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612964

FORM 1

AMENDED

SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

CONVENTION APPLICATION FOR A STANDARD PATENT

We, **NOVO NORDISK A/S**, of Novo Alle, DK-2880 BAGSVAERD, DENMARK, hereby apply for the grant of a standard patent for an invention entitled:

"Insulin Derivatives Having A Charge Which Is Positive Compared With The Charge of Human Insulin At Neutral pH".

which is described in the accompanying complete specification.

DETAILS OF BASIC APPLICATION

Number of Basic Application:-
1135/85

Name of Convention Country in which Basic Application were filed:-
Denmark

Date of Basic Application:-
12 March 1985

The address for service is:-

Spruson & Ferguson
Patent Attorneys
Level 33 St Martins Tower
31 Market Street
Sydney New South Wales Australia

DATED this FOURTEENTH day of MARCH 1991

NOVO NORDISK A/S

By: M.J. Anderson

Registered Patent Attorney.

TO: THE COMMISSIONER OF PATENTS

AUSTRALIAN PATENT OFFICE MB

7797U/LPR

SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA
THE PATENTS ACT 1952

AUSTRALIA
CONVENTION
STANDARD
& PETTY PATENT
DECLARATION

DECLARATION IN SUPPORT OF A CONVENTION APPLICATION FOR A PATENT

In support of the Convention Application made for a patent for an invention entitled:

Title of Invention: "NOVEL PEPTIDES"

I/We William Andersen

Full name(s) and address(es) of Declarant(s)

of 58, Nybro Vænge, DK-2800 Lyngby, Denmark

do solemnly and sincerely declare as follows:-

Full name(s) of Applicant(s)

~~1. I am/We are the applicant(s) for the patent~~

(or, in the case of an application by a body corporate)

1. I am/We are authorised by NOVO INDUSTRI A/S

the applicant(~~s~~) for the patent to make this declaration on its/~~their~~ behalf.

2. The basic application(~~s~~) as defined by Section 141 of the Act was/~~were~~ made

Basic Country(ies): in Denmark

Priority Date(s): on 12 March 1985

Basic Applicant(s): by NOVO INDUSTRI A/S

~~3. I am/We are the actual inventor(s) of the invention referred to in the basic application(s)~~

Full name(s) and address(es) of inventor(s)

(or where a person other than the inventor is the applicant)

3. JAN MARKUSSEN

of 7 Kikudbakken, DK-2730 Herlev, Denmark

(~~respectively~~)

is/~~are~~ the actual inventor(~~s~~) of the invention and the facts upon which the applicant(~~s~~) is/~~are~~ entitled to make the application are as follows: NOVO INDUSTRI A/S is entitled by Contract of Employment between the inventor as employee and NOVO INDUSTRI A/S as employer, as a person who would be entitled to have the patent assigned to it if a patent were granted upon an application made by the inventor.

Set out how Applicant(s) derive title from actual inventor(s) e.g. The Applicant(s) is/are the assignee(s) of the invention from the inventor(s)

4. The basic application(~~s~~) referred to in paragraph 2 of this Declaration was/were the first application(~~s~~) made in a Convention country in respect of the invention the subject of the application.

Declared at Bagsvaerd this 24th day of February 1986

William Andersen

Signature of Declarant(s)

To: The Commissioner of Patents

REP4

7/79

(12) PATENT ABRIDGMENT (11) Document No. AU-B-54495/86
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 612964

