesulphonic acid. Similar synthetic strategies with the symmetrical protected amino acid 15 anhydrides or the pentachlorophenylesters led to the preparation of identical products but in different yields. After the final coupling reaction was completed, the fully protected resin-bound peptide was treated with a reagent grade trialkylamine such as triethylamine (15- 20 ml per g resin) in 20 order to convert the L-aspartyl-L-asparaginyl moiety to the stable β -imido form. The resin-bound peptide was side chain deprotected with trifluoroacetic acid, and the deprotected β -imido-L-aspartyl-L-asparaginyl peptide analogue cleaved from the resin using liquid hydrogen fluoride as the acid and 25 dimethylsulphide/p-cresol or thioanisole as the scavenger. Reaction times were between 30 and 120 min at 0°C. Workup following cleavage involved standard procedures of peptide synthesis. The deprotected peptide was removed from the resin by washing with trifluoroacetic acid, then water, then 30 water/acetonitrile mixtures.

The washes were then combined and lyophilised.

The crude dried product was chromatographed by anion exchange chromatography on Whatman DE 52 and then desalted on BiogelTM P2 gels. Further purification was achieved using 35 reversed phase HPLC with octadecylsilica as stationary phase and gradients of 0-75% water-acetonitrile - 0.1% trifluoroacetic acid as mobile phase.

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A similar synthetic strategy using f-MOC (9-fluorenylmethyloxycarbonyl group) protection has also been employed.

The synthetic peptides were characterised by amino acid compositional analysis, analytical reversed phase HPLC with several elution systems, fast atom bombardment mass spectroscopy (FAB-MS), paper electrophoresis at pH6.0, FTIR-infrared spectroscopy, aminopeptidase M cleavage, H^1 -NMR spectroscopy and circular dichroism (CD) spectroscopy.

10 It was found that the synthesis protocol could be manipulated in order to produce all α or all imide form of the peptide. In the absence of alkylamine treatment, all α was formed, and vice versa.

Representative results from these characterizations
15 are illustrated for the peptide

leu-ser-arg-leu-phe-asn-ala
(α -form, inactive), and for the peptide
leu-ser-arg-leu-phe- β -imidoasp-asn-ala
(β -imido form I, active).

20 Table 1 shows representative results of amino acid composition analysis following acid hydrolysis and aminopeptidase M cleavage.

TABLE 1

Amino Acid Sequence	Leu	Ser	Arg	Leu	Phe	Asp	Asn	Ala
5 α -form Acid Hydrolysis ^a	1.03	0.78	0.93	1.03	0.98	0.98	0.98	0.96
10 Enzymic Hydrolysis ^b	1.02	0.98	-	1.02	0.98	0.98	0.92	1.00
aspartimide Acid Hydrolysis ^a	1.02	0.86	0.86	1.02	0.97	0.99	0.99	1.00
15 Enzymic Hydrolysis ^b	1.06	0.99	-	1.06	1.00	-	-	-

a) 24 h acid hydrolysis at 110°C in 6M HCl.

b) 24 h aminopeptidase M digestion.

Results of circular dichroism spectroscopy,
 20 infra-red spectroscopy, FAB-MS, and ¹H-NMR spectroscopy are
 illustrated in Figures 2 to 6 respectively. Without wishing
 to be bound by any proposed mechanism for the observed
 effects, it appears that the α form is in an extended
 configuration, while the β -aspartimido form I has one
 25 completely hydrophobic face, with the β -imido group at 90° to
 this hydrophobic face.

This conclusion is in accord with results obtained
 from correlation Overhauser spectroscopy (COSY) and nuclear
 Overhauser enhanced spectroscopy (NOESY) NMR spectroscopy for
 30 all cases of the representative active β -imide analogues.

Figure 8 shows results of reversed phase HPLC on
 ODS-silica with shallow gradients of 0.1% trifluoroacetic acid
 - acetonitrile-water.

2. BIOLOGICAL ACTIVITIES

Considering the physiology of hypoglycaemia and the possible molecular actions of peptide analogues with hypoglycaemic and insulin potentiating properties, the following in vitro and in vivo assays were chosen to assess the potency of the various synthetic β -imido-L-aspartyl-L-asparaginyl peptide analogues.

(a) In vitro bioassays

- (i) Activation of glycogen synthase
- 10 (ii) CO_2 production from U^{14}C -glucose and ^{14}C -pyruvate via pyruvate dehydrogenase
- (iii) Glucose uptake

In these assays collagenase-dispersed rat adipocytes, cultured muscle cells (L6 rat skeletal muscle cell 15 line) and hemidiaphragm preparations were employed.

(b) In vivo bioassays

- (i) Effect on basal blood glucose levels
- (ii) Effect on insulin release
- (iii) Intravenous insulin tolerance test
- 20 (iv) Activation of glycogen synthase

Representative data on the biological properties of the active β -imidoaspartylasparaginyl form I peptides and their inactive α -form peptide analogues are presented.

Table 2 shows the decrease in blood glucose level in 25 rats following intravenous injection of peptides according to the invention. Injection of the α -form had no significant effect; however, injection of the β imide form of Pep (6-13) or Pep (6-15) resulted in a significant hypoglycaemic effect.

TABLE 2

Peptide	Conc. of Peptide $\mu\text{mol}/100\text{g}$	No. of animals	Decrease in blood from basal	Difference from control mean	2P Value t-test
Control	-	28	1.05		
Pep (6-11)	0.5	5	1.28	0.23	N.S.
Pep (6-12)	0.5	5	1.00	-0.05	N.S.
10 Pep (6-13)	0.5	14	1.05	0.00	N.S.
Pep (6-13) imide	0.5	22	1.90	0.85	< 0.001
Pep (6-15)	0.5	5	1.26	0.21	N.S.
Pep (6-15) imide	0.5	5	2.00	0.95	< 0.001
Pep (9-13)	0.5	7	1.12	0.07	N.S.
15 Pep (9-13) imide	0.5	9	1.30	0.25	N.S.

Values are changes (means $\pm$ SEM) of blood glucose from the basal level (4.0 - 4.4 mmol glucose/l) at time 0. Differences between the control and test groups were statistically evaluated according to Student's t-test. N.S. indicates $2P > 0.05$.

Similar results are illustrated graphically in Figure 7. The α form of Pep (6-13) was inactive for several variants tested (Ala^{11} , Gly^{10} , $\text{Gly}^{10}\text{Pro}^{11}$, Pro^{11} , Tyr^{10}) whereas the β imide form was always active. Similarly Pep (6-11), Pep (6-12), Pep (6-13), Pep (6-15) were active only in the β imide form. However, for Pep (9-13) neither the α form nor the β imide form was active in this test.

The β imide form of peptides according to the invention was also found to potentiate the action of insulin during intravenous insulin sensitivity tests carried out on rats trained to eat on a scheduled time basis. Rats were anaesthetised with pentobarbitone, and blood samples taken at various times after intravenous infusion of insulin. Control animals were untreated or were given insulin only; test

animals received insulin plus the β imide form of Pep (6-13). The results are shown in Figure 10. The effect depended on the dose of peptide.

Figure 11 shows that Pep (6-13) β imide accelerates the return of blood glucose levels in rats to basal level in the glucose tolerance test. The dose of Pep (6-13) was 200 $\mu\text{g/Kg}$ body weight. Similarly, various forms of the peptide according to the invention in its β imide form potentiate the hypoglycaemic effect of insulin on "untrained" male and female mice. The effect on trained male mice trained to feed on schedule as described above was less striking, but still significant in some cases. These results are illustrated in Figures 12 and 13.

As well as showing activity in the in vitro assays described above, peptides according to the invention showed hypoglycaemic activity in in vitro bioassays. Table 3 shows the effect of Pep (6-13) on glycogen deposition by isolated rat hemidiaphragms. There was no effect on the basal level in the absence of added insulin. At low or high insulin concentrations (10^2 and 10^4 $\mu\text{U/ml}$) respectively, Pep (6-13) β imide significantly increased glycogen deposition. The α form and the ring opened, hydrolysed β imide form were both inactive.

TABLE 3

In Vitro Effect of Pep (6-13) Imide on Glycogen Deposition
In Rat Hemidiaphragms.

	Insulin Conc. (μ U/ml)	Peptide Conc. (μ mol/ml)	No. of hemidiaphragms	14 C - glycogen deposition (μ mol/g) Mean $\pm$ SEM	Difference from controls	P (Student's t - test)	P (Wilcoxon Rank Sum Test)
Basal			14	2.16 $\pm$ 0.24			
Pep(6-13) aspartimide	0	0.1	14	2.45 $\pm$ 0.19	0.29	NS	NS
Basal			12	2.57 $\pm$ 0.23			
Pep(6-13).	0	0.1	12	2.30 $\pm$ 0.17	-0.27	NS	NS
Basal			13	2.42 $\pm$ 0.19			
Pep(6-13) imide hyd	0	0.1	12	2.40 $\pm$ 0.24	-0.02	NS	NS
Low			14	2.75 $\pm$ 0.15			
Pep(6-13) aspartimide	10 ²	0.1	12	3.47 $\pm$ 0.21	0.72	0.01	Sig
Low			13	2.67 $\pm$ 0.14			
Pep(6-13).	10 ²	0.1	13	2.35 $\pm$ 0.15	-0.32	NS	NS
Low			11	2.71 $\pm$ 0.15			
Pep(6-13) imide hyd	10 ²	0.1	13	2.38 $\pm$ 0.13	-0.33	NS	NS
HIGH			13	5.53 $\pm$ 0.32			
Pep(6-13) aspartimide	10 ⁴	0.1	14	6.01 $\pm$ 0.54	-0.48	0.05	Sig
HIGH			12	4.00 $\pm$ 0.30			
Pep(6-13).	10 ⁴	0.1	13	4.36 $\pm$ 0.46	0.36	NS	NS
HIGH			13	4.62 $\pm$ 0.66			
Pep(6-13) imide hyd	10 ⁴	0.1	14	4.10 $\pm$ 0.57	-0.52	NS	NS

imide hyd = hydrolysed β imide form i.e. inactive ring
 opened form.

The β imide form of the peptide also stimulated metabolism of glucose by isolated rat adipocytes, both in the presence and in the absence of added insulin. This is shown in Figure 14. The degree of stimulation depended on insulin dose. The dose of peptide was 10 μ g per 3 ml culture.

The effect of the β imide form peptides on glucose metabolism is further shown by their ability to stimulate glucose uptake and glycogen synthesis by cultured muscle cells and isolated rat hemidiaphragms.

10 The results are presented in Tables 4 and 5.

TABLE 4
In Vitro Effect of β -Imido Linked L-Aspartyl-L-Asparaginy] Peptides
On Glucose Uptake In Rat Hemidiaphragms.

Peptide	No. of Pairs	Peptide concentration ($\mu\text{mol/ml}$)	Glucose Uptake ($\mu\text{mol/g muscle/h}$)			p
			Control Mean $\pm$ SEM	+ Peptide Mean $\pm$ SEM	Mean Difference	
1-15	12	0.14	22.06 $\pm$ 1.39	30.39 $\pm$ 1.72	8.33 $\pm$ 1.50	<0.001
6-13	15	0.16	35.34 $\pm$ 1.54	38.49 $\pm$ 1.53	3.15 $\pm$ 0.75	<0.001
7-13	9	0.22	42.16 $\pm$ 1.88	46.79 $\pm$ 1.10	4.64 $\pm$ 1.63	<0.25
8-13	7	0.20	38.70 $\pm$ 0.77	44.11 $\pm$ 0.69	5.41 $\pm$ 0.65	<0.001
9-13	8	0.20	31.50 $\pm$ 1.01	32.16 $\pm$ 0.97	0.68 $\pm$ 0.80	nsd
10-13	9	0.20	33.86 $\pm$ 1.19	33.07 $\pm$ 1.01	0.80 $\pm$ 1.16	nsd

TABLE 5

Results of Cultured Cell Assays for the Stimulation of
Glucose Uptake by Synthetic Peptides (10 μ g/ml)

<u>Peptide</u>	<u>Rate of Increase from Basal</u> <u>(μmol glucose/hour)</u>
P015 Ser-Arg-Leu-Phe-Asp-Asn-Ala	1.5
P051 Leu-Ser-Arg-Leu-Phe-Asp-Asn-Ala	3.3
P057 Leu-Ser-Arg-Leu-dPhe-Asp-Asn-Ala	2.0
P063 Leu-Ser-Arg-Leu-Phe-Asp-Asn-Ala-Gly	1.9
P086 Leu-Ser-Arg-Val-Phe-Asp-Asn-Ala	4.9
P168 Cys-Tyr-Leu-Ser-Arg-Leu-Phe-Asp-Asn-Ala	4.0
P202 Cys-Tyr-Leu-Ser-Arg-Leu-Phe-Asp-Asn-Ala	1.9

Results of Isolated Muscle Assays for the Stimulation of
Glycogen Synthesis by Synthetic Peptides (100 μ g/ml)

<u>Peptide</u>	<u>Rate of Increase from Basal</u> <u>(μmol glycogen/g tissue/h)</u>
P051 Leu-Ser-Arg-Leu-Phe-Asp-Asn-Ala	0.74
P076 Leu-Ser-Arg-Leu-Phe-Asp-Gln-Ala	0.30
P078 Leu-Ser-Arg-Leu-His-Asp-Asn-Ala	0.11
P087 Leu-Ser-Arg-Ile-Phe-Asp-Asn-Ala	0.36
P090 Leu-Ser-Lys-Leu-Phe-Asp-Asn-Ala	0.16
P197 Cys-Tyr-Leu-Ser-Arg-Leu-Phe-Asp-Asn-Ala	0.17
P202 Cys-Tyr-Leu-Ser-Arg-Leu-Phe-Asp-Asn-Ala	0.44

Of the peptides tested, Pep (1-15), (6-13), and (8-13) showed a highly significant stimulation; Pep (7-13) was on the borderline of significance, while Pep (9-13) and Pep (10-13) showed no effect.

5 The results show that peptides of general formula I induce in a dose dependent manner a significant lowering of blood glucose levels at or below the level of 10 μ M/Kg body weight with the intravenous insulin tolerance test, and enhance in a dose dependent manner glucose utilisation via
10 activation of glycogen synthase, incorporation of glucose into glycogen and oxidation of glucose via pyruvate to carbon dioxide. A dose of as little as 0.05 μ M/Kg body weight was effective with some peptides.

APPLICATIONS OF THE INVENTION

15 The β -imido-L-aspartyl-L-asparaginyl peptides of general formula I induce

(a) dose-dependent lowering of blood glucose and a potentiation of glycogen synthesis in vivo

(b) dose-dependent oxidation of glucose and the
20 activation of glycogen synthase in vitro.

This suggests that the β -imido-L-aspartyl-L-asparaginyl peptide analogues are potent hypoglycaemic agents, and may be useful as therapeutic agents for the control of glucose metabolism, since normalisation of blood
25 glucose levels in insulin-independent diabetes mellitus (Type II) mammals with glucose intolerance and insulin resistance is achieved by control of carbohydrate metabolism via glucose utilisation, and since glucose utilisation can be achieved via two pathways, namely oxidation of glucose or incorporation of
30 glucose into glycogen.

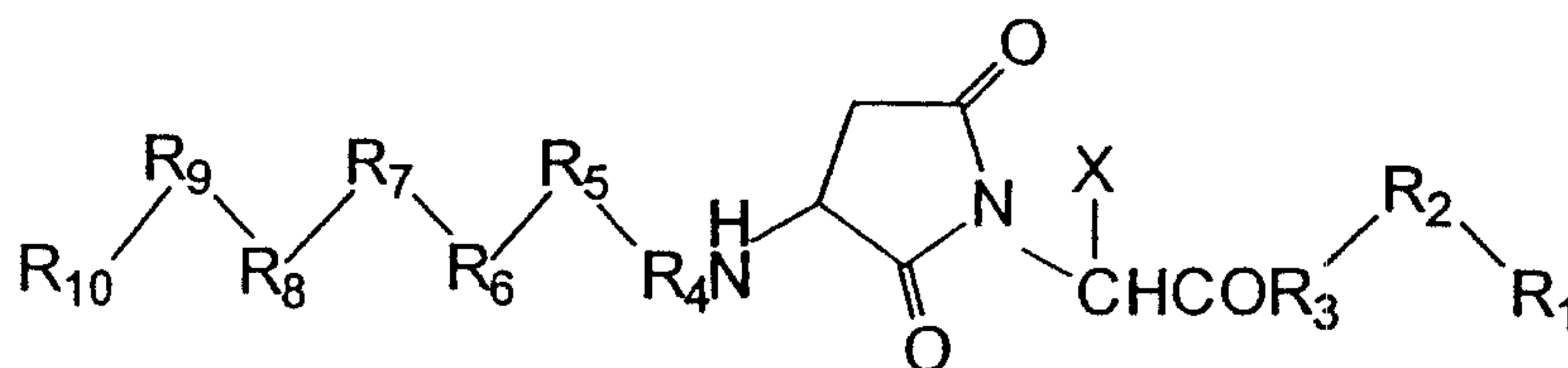
Since potentiation of insulin action results in an decreased requirement for endogenous insulin, the use of the β -imido-L-aspartyl-L-asparaginyl peptide analogues may permit control of hyperinsulinaemia in insulin-independent Type II
35 diabetes in humans or streptozotocin-treated animals.

(f) By analogy, the use of other synthetic peptide analogues containing the β -imido-L-aspartyl-L-asparaginyl moiety of general formula I is likely to exhibit either agonistic or antagonistic properties in vitro or in vivo 5 related to glucose utilisation and so provide methods for control of hypoglycaemia or hyperglycaemia, depending on their respective agonistic or antagonistic properties in control of glucose metabolism.

It will be clearly understood that the invention in 10 its general aspects is not limited to the specific details referred to hereinabove.

The following words used hereinabove are trade marks: BIOGEL, NUJOL.

1. A compound of Formula I:



where in X is hydrogen, -CH₂CONH₂ or -CH₂CH₂CONH₂;

each of R₁, R₂, and R₃ is independently an L -α-amino acid selected from the group consisting of alanine, glycine, and phenylalanine, a δ-amino acid or an ε-amino acid;

R₄ is an L or D α-amino acid, a δ-amino acid or an ε-amino acid; and

each of R₅ to R₁₀ is independently hydrogen or an L or D α-amino acid, a δ-amino acid or an ε-amino acid,

wherein one or more of R₁ or R₂ may be absent and wherein one or more of R₅, R₆, R₇, R₈, R₉, and R₁₀ may be absent, with the proviso that only the outermost R substitution chosen from R₅-R₁₀ can be H;

and wherein at least one of the following must exist;

R₄ is selected from the group consisting of L-phenylalanine, L-tyrosine, L-tryptophan, and L-histidine; and

R₅ is selected from the group consisting of L- or D-leucine, isoleucine, and histidine;

R_6 is selected from the group consisting of L- or D-arginine, lysine, and histidine;

R_7 is a hydrophobic amino acid;

R_7 is selected from the group consisting of L- or D-serine, leucine, and isoleucine;

R_8 is selected from the group consisting of L- or D-leucine, phenylalanine, proline, isoleucine;

R_8 is selected from the group consisting of 6-aminohexanoic acid, 4-aminocyclohexane-1-carboxylic acid;

each of R_9 and R_{10} is a hydrophobic amino acid; and

each R_9 and R_{10} are independently selected from the group consisting of L- or D-leucine, phenylalanine, proline, isoleucine and tyrosine;

wherein the cyclic imide structure in general formula I is a type II' β -turn structure;

or a pharmaceutically acceptable salt thereof.

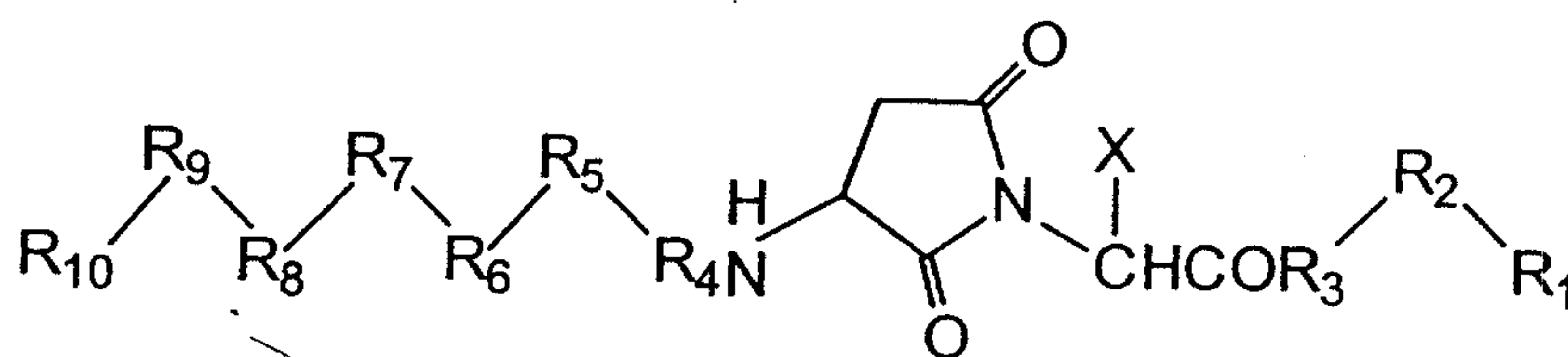
2. A compound according to Claim 1 in which R_5 is a L or D α -amino acid, a δ -amino acid or an ϵ -amino acid.
3. A compound according to Claim 1 in which each of R_1 , R_2 and R_3 is selected from the group consisting of alanine, glycine and phenylalanine.
4. A compound according to Claim 1, in which R_4 is a hydrophobic amino acid.

5. A compound according to Claim 4 in which R_4 is selected from the group consisting of L-phenylalanine, L-tyrosine, L-tryptophan and L-histidine.
6. A compound according to any one of ^{1 to 5} ~~the preceding claims~~, in which R_5 is a hydrophobic amino acid.
7. A compound according to Claim 6 in which R_5 is selected from the group consisting of L- or D-leucine, isoleucine and histidine.
8. A compound according to claim 1, in which R_6 is a basic amino acid.
9. A compound according to Claim 8, in which R_6 is selected from the group consisting of L- or D- arginine, lysine, and histidine.
10. A compound according to claim 1, in which R_7 is a hydrophobic amino acid.
11. A compound according to Claim 10 in which R_7 is selected from the group consisting of L- or D- serine; leucine and isoleucine.
12. A compound according to claim 1, in which R_8 is selected from the group consisting of L- or D- leucine, phenylalanine, proline, isoleucine, and an ϵ -amino acid.

13. A compound according to claim 1, in which each of R_9 and R_{10} is a hydrophobic amino acid.
14. A compound according to Claim 13, in which each of R_9 and R_{10} is selected from the group consisting of L- or D- leucine, phenylalanine, proline, isoleucine and tyrosine.
15. A compound according to claim 1, in which one of R_8 , R_9 or R_{10} is coupled to R_1 via a bifunctional agent.
16. A compound according to Claim 15, in which the bifunctional agent is 6-aminohexanoic acid.
17. A compound according to claim 1, in which R_1 , R_2 , R_8 , R_9 , or R_{10} is absent from the peptide, allowing coupling between C-terminal and N-terminal amino acids via a bifunctional agent.
18. A pharmacologically active analogue or derivative of a compound according to Claim 1, in which the aspartimide ring is replaced by a group selected from α -lactams and δ -lactams.

19. A pharmaceutical composition comprising a compound according to claim 1, together with a pharmaceutically acceptable carrier, diluent or excipient.

20. A compound of Formula I:



wherein R_1 is absent, R_2 is glycine, R_3 is alanine, X is $-\text{CH}_2\text{CONH}_2$, R_4 is phenylalanine; R_5 is leucine, R_6 is arginine, R_7 is serine, R_8 is leucine, R_9 is hydrogen, and R_{10} is absent; or

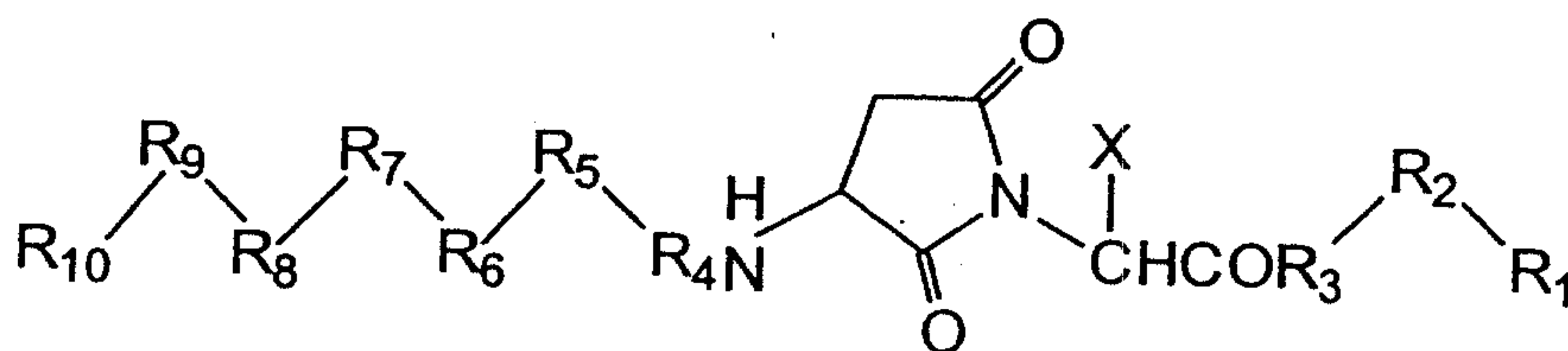
or a pharmaceutically acceptable salt thereof.

21. A method of solid phase ^{synthesis} ~~synthesis~~ of a peptide of general formula I, ^{as defined in claim 1} in which the peptide is bound to a resin, comprising the steps of:

(a) protecting the α -amino functionality of added amino acids using t-BOC or f-MOC,

- (b) treating the resin-bound protected α / straight chain L-aspartyl-L-asparaginyl peptide with a base to produce the stable β -imido form of the peptide, and
- (c) recovering the peptide.

22. A method according to Claim 21, in which the base is a dialkylamine or a trialkylamine.
23. A method according to Claim 22, in which the base is piperidine or triethylamine.
24. A compound according to claim 12, wherein R_8 is 6-aminohexanoic acid or 4-aminocyclohexane-1-carboxylic acid.
25. A compound according to claim 17, wherein the bifunctional agent is 6-aminohexanoic acid.
26. A compound of Formula I:



wherein X is hydrogen, $-CH_2CONH_2$ or $-CH_2CH_2CONH_2$;

each of R_1 , R_2 , and R_3 is selected from the group consisting of alanine, glycine, and phenylalanine; and

R_4 is selected from the group consisting of L-phenylalanine, L-tyrosine, L-tryptophan, and L-histidine; and

R_5 is absent or is selected from the group consisting of H, L- or D-leucine, isoleucine, and histidine; and

R_6 is absent or is selected from the group consisting of H, L- or D-lysine and histidine; and

R_7 is absent or is selected from the group consisting of H, L- or D-serine, leucine, and isoleucine; and

R_8 is absent or is selected from the group consisting of H, L- or D-leucine, phenylalanine, proline, isoleucine, 6-aminohexanoic acid, 4-aminocyclohexane-1-carboxylic acid; and

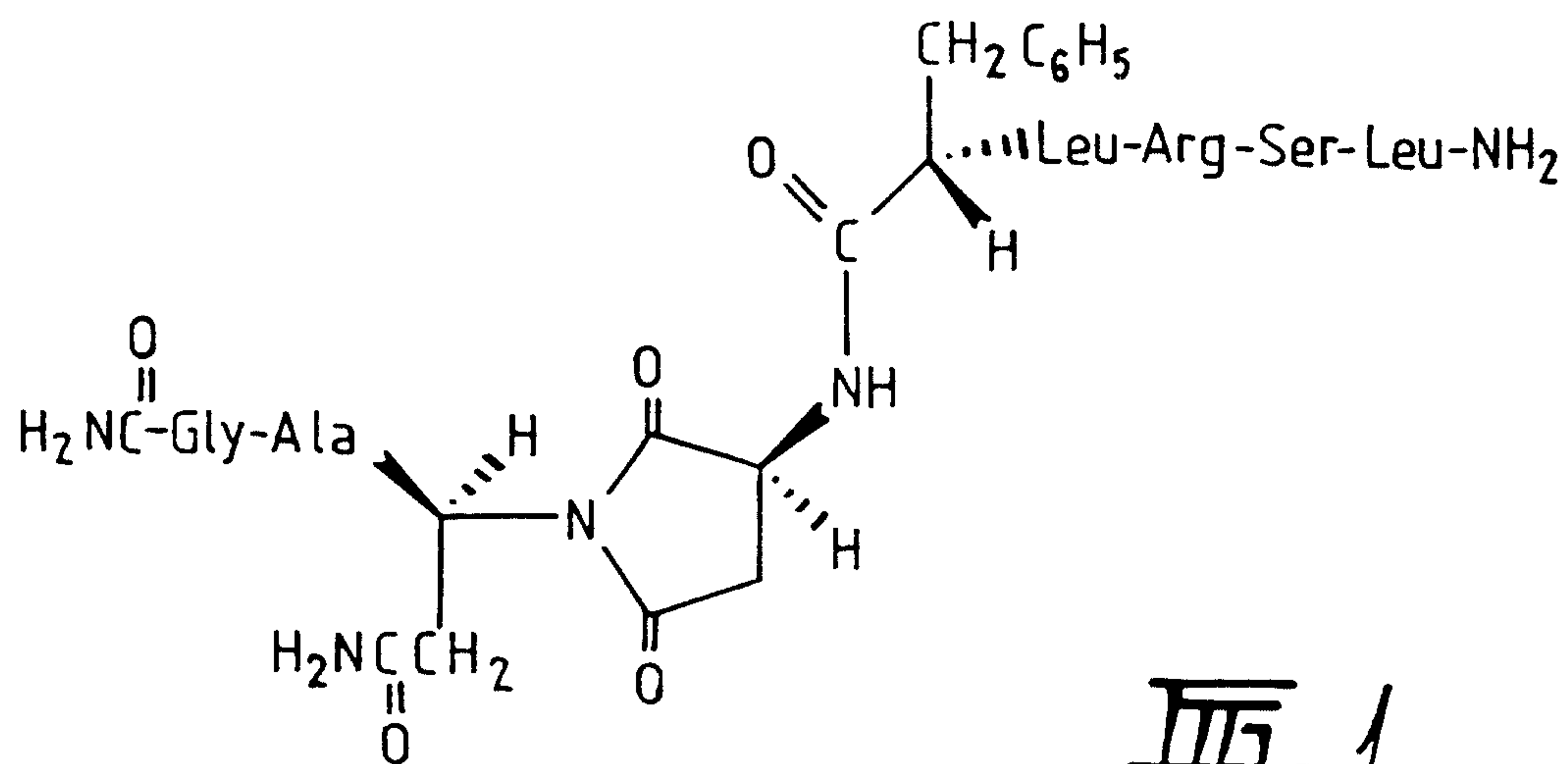
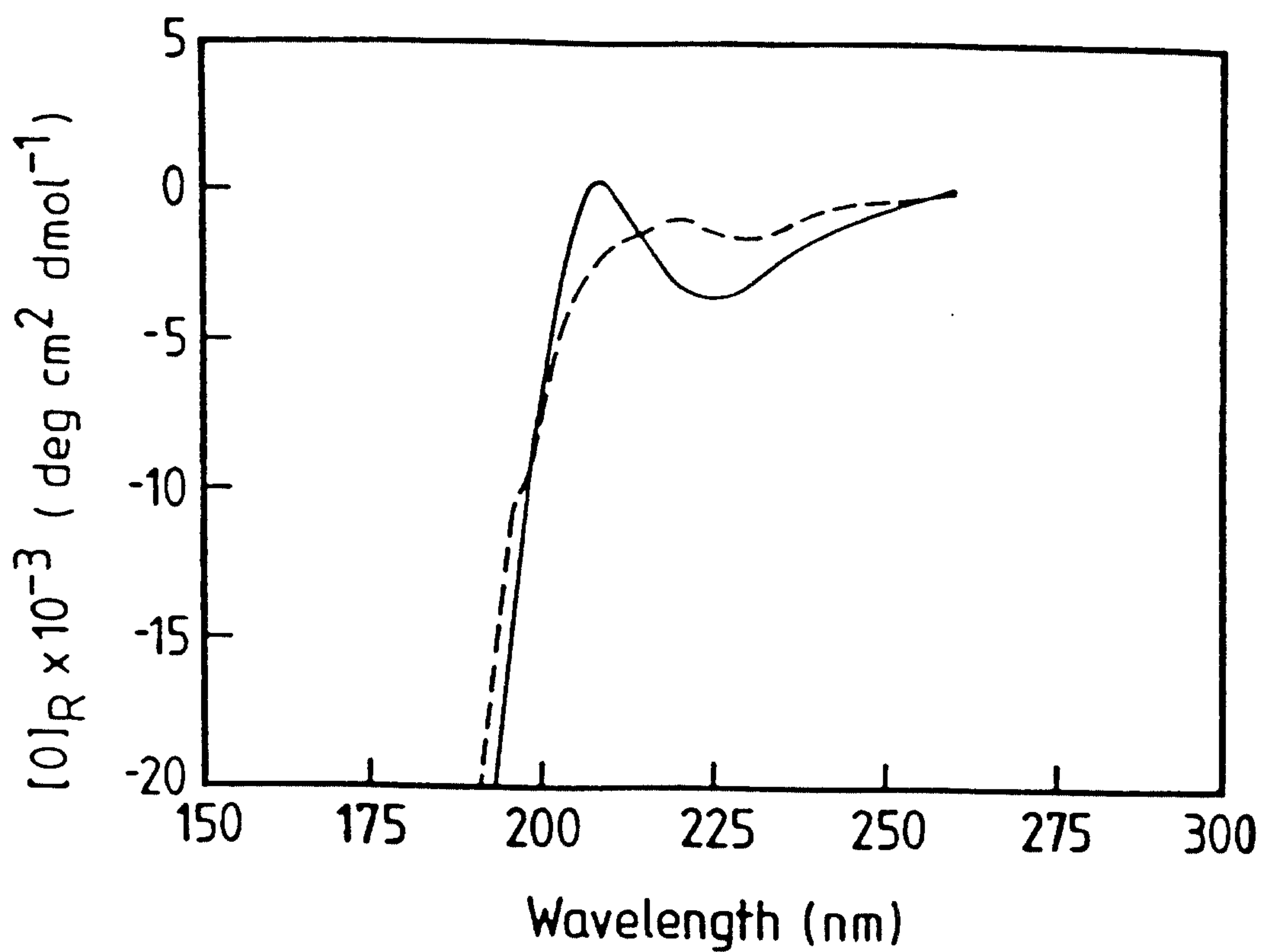
each of R_9 and R_{10} are absent or are selected from the group consisting of H, L- or D-leucine, phenylalanine, proline, isoleucine and tyrosine;