(54) Title
INSULIN DERIVATIVE

International Patent Classification(s)
(51)[4] C07K 007/40 A61K 037/26

(21) Application No. : 54495/86 (22) Application Date : 11.03.86

(30) Priority Data

(31) Number (32) Date (33) Country
1135/85 12.03.85 DK DENMARK

(43) Publication Date : 18.09.86

(44) Publication Date of Accepted Application : 25.07.91

(71) Applicant(s)
NOVO NORDISK A/S

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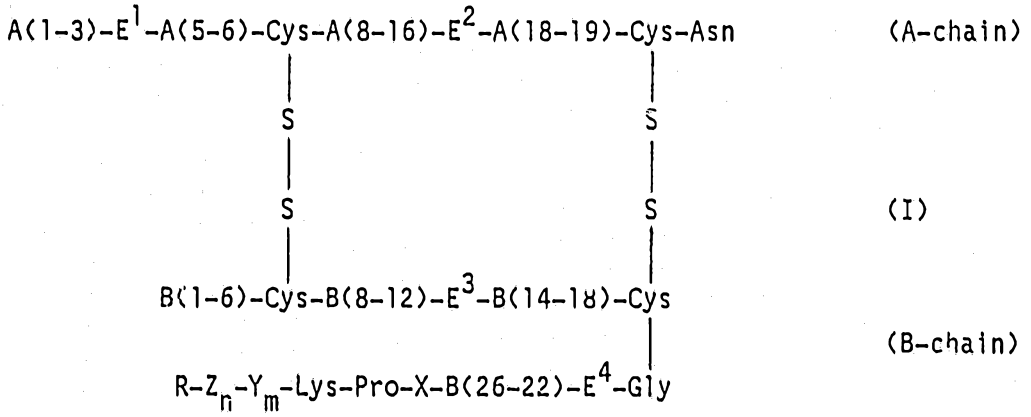
(56) Prior Art Documents
EP 45187
AU 33072/84 C07C 103/52
AU 56227/86 C07K 007/40

(57) Claim

1. A compound of the general formula I



wherein the letters A and B followed by figures in parentheses designate the peptide fragments of the A- and B-chains, respectively, indicated by the figures in parentheses, E^1, E^2, E^3 and E^4 are the same or different each representing glutamic acid or a neutral amino acid residue which can be coded for by nucleotide sequences, X represents an L-threonine, L-arginine or L-lysine residue, Y and Z are the same or different and each represent an amino acid residue wherein any side chain hydroxy group may be alkylated, and m and n are the same or different and each represent zero or one, and R represents an amido or ester residue which blocks the C-terminal carboxyl group of the B-chain, with the proviso

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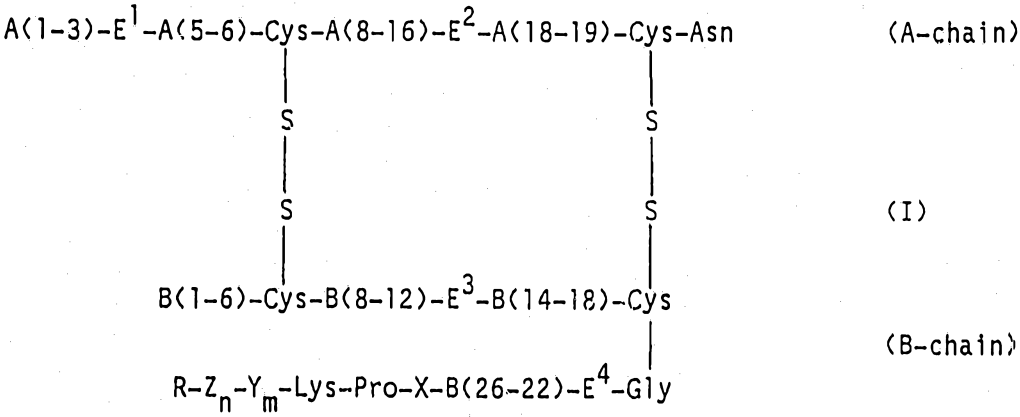
that not all of E^1, E^2, E^3 and E^4 are glutamic acid residues when X is a threonine residue, or, when E^1, E^2, E^3 and E^4 each is a glutamic acid residue and X is a threonine residue, the group of formula $-Y_m-Z_n-R$ represents $-NH_2$, $-Arg-NH_2$, $-Arg-Arg-NH_2$, $-Arg-Lys-NH_2$, $-Dab-Dab-NH_2$, $-Dap-Dap-NH_2$, $Lys-NH_2$, $-Lys(Lau)-NH_2$, $-Lys-Arg-NH_2$, $-Lys-Lys-NH_2$, $-Orn-NH_2$ or $-Orn-Orn-NH_2$, with the further proviso that the compound of formula I has at least one charge more than human insulin at a pH value of 7.