with the proviso that only the outermost R substitution chosen from R_5 to R_{10} can be H;

or a pharmaceutically acceptable salt thereof.

27. The use of a compound of Claim 1 for lowering the level of blood glucose in a mammal.

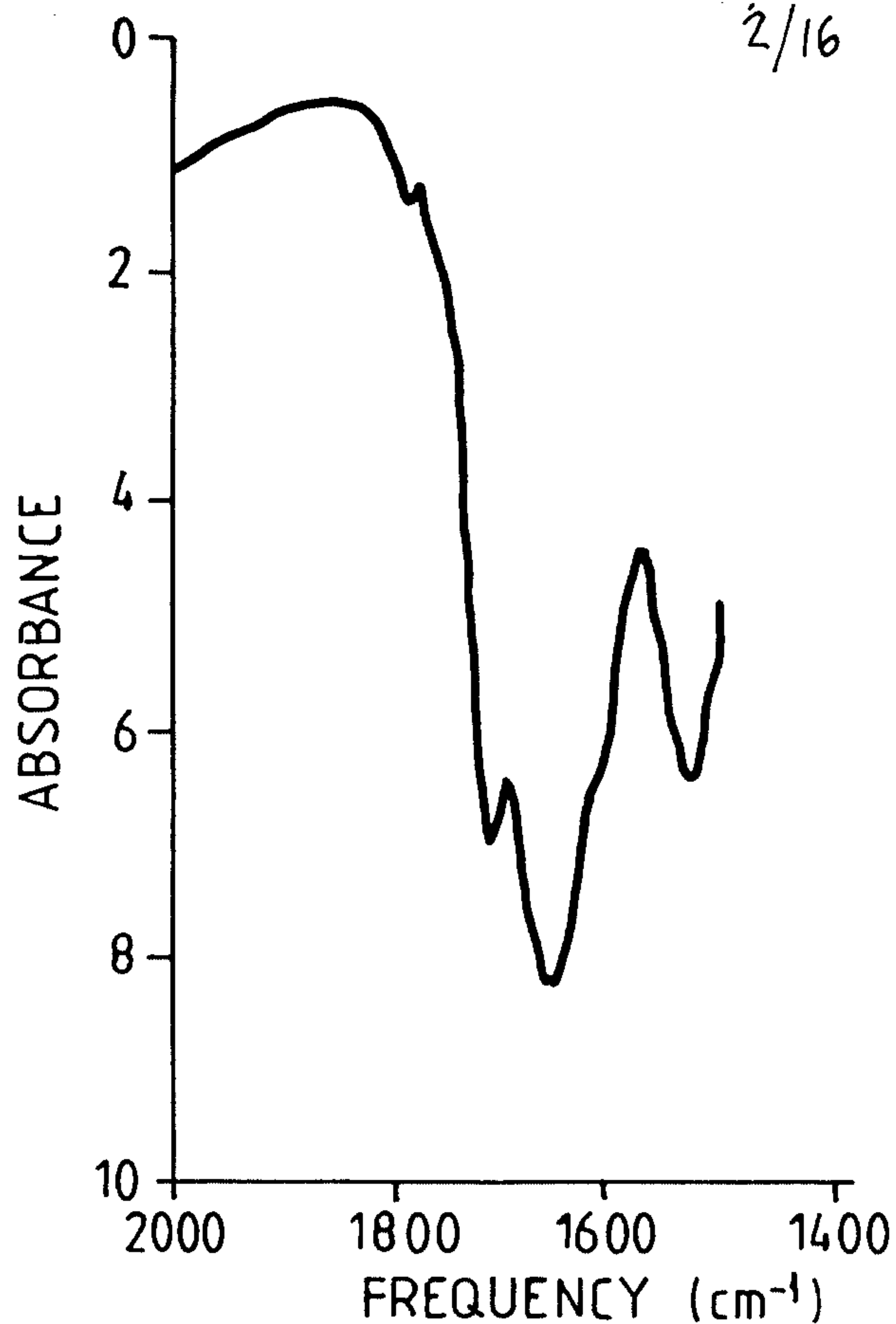
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III.1.III.2.

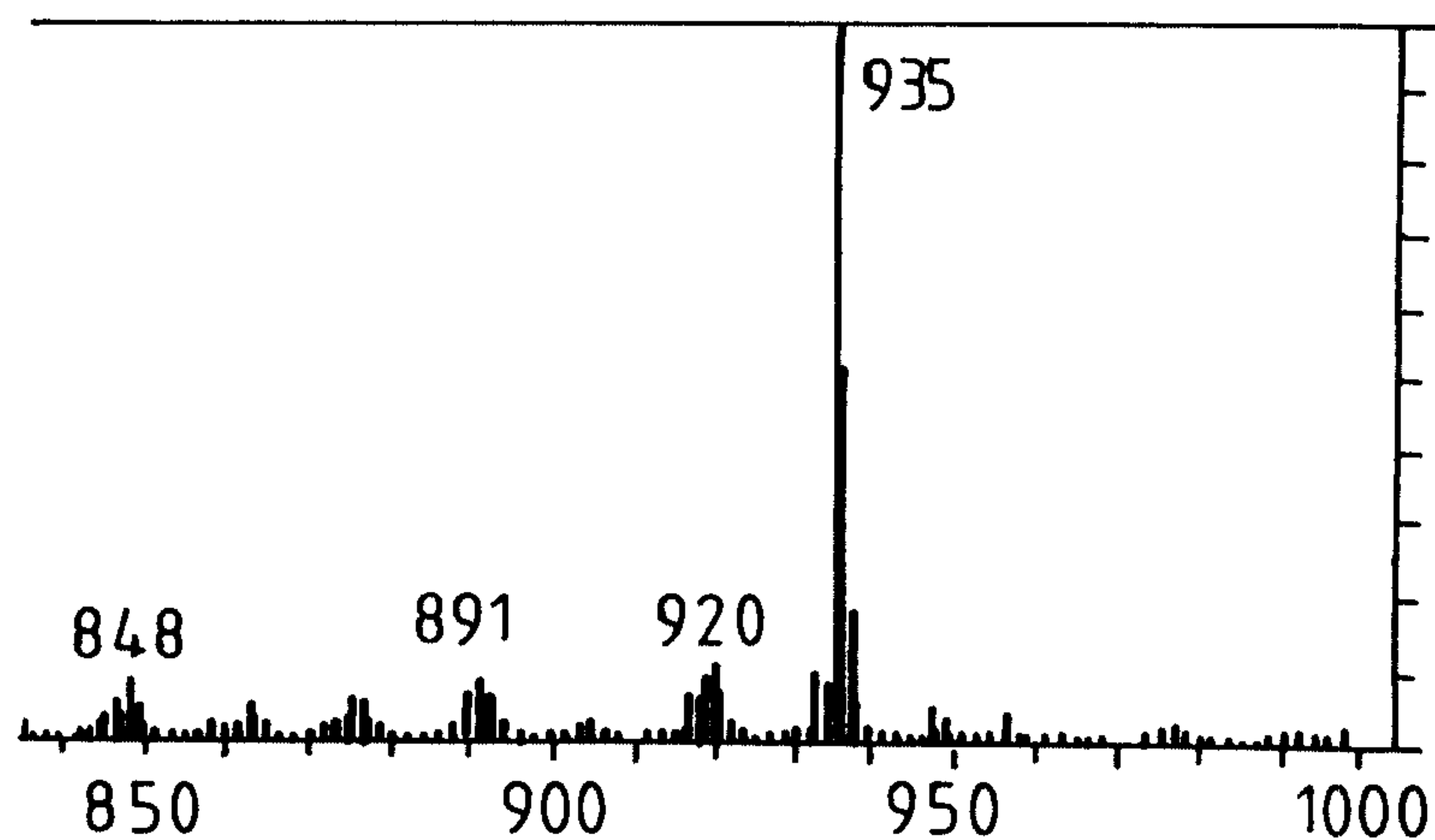
Andrew Sage Dubuc & Martin Walker

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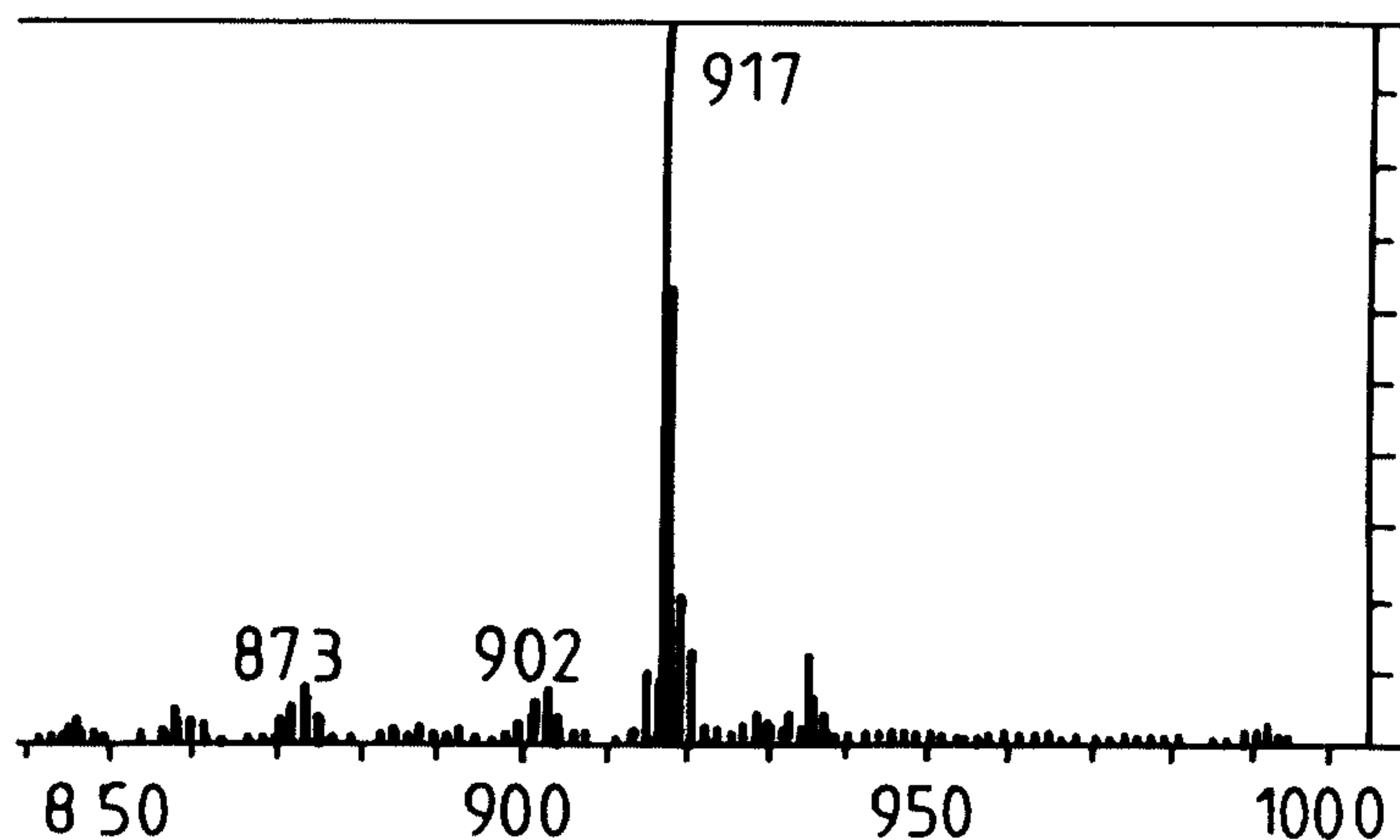


III. 3.



α -form

FAB-MS

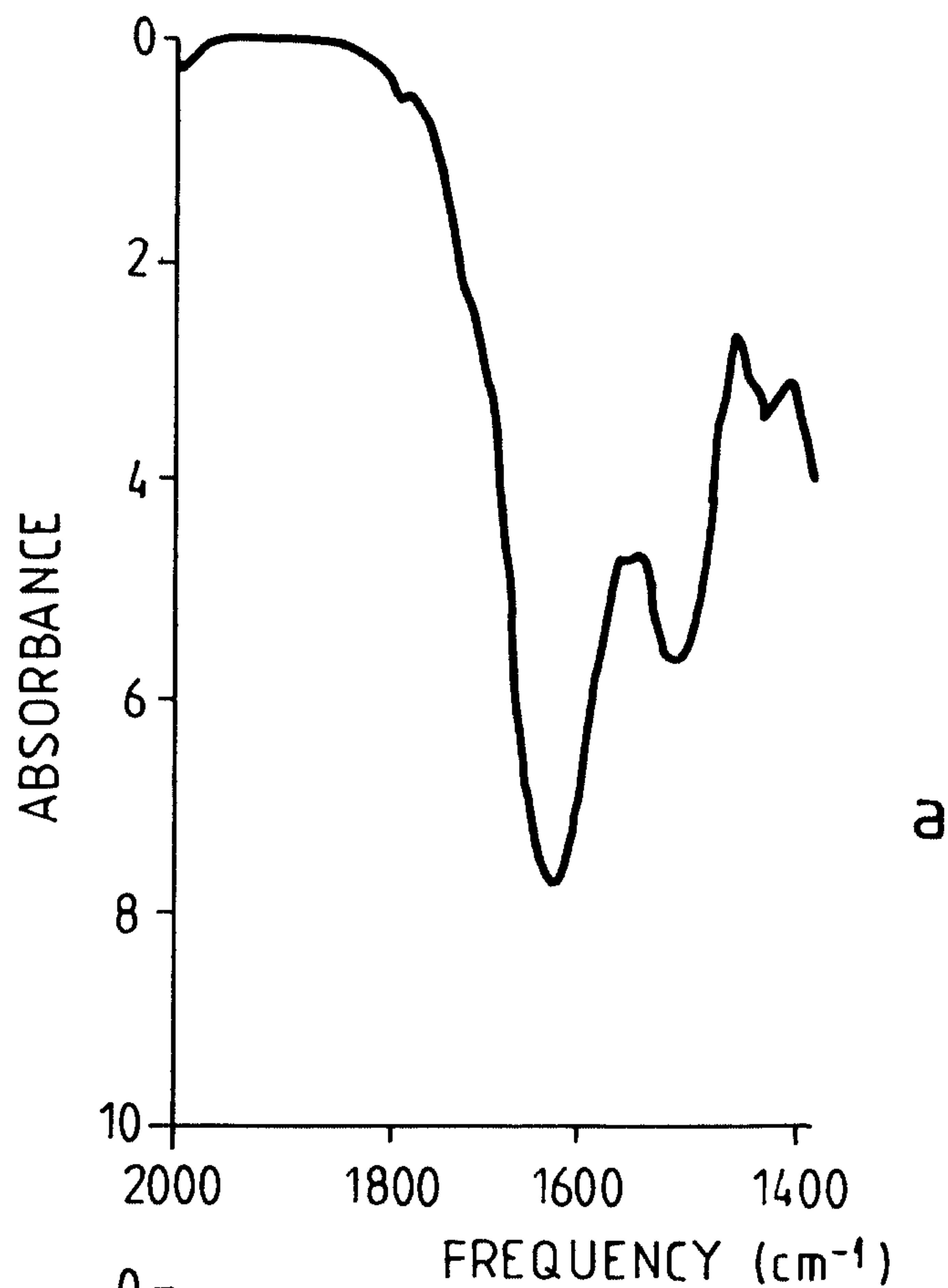
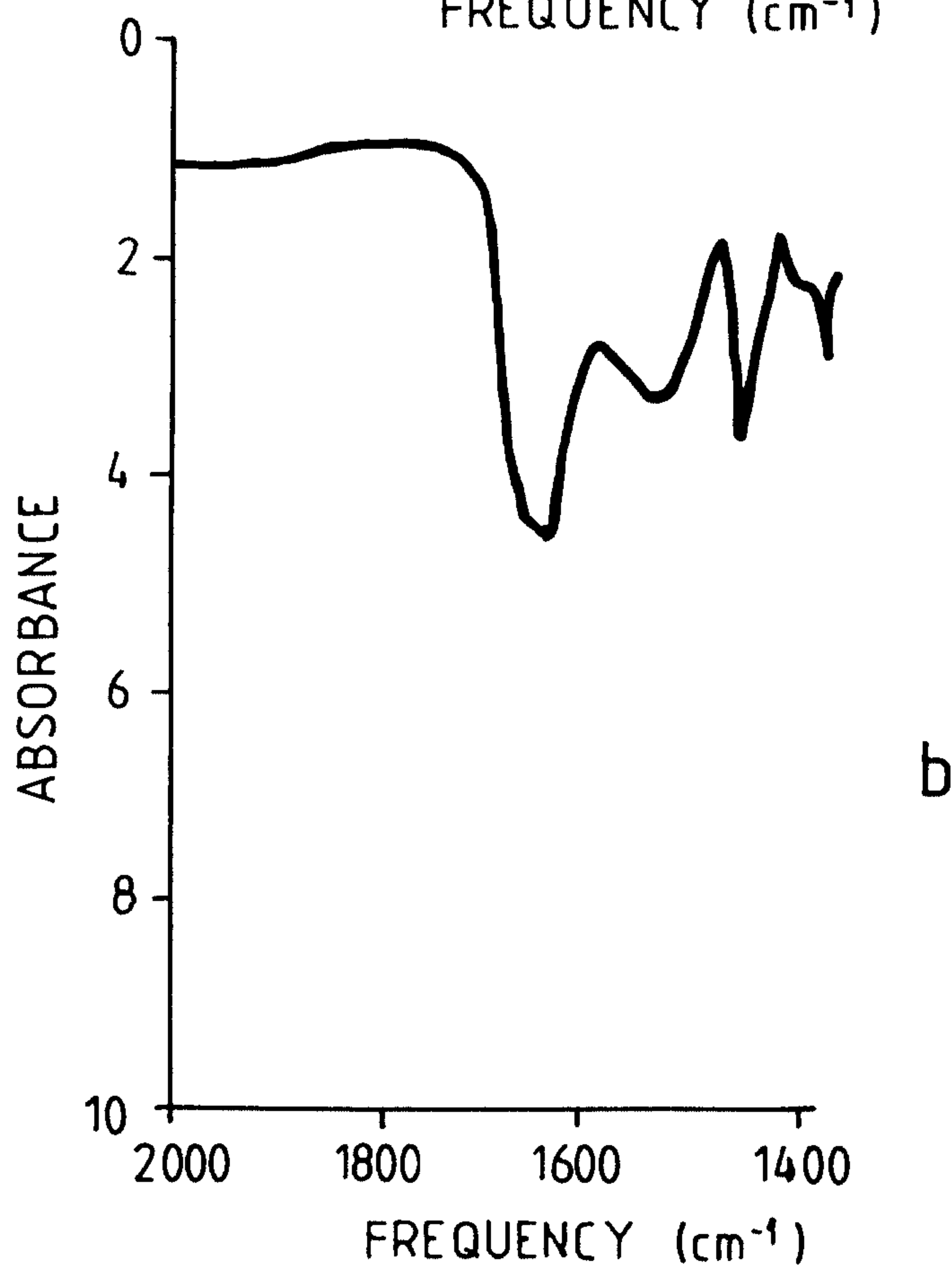


β -imide

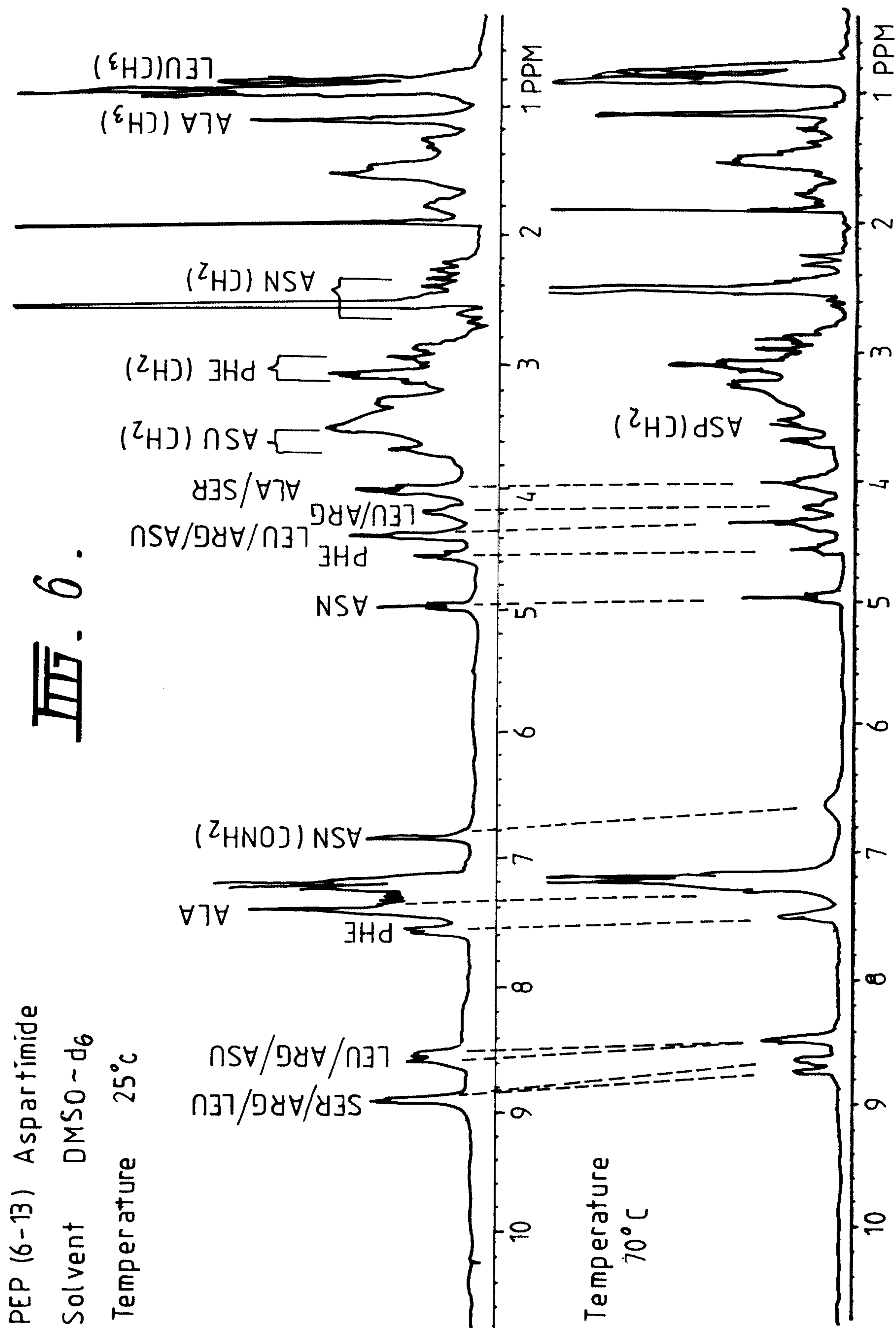
III. 5.

Gudrun Lage Dubuc & Martin Walker

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INFRARED SPECTRA OF PEP (6-13) α -FORMIII. 4.*Gordon R. Dube & Martin Walker*

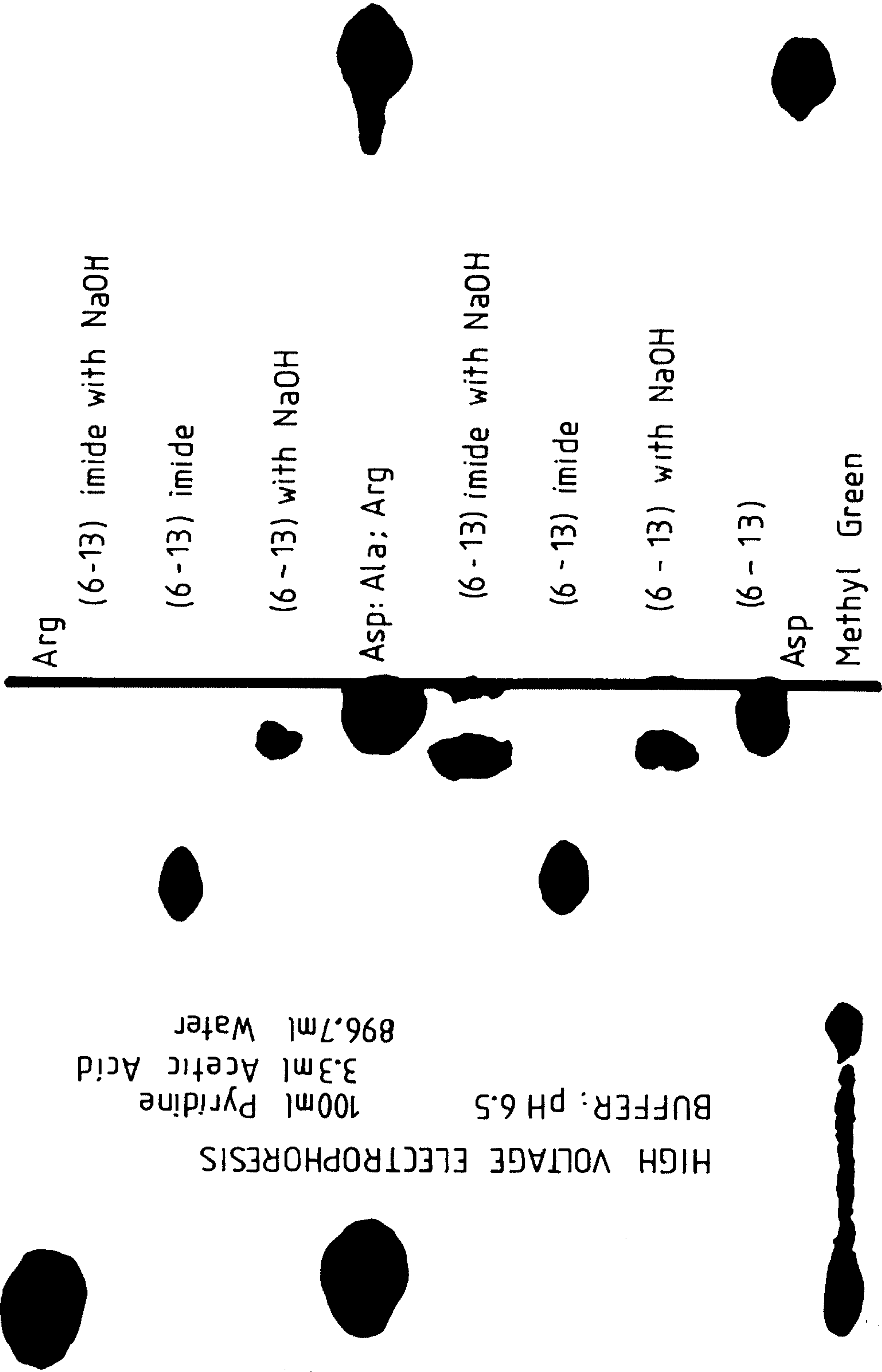
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Gunderson, Roger, Debus & Martinian Walker