15. An injectable solution with prolonged insulin action, characterized in that it contains a compound of the general formula I



wherein the letters A and B followed by figures in parentheses designate the peptide fragments of the A- and B-chains, respectively, indicated by the figures in parentheses, E^1, E^2, E^3 and E^4 are the same or different each representing glutamic acid or a neutral amino acid residue which can be coded for by nucleotide sequences, X represents an L-threonine, L-arginine or L-lysine residue, Y and Z are the same or different and each represent an amino acid residue wherein any side chain hydroxy group may be alkylated, and m and n are the same or different and each represent zero or one, and R represents an amido or ester residue which blocks the C-terminal carboxyl group of the B-chain, with the proviso that not all of E^1, E^2, E^3 and E^4 are glutamic acid residues when X is a threonine residue, or, when E^1, E^2, E^3 and E^4 each is a glutamic acid residue and X is a threonine residue, the group of formula $-Y_m-Z_n-R$ represents $-NH_2$, $-Arg-NH_2$, $-Arg-Arg-NH_2$, $-Arg-Lys-NH_2$, $-Dab-Dab-NH_2$, $-Dap-Dap-NH_2$, $Lys-NH_2$, $-Lys(Lau)-NH_2$, $-Lys-Arg-NH_2$, $-Lys-Lys-NH_2$, $-Orn-NH_2$ or $-Orn-Orn-NH_2$, with the further proviso that the compound of formula I has at least one charge more than human insulin at a pH value of 7, together with a pharmaceutically acceptable diluent.

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29. A method of treatment of diabetes mellitus in a patient requiring said treatment which method comprises administering to said patient an effective amount of either a compound according to any one of Claims 1 to 11 or 14 or of a solution according to Claim 20.

612964

FORM 10 SPRUSON & FERGUSON

COMMONWEALTH OF AUSTRALIA

PATENTS ACT 1952

COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE:

54495/86

Class Int. Class

Complete Specification Lodged:

Accepted:

Published:

Priority:

Related Art:

Name of Applicant: Novo-Nordisk A/S
NOVO-INDUSTRI A/S

Address of Applicant: Novo Alle, 2880 Bagsvaerd, Denmark

Actual Inventor: JAN MARKUSSEN

Address for Service: Spruson & Ferguson, Patent Attorneys, Level 33 St Martins Tower, 31 Market Street, Sydney, New South Wales, 2000, Australia

Complete Specification for the invention entitled:

"Insulin Derivatives Having A Charge Which Is Positive Compared With The Charge of Human Insulin At Neutral pH".

The following statement is a full description of this invention, including the best method of performing it known to us

SBR/TGK/65W

ToN/VMN, 1986-02-13, 3j, D-328B, IL 2771.200

TITLE: "Insulin Derivatives Having A Charge Which Is Positive Compared With The Charge of Human Insulin At Neutral pH".

ABSTRACT

Insulin derivatives having a charge which is positive compared with the charge of human insulin at neutral pH, can be used to prepare solutions having prolonged insulin action. In the novel insulin derivatives, a basic amino acid has been substituted in the B27-position and/or a neutral amino acid has been inserted in the A4-, A17-, B13- and/or B21-position. Furthermore, the C-terminal carboxyl group of the B-chain has been blocked with an amido or ester residue.

BACKGROUND OF THIS INVENTION

The present invention relates to novel insulin compounds and to novel injectable solutions having prolonged insulin action.

In the treatment of diabetes mellitus, many varieties of insulin preparations have been suggested and used. Some of the preparations are fast acting and other preparations have more or less prolonged actions. Usually, pharmaceutical insulin preparations with more or less prolonged action are desirable. Such a prolonged action may be obtained by administering the insulin as a suspension of insulin crystals. The crystalline preparations can be obtained by crystallization of insulin in the presence of zinc (such as Lente™, see Schlichtkrull: Insulin Crystals, Chemical and Biological Studies on Insulin Crystals and Insulin Zinc Suspensions, Munksgaard, 1958) or by crystallization of insulin in the presence of zinc and protamine (such as NPH-insulin, see Rep.Steno Mem.Hosp. 1 (1946), 60).

One disadvantage in the use of the known suspensions of zinc insulin crystals or of zinc protamine insulin is the necessity of shaking the vial in order to ensure that the correct amount of insulin is being injected and to ensure that the concentration of insulin in the vial remains constant throughout its use. In Penfill™ cartridges where air must be absent, prolonged acting insulin suspensions require the incorporation of a solid body in the cartridge to enable agitation. The shaking of insulin suspensions and insulin solutions with air is in itself an undesirable process, as insulin has a tendency to denature under formation of fibrills at water-air interfaces. Consequently, solutions of insulins with prolonged action are desirable.