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III - 7a



Gordon Sage Dubuc & Martin Walker

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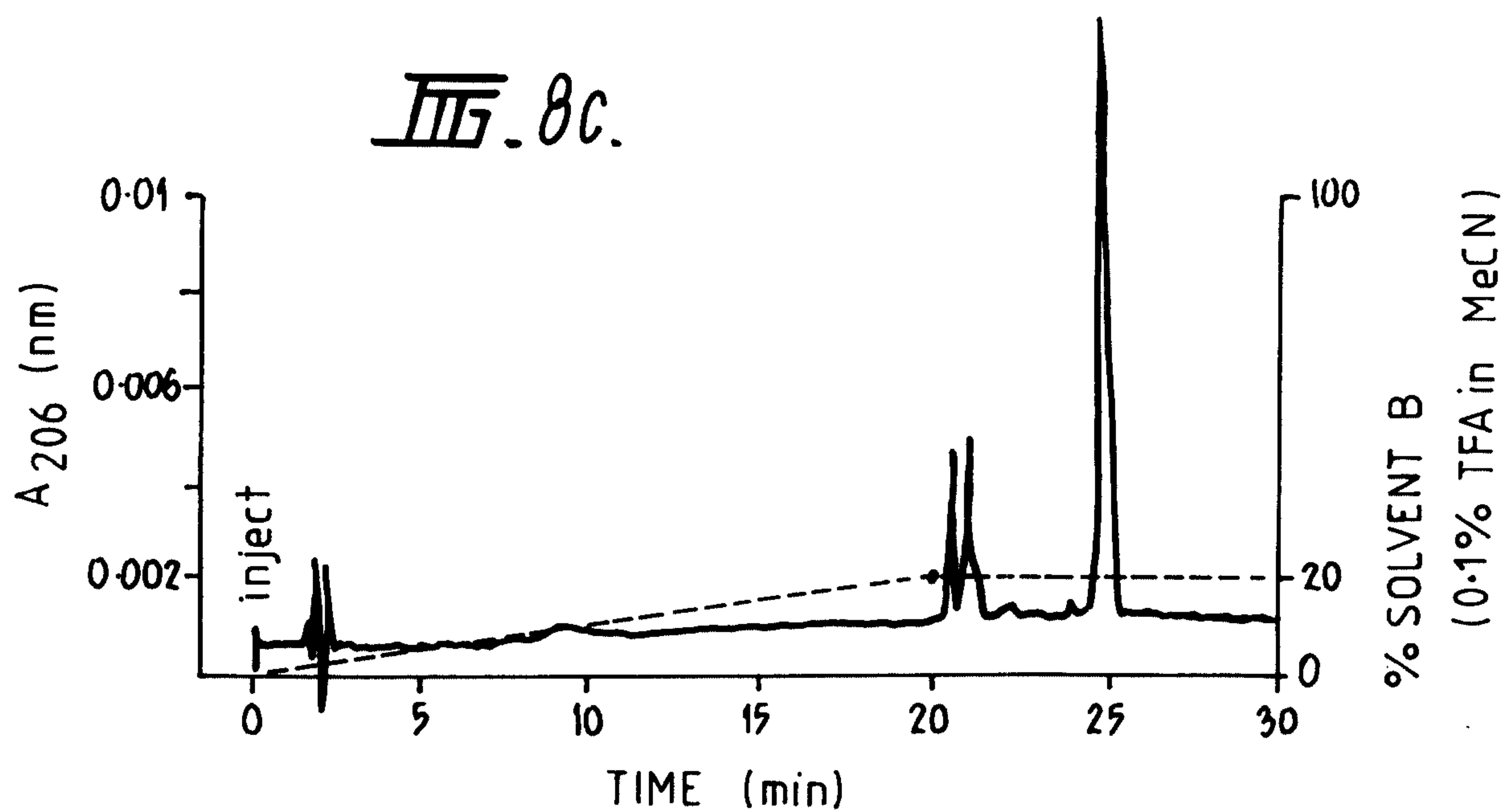
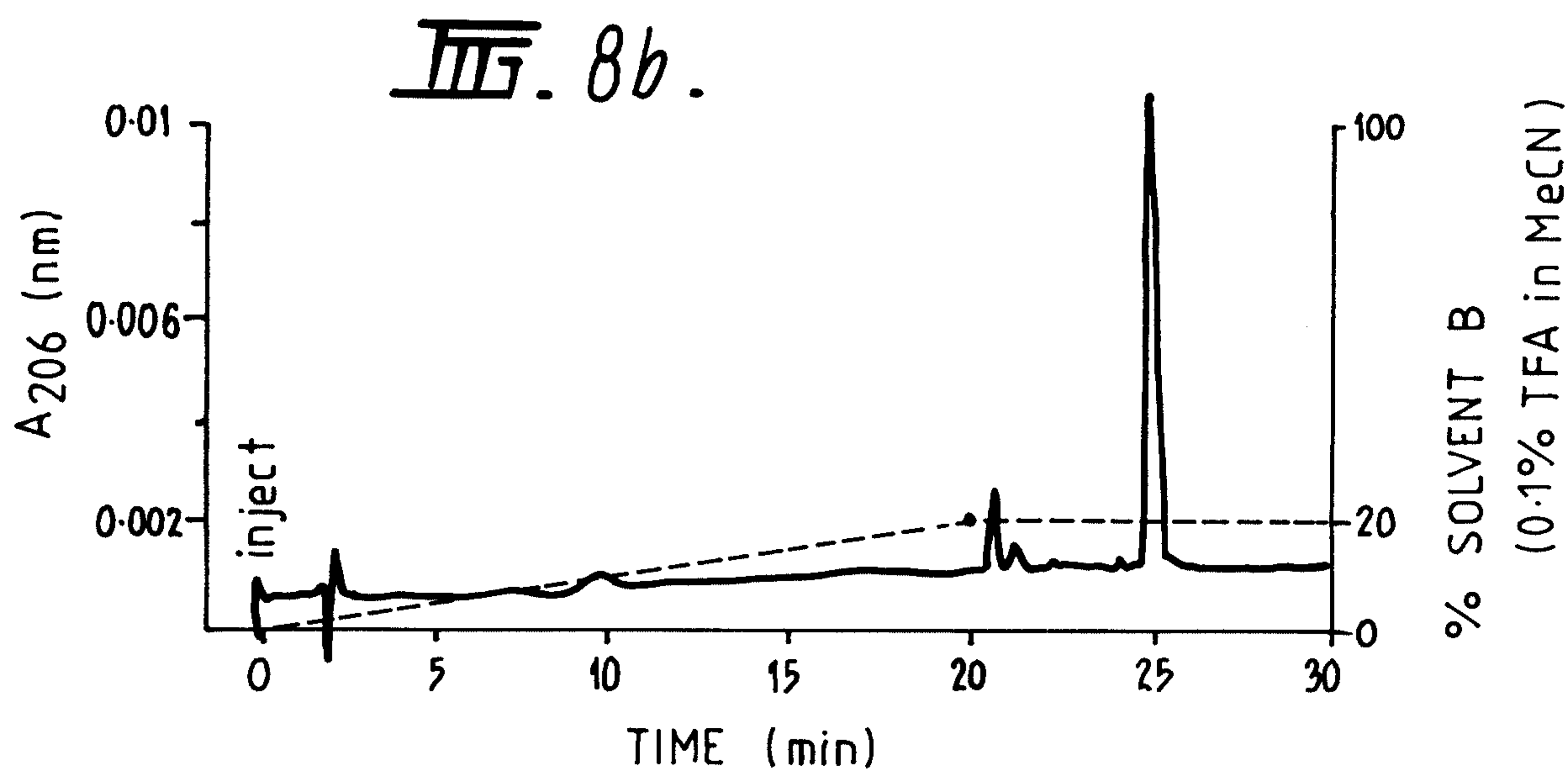
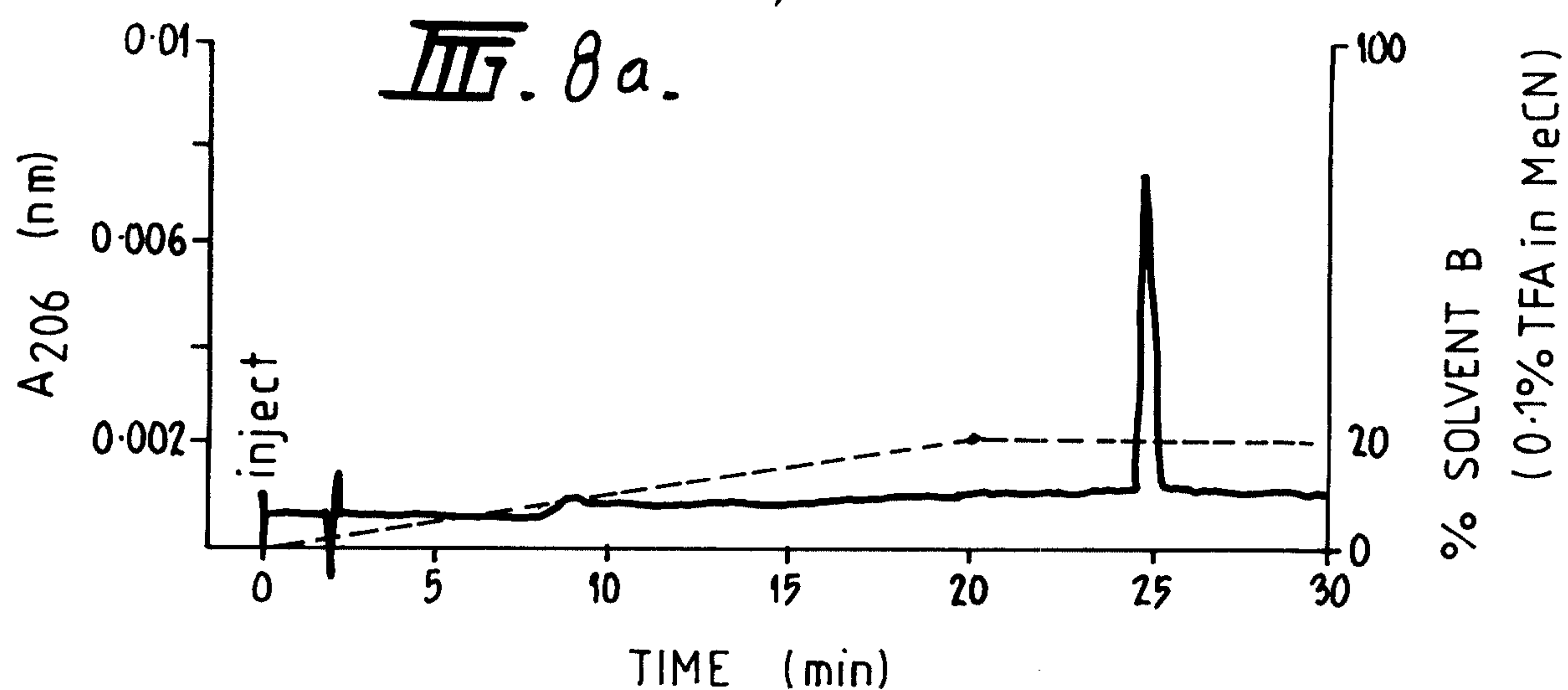
Arg
(6-13) α form
(6-13) imide treated with NaOH
(6-13) imide
Asp:Ala:Arg
(6-13) α form
(6-13) imide treated with NaOH
(6-13) imide
Asp
Ala
Methyl Green

III. 7b.

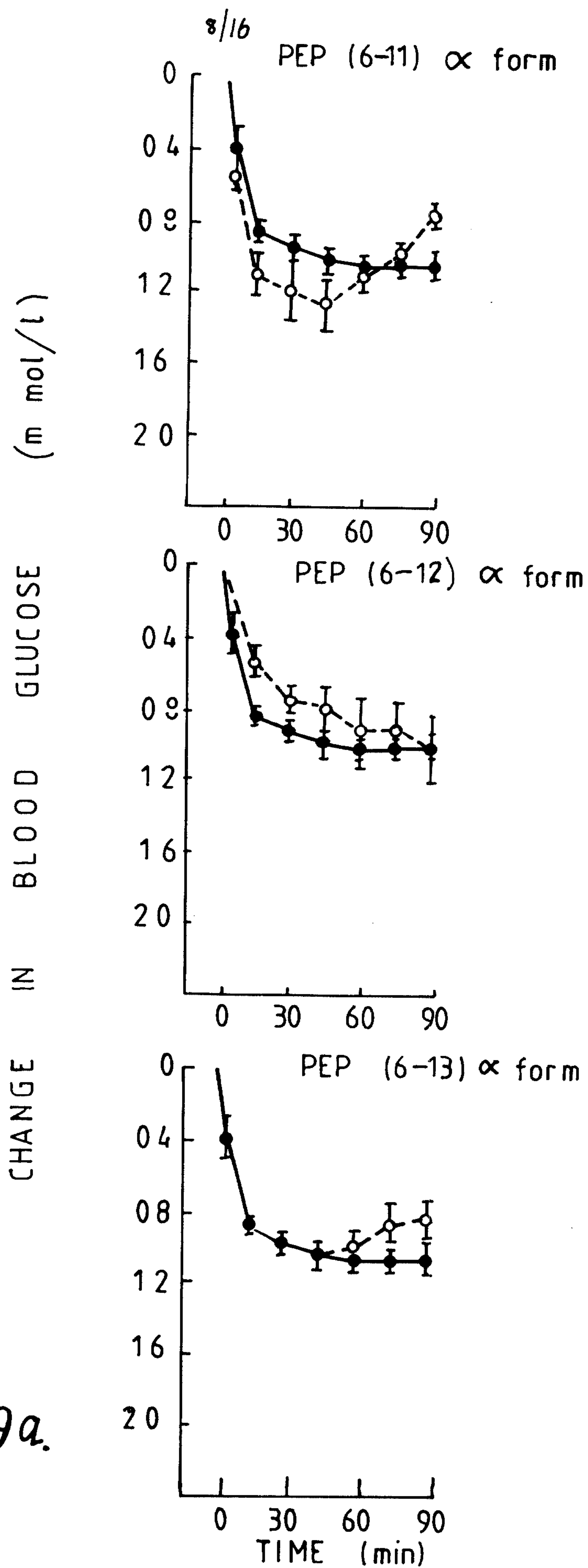
HIGH VOLTAGE ELECTROPHORESIS
BUFFER pH 3.0 50ml HCOOH
~ 28ml Pyridine made up
0.2% Ninhydrin Stain to 11t H₂O
2.5H 1500v

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Andrew Sage Dutta & Martin Walker



III. 9a.

Andrian Sage Dubus & Martine Walker

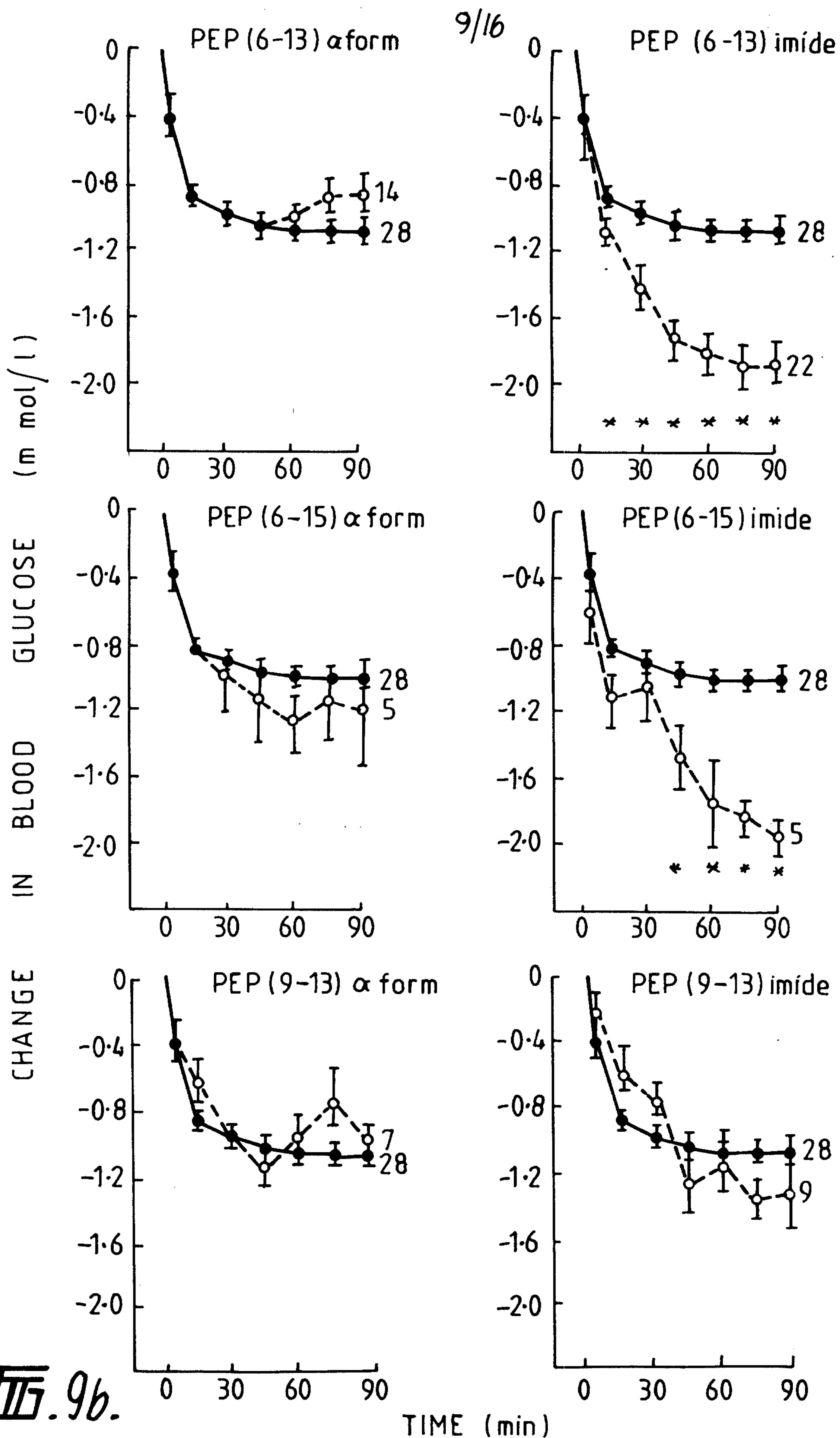
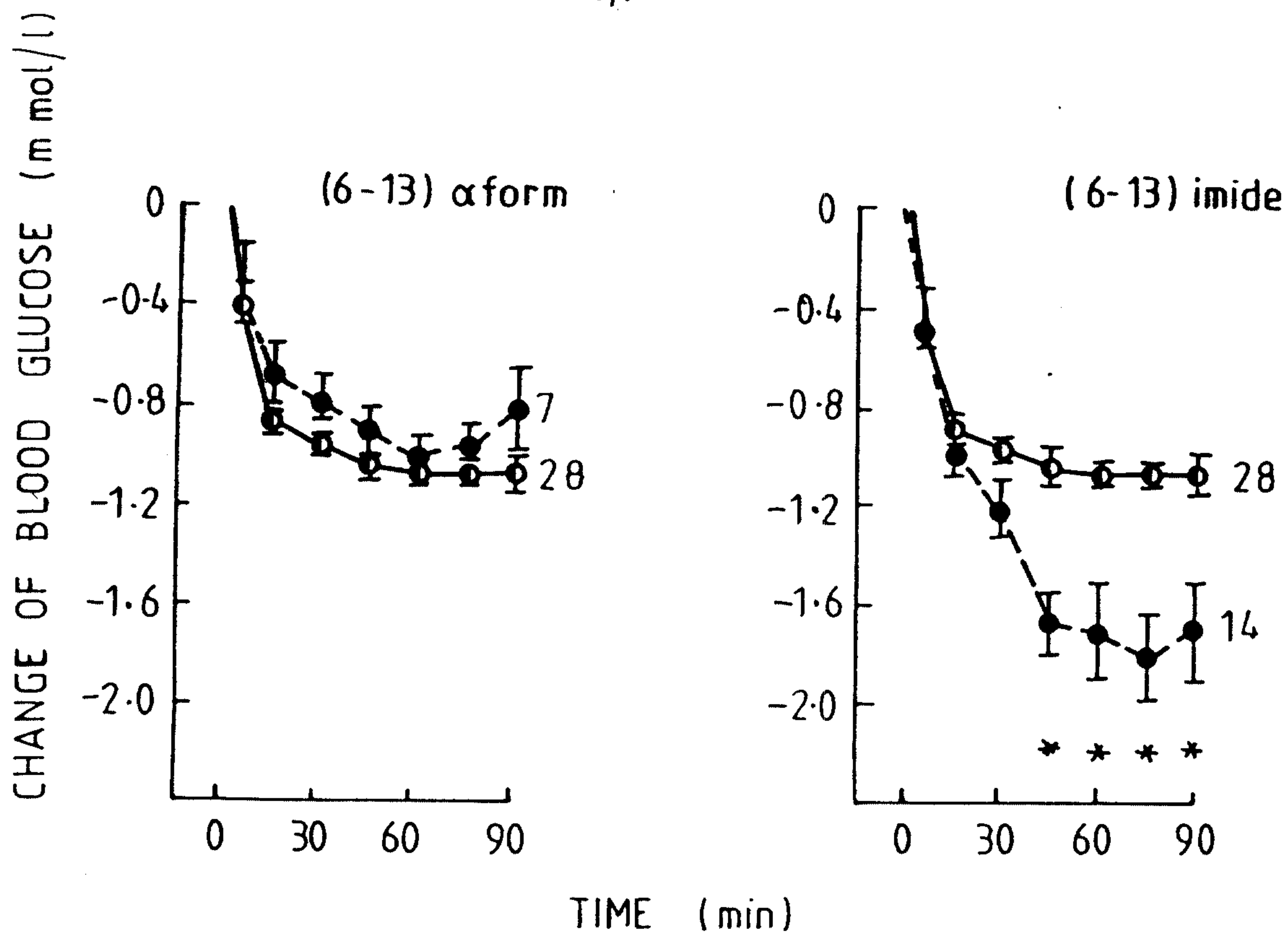
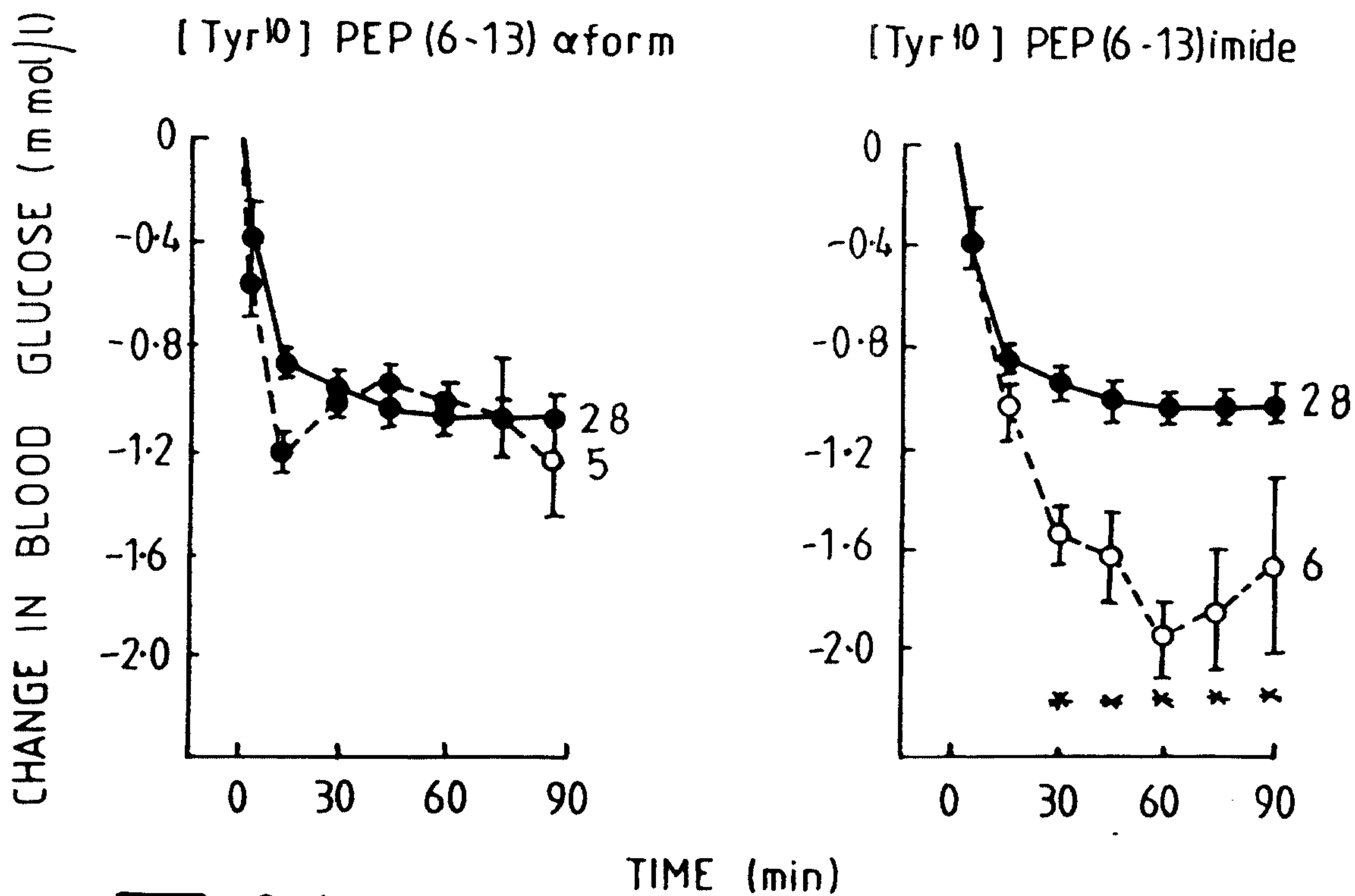


Fig. 96.

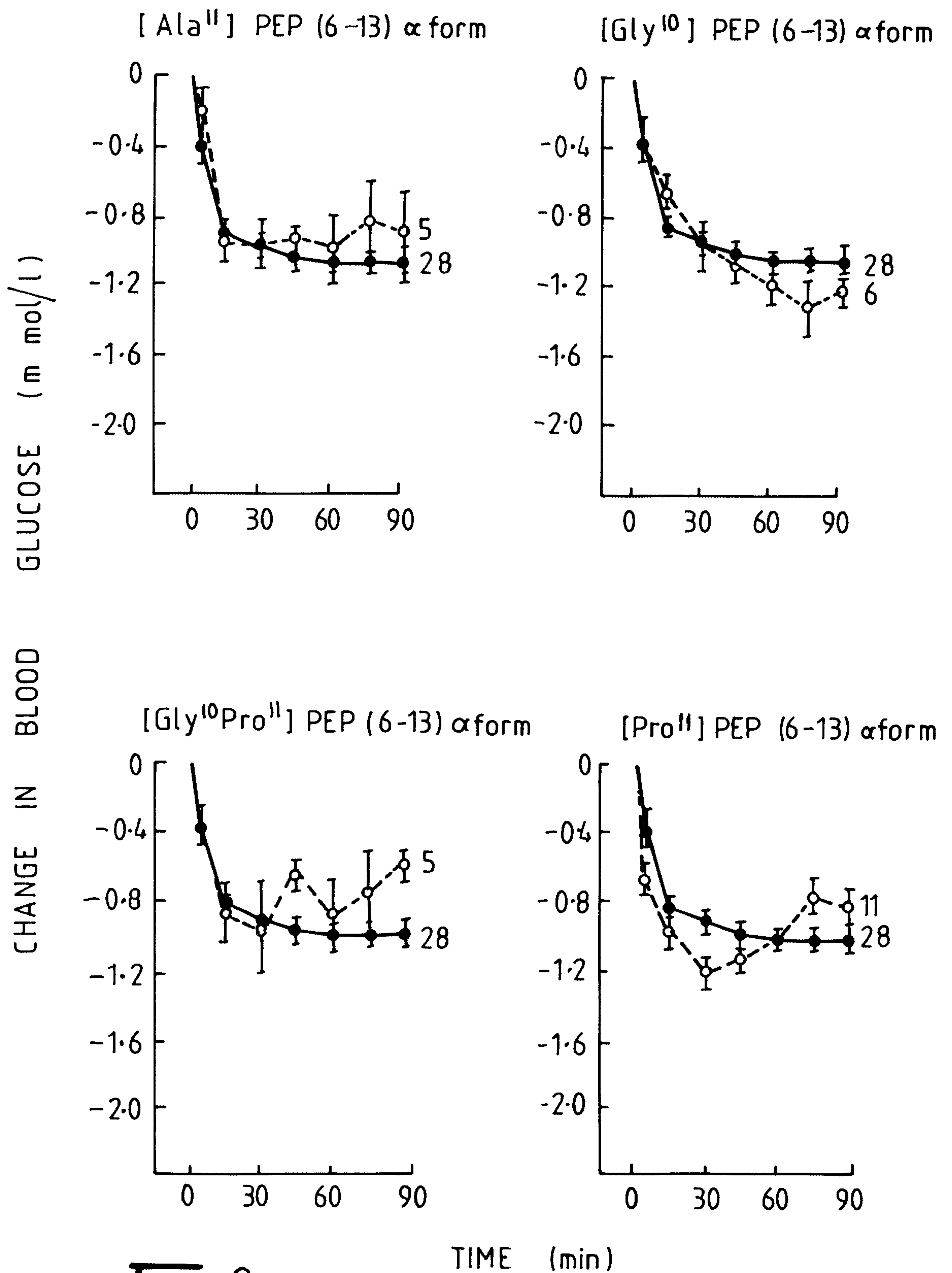
Andrew Sage Dubuc & Martin Walker

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Fig. 9c.Fig. 9d.

Judson Sage Oshes & Martin Walker

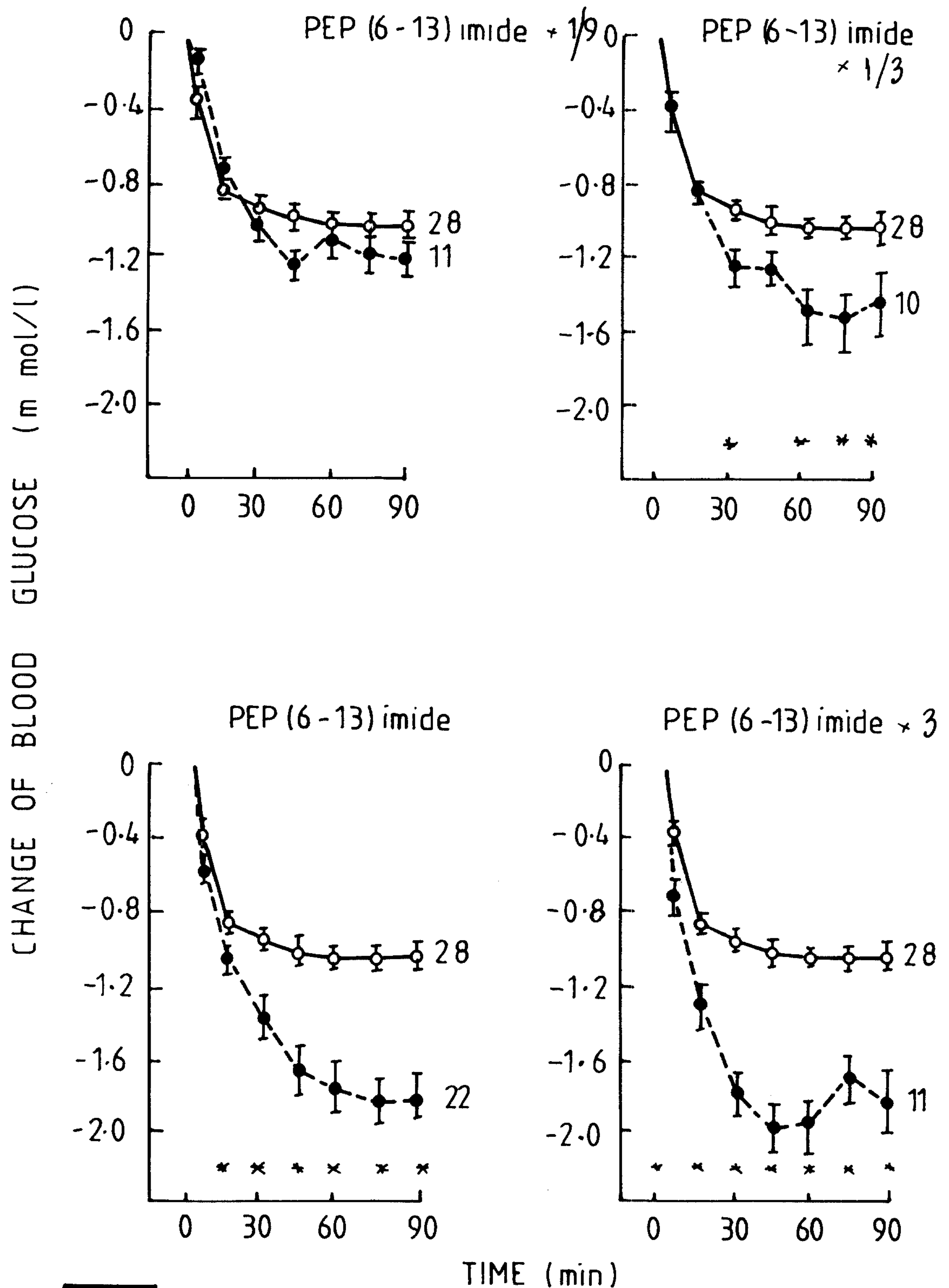
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III. 9e.