Solutions of insulin derivatives having a prolonged action was obtained from insulin that had been modified in its amino groups by reaction with phenylisocyanate (so-called Iso-insulin, see Hallas-Moeller: Chemical and Biological Insulin

Studies based upon the Reaction between Insulin and Phenylisocyanate, Copenhagen 1945). Similarly, A1,B29-di-Boc substituted insulin (Boc designates tertiary butyloxycarbonyl) was reported to show a prolonged insulin action after subcutaneous administration (see Geiger & Enzmann in: Proinsulin, Insulin, C-peptide; Proceedings of the Symposium on Proinsulin, Insulin and C-Peptide, Tokushima 1978; Amsterdam-Oxford 1979, 306 - 310). The A1,B29-di-Boc substituted insulin was found to exhibit a too slightly prolonged action to be clinically useful.

Solutions of unmodified insulins require large amounts of zinc ions (for example, 0.4 - 1 mg/U insulin) in order to exhibit a prolonged action (see J.Pharmacol. 55 (1935), 206). Injection of such large doses of zinc ions will probably cause pain and such solutions have, therefore, never been used in therapy.

The isoelectric point of insulin is about 5.5 and attempts have been made to decrease the solubility of insulin derivatives at neutral pH by shifting the isoelectric point upwards, for example, through additions, in the N-terminus of the B-chain, of basic amino acids like lysine or arginine (see, for example, German Offenlegungsschrift No. 2,042,299) or with the basic dipeptide arginyl-arginine (see Geiger & Enzmann cited above). The solubility of the latter compound, $Arg^{B(-1)}$-Arg^{B0} insulin, near its isoelectric point was, however, much higher than that of the parent insulin.

Japanese patent application No. 55-144032 relates to analogues to human insulin wherein the B30-amino acid has been replaced by an amino acid having at least five carbon atoms, and amides and esters thereof. These insulin analogues were to be used in patients who had developed antibodies against mammalian insulins. In the Japanese patent application, six specific compounds are described, none of which were stated to have prolonged action. No specific injectable preparations are described in the Japanese patent application.

European patent application No. 84108442.9 relates to insulin analogues wherein a basic, organic group is attached to the B30-amino acid thereby introducing a positive charge at neutral pH. In these analogues, the B30-amino acid is neutral and, preferably, threonine as in human insulin. German patent application No. 3,327,709.5 relates to a suspension of crystals of the derivatives described in the above-noted European patent application as well as an aromatic hydroxy compound. German patent application No. 3,326,473.2 relates to a medicament containing a mixture of insulin compounds, of which at least one is described in the above-noted European patent application.

BRIEF STATEMENT OF THE INVENTION

The present invention comprises novel analogs of human insulin that differ from human insulin by:

a) presence of an amide or ester residue on the C-terminal carboxyl group of the B-chain and

b) having at least one charge more than human insulin at pH 7, preferably not more than 4 charges more than human insulin at pH 7.

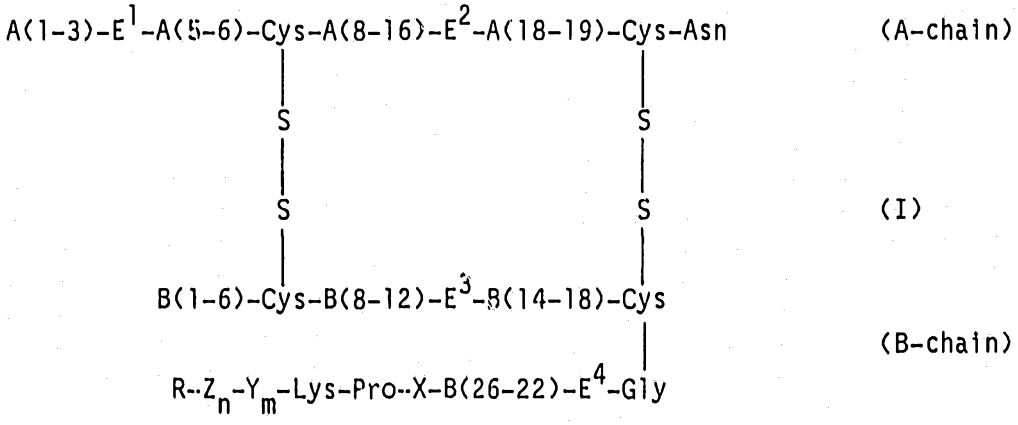
The change in charge is achieved by the blocking of the carboxylic group in the B30 amino acid and, if desired, by substituting one or more of the amino acids compared with human insulin.