Andreas Sage Oubus & Martin Walker

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Effect of various doses of PEP (6-13) imide during intravenous insulin sensitivity tests on normal rats

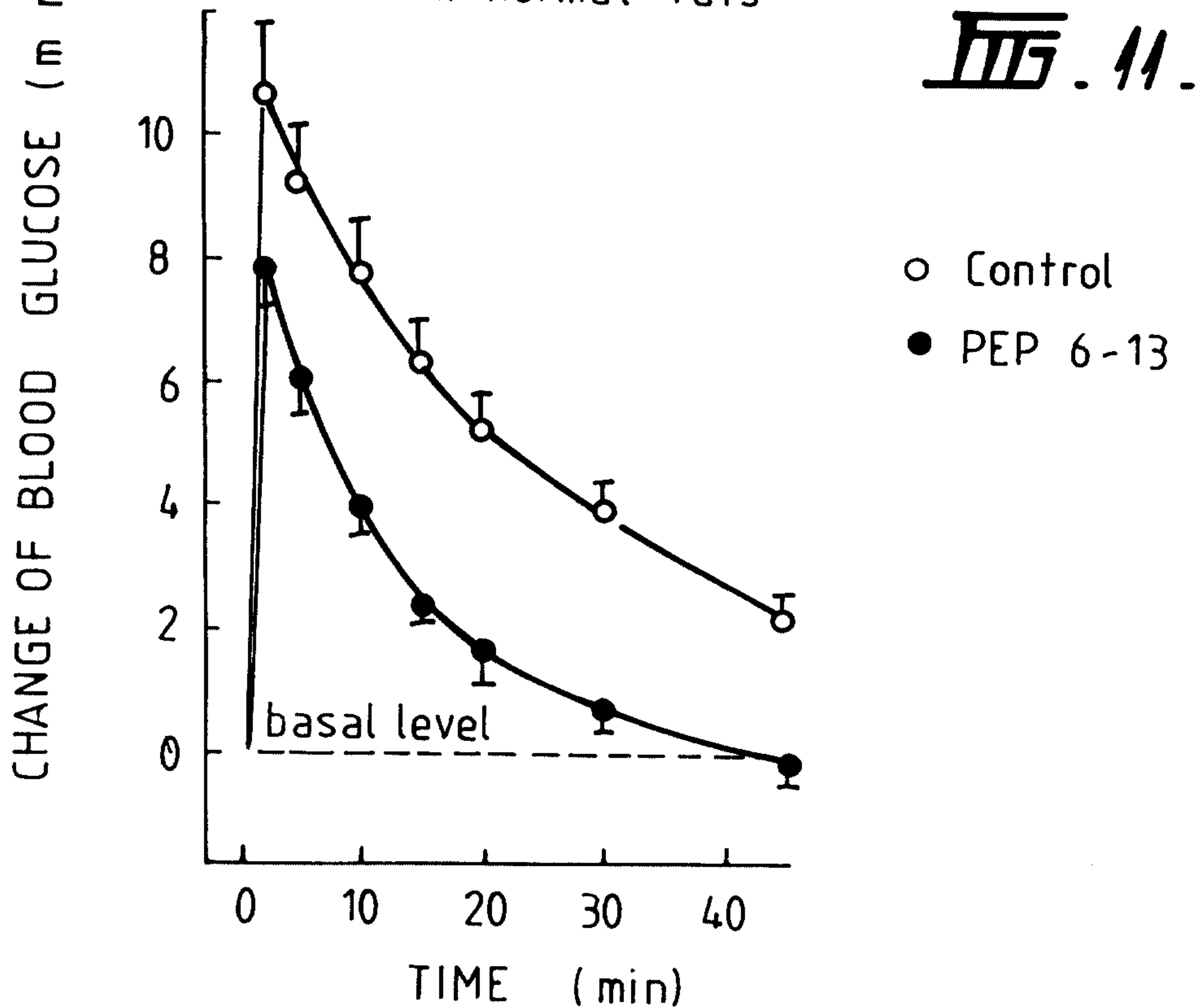


III. 10.

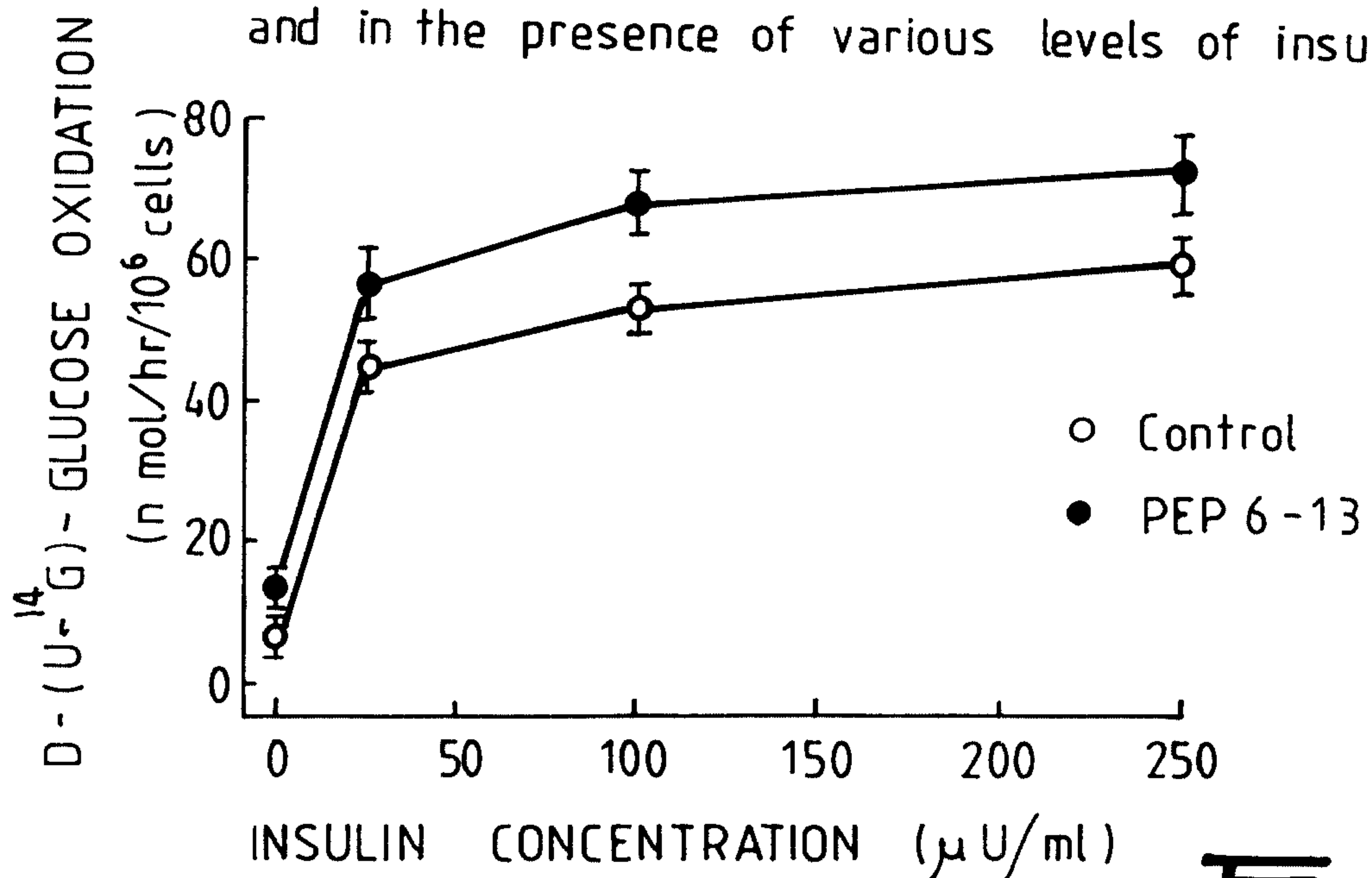
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Effect of PEP (6-13) on glucose tolerance tests
in normal rats



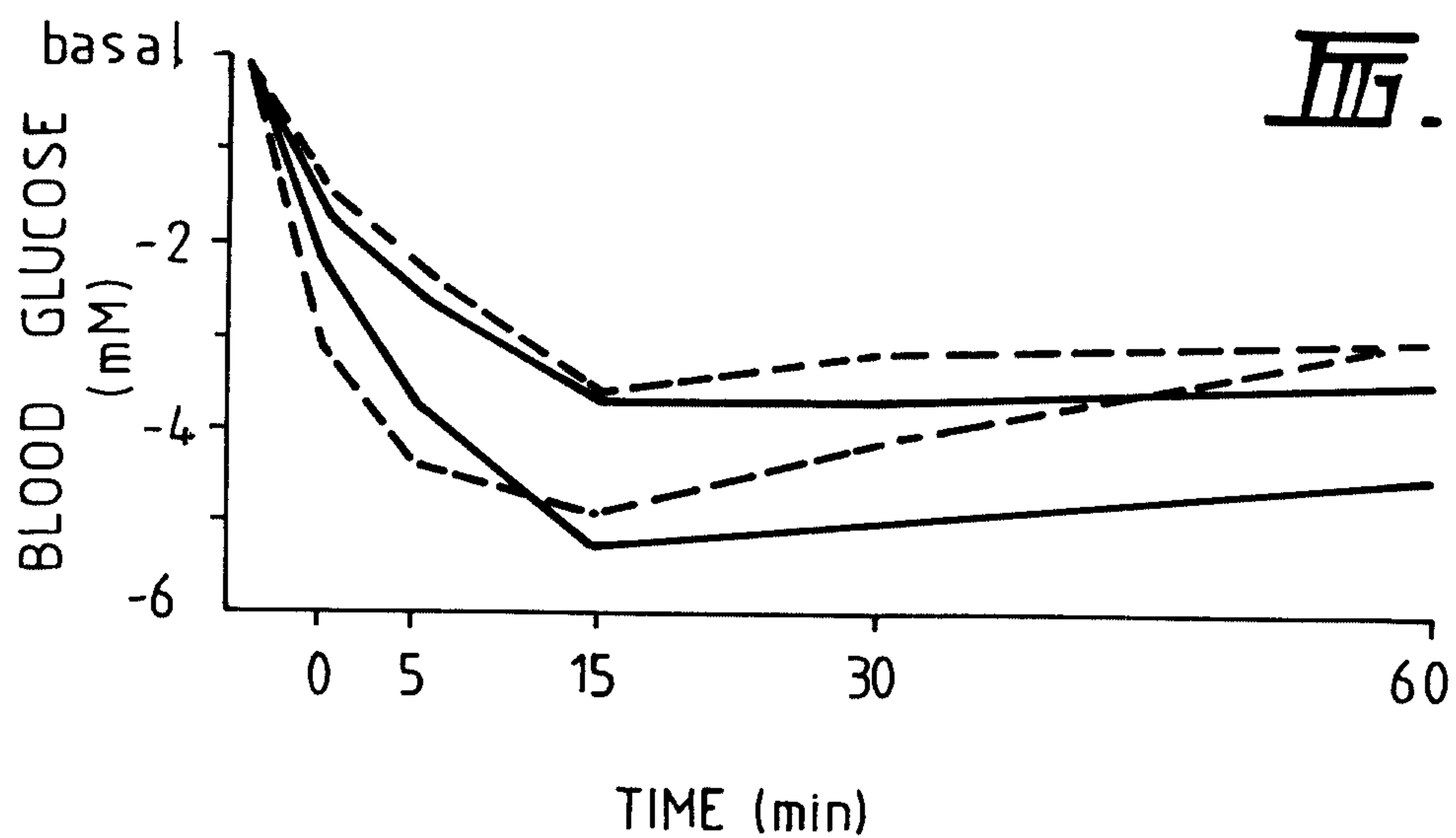
In vitro effect of PEP (6-13) imide on glucose oxidation in isolated rat adipocytes in the absence and in the presence of various levels of insulin.



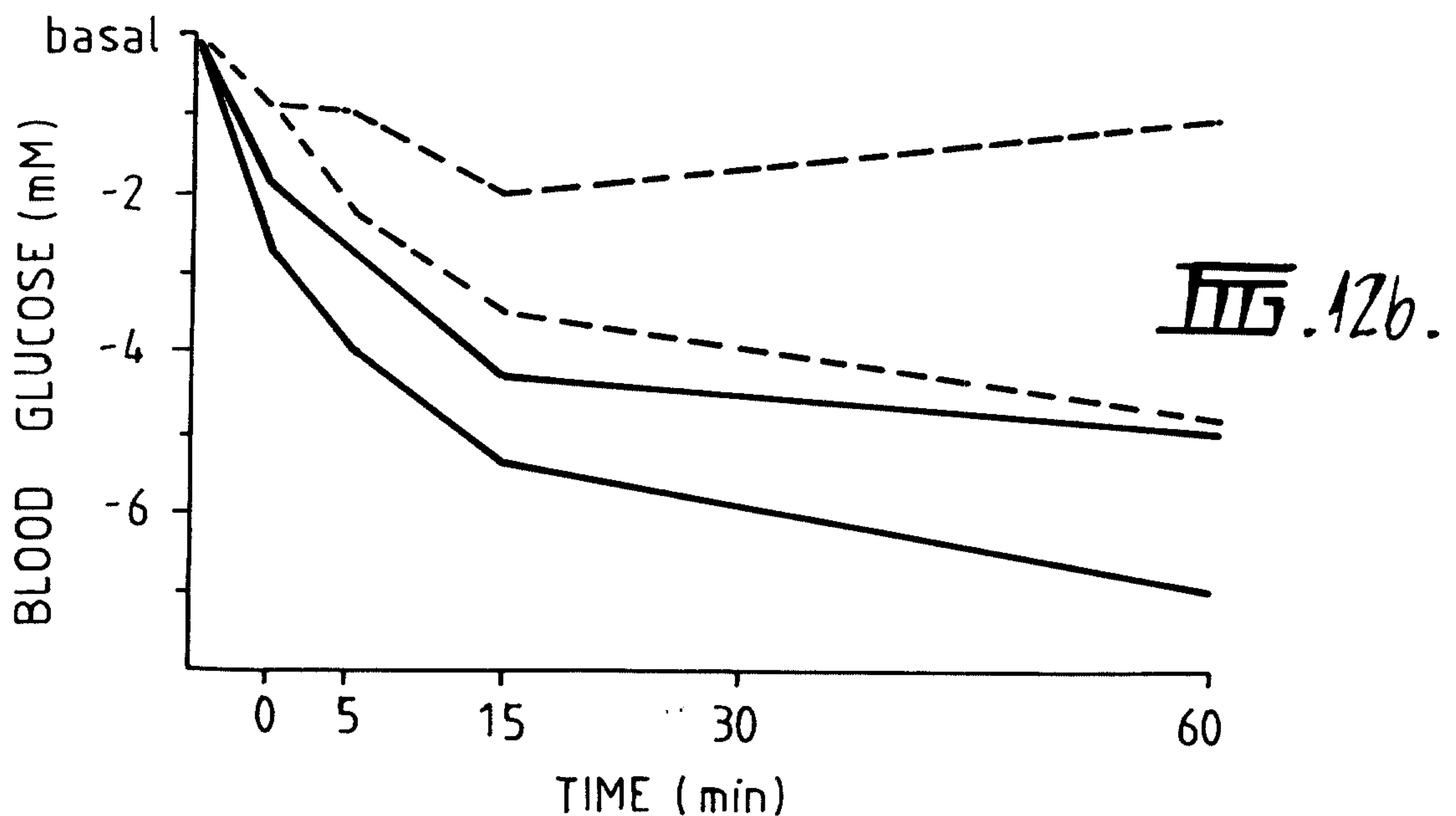
Andrew Lloyd Davies & Martin Walker

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In vivo testing of PEP6-13 (Asn¹¹ Asp¹²) for insulin-potentiating activity on "untrained" female mice.



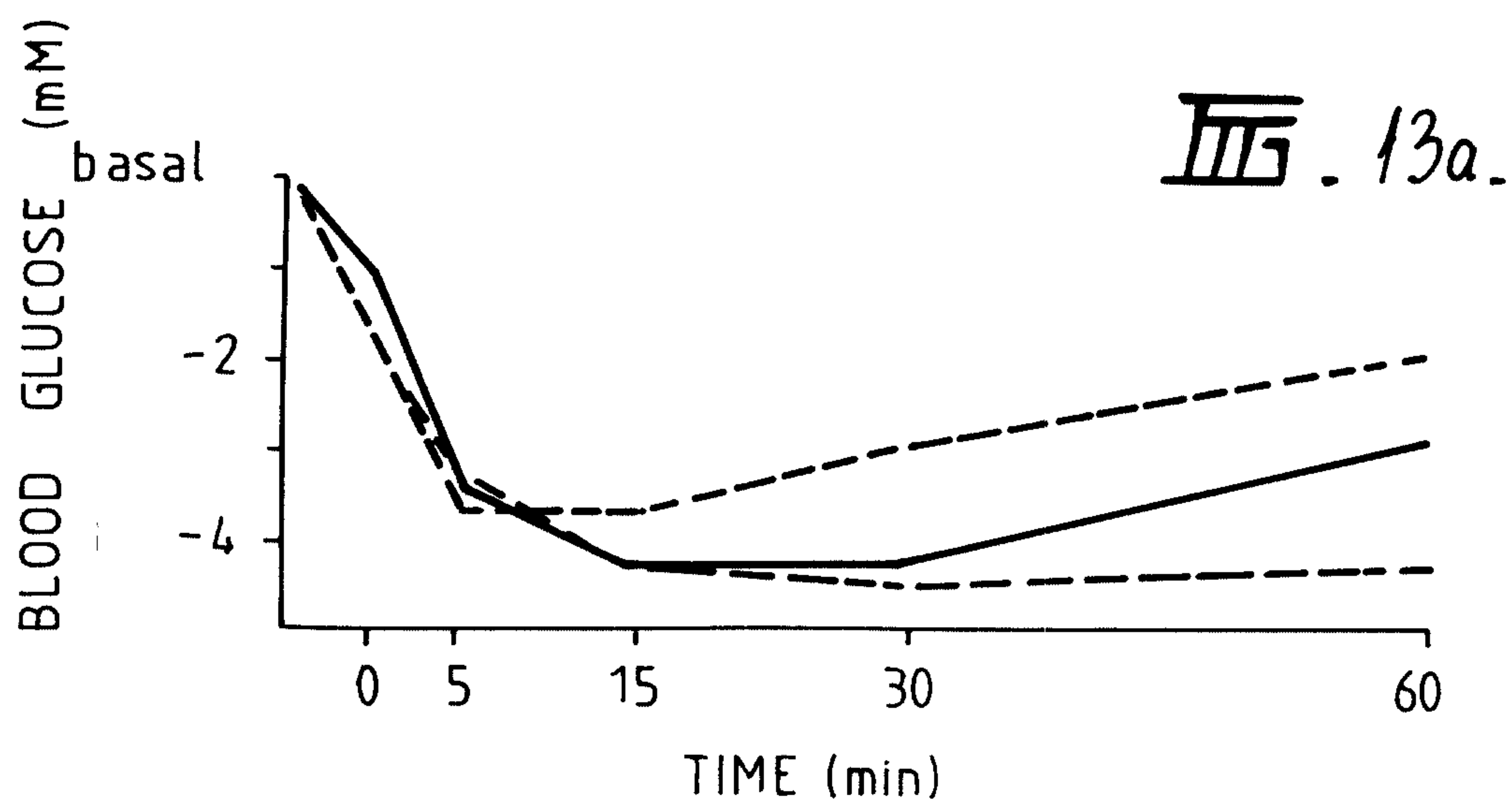
In vivo testing of PEP6-13 (Asp¹²) for insulin potentiating activity on "untrained" female mice



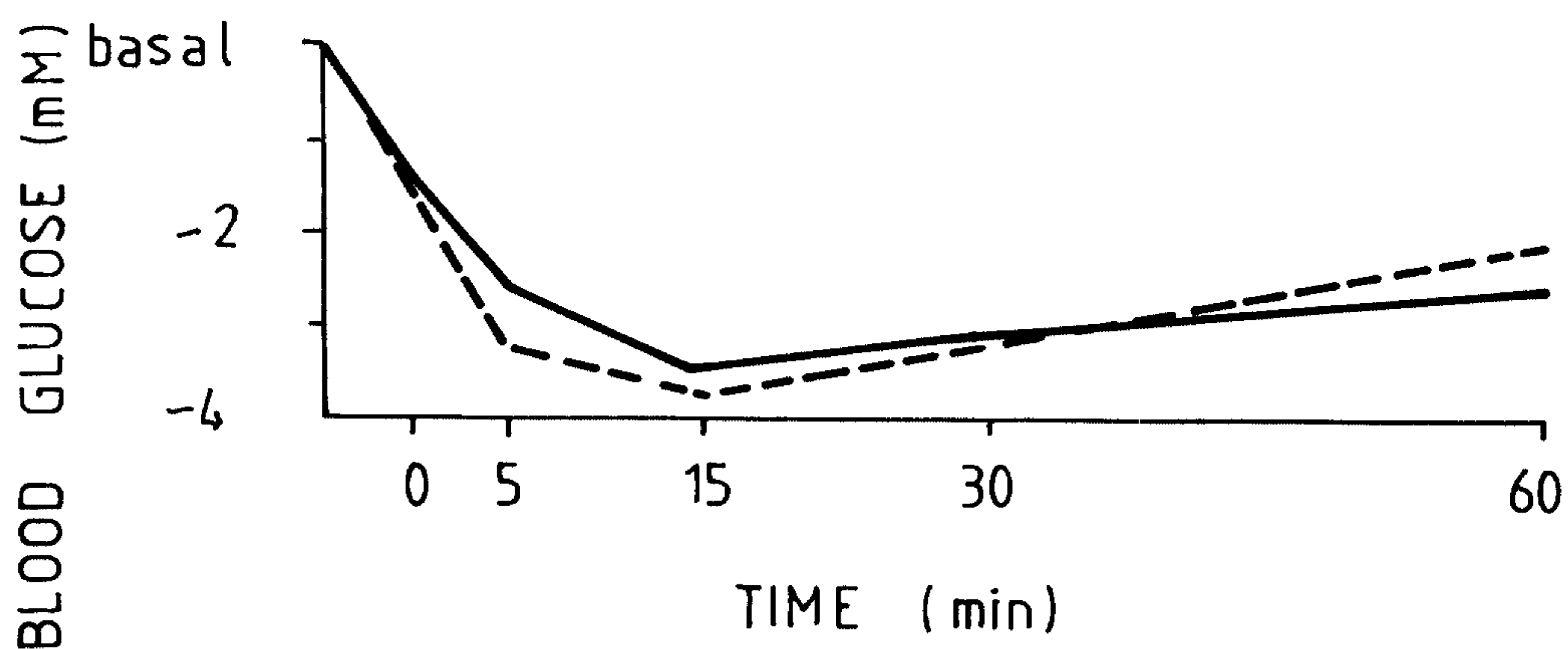
Goudreau, Sage, Dubuc & Martineau Walker

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In vivo testing of PEP6-13 (Gln¹¹ Asp¹²) for
insulin-potentiating activity on "untrained"
male mice



In vivo testing of PEP6-13 (Gln¹¹ Asp¹²) for
insulin potentiating activity on "trained"
male mice : mean fall in blood glucose

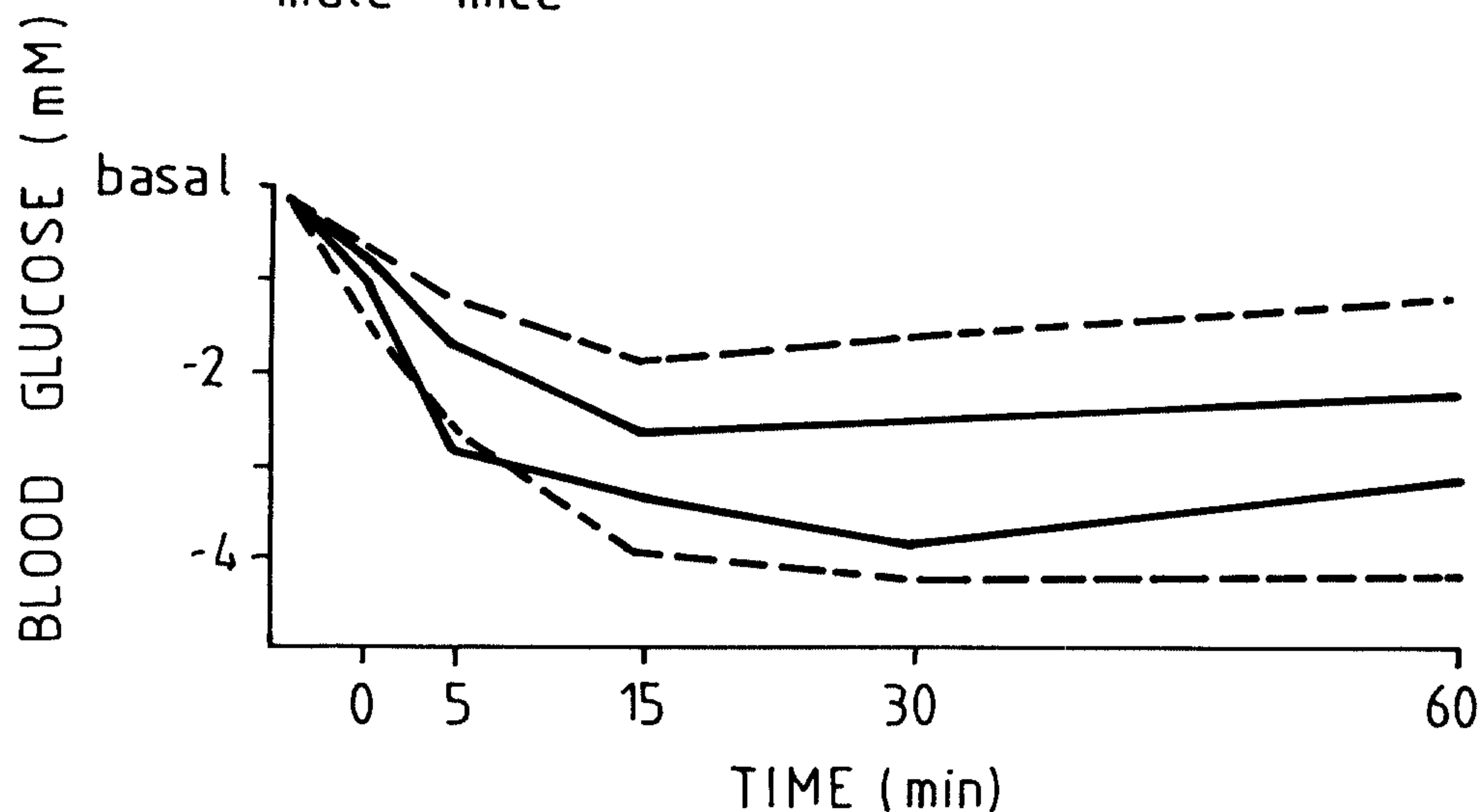


III. 13b.

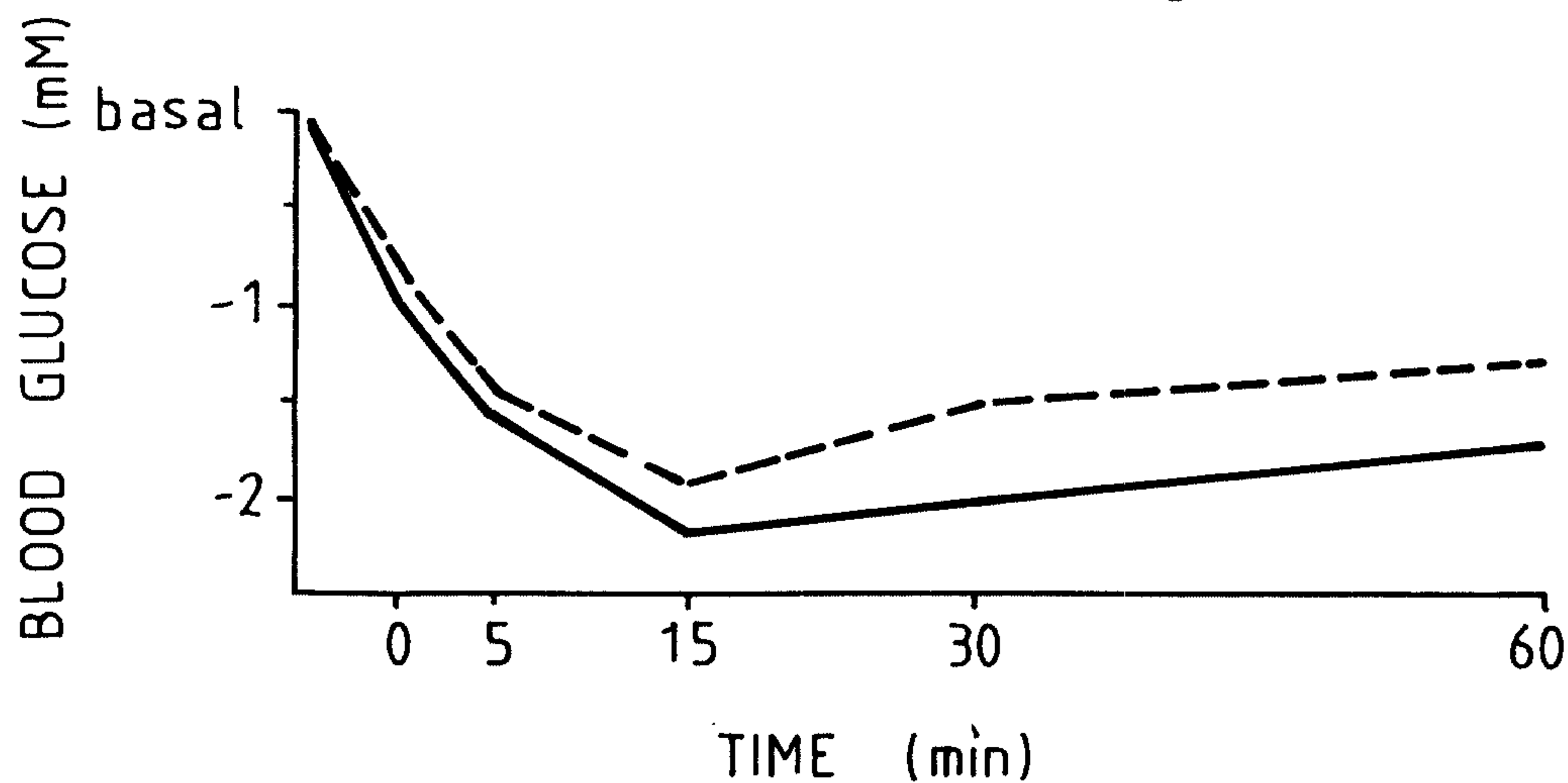
Andrew Sage Oshur & Martin Walker

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In vivo testing for PEP6-13 (Asn¹¹ Asp¹²) for
insulin-potentiating activity on "trained"
male mice

FIG. 13c.

In vivo testing of PEP6-13 (Asp¹²) for
insulin-potentiating activity on "trained" male
mice: mean fall in blood glucose

FIG. 13d

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