In specific, the compounds of interest to practice of this invention are characterizable as follows: One or more of the four glutamic acid residues at A4, A17, B13, B21 is instead another naturally occurring neutral amino acid, preferably glutamine; and/or the threonine residue at B27 is instead a naturally occurring basic amino acid residue, preferably, L-arginine or L-lysine; and/or the threonine residue at B30 is

instead one or two basic amino acid residues, one being preferred, and the N terminal carboxylic group in the B chain being protected.

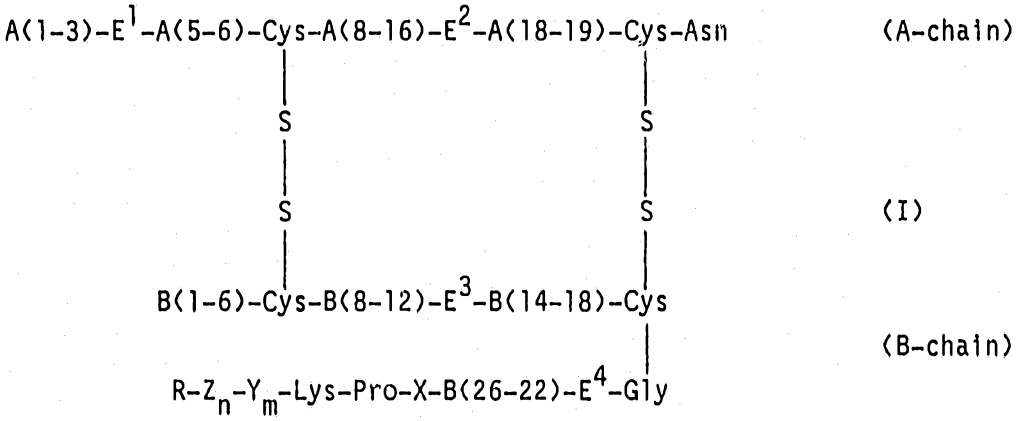
The invention also comprises solutions of the above categorized human insulin analogs with a controlled level of zinc ions therein. The degree of prolongation of insulin action is enhanced and controlled thereby.

According to a first embodiment of this invention, there is provided a compound of the general formula I

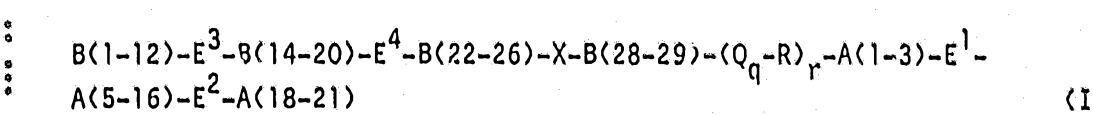


wherein the letters A and B followed by figures in parentheses designate the peptide fragments of the A- and B-chains, respectively, indicated by the figures in parentheses, E^1, E^2, E^3 and E^4 are the same or different each representing glutamic acid or a neutral amino acid residue which can be coded for by nucleotide sequences, X represents an L-threonine, L-arginine or L-lysine residue, Y and Z are the same or different and each represent an amino acid residue wherein any side chain hydroxy group may be alkylated, and m and n are the same or different and each represent zero or one, and R represents an amido or ester residue which blocks the C-terminal carboxyl group of the B-chain, with the proviso that not all of E^1, E^2, E^3 and E^4 are glutamic acid residues when X is a threonine residue, or, when E^1, E^2, E^3 and E^4 each is a glutamic acid residue and X is a threonine residue, the group of formula $-Y_m-Z_n-R$ represents $-NH_2$, $-Arg-NH_2$, $-Arg-Arg-NH_2$, $-Arg-Lys-NH_2$, $-Dab-Dab-NH_2$, $-Dap-Dap-NH_2$, $Lys-NH_2$, $-Lys(Lau)-NH_2$, $-Lys-Arg-NH_2$, $-Lys-Lys-NH_2$, $-Orn-NH_2$ or $-Orn-Orn-NH_2$, with the further proviso that the compound of formula I has at least one charge more than human insulin at a pH value of 7.

According to a second embodiment of this invention, there is provided a process for preparing a compound of the general formula I



wherein the letters A and B followed by figures in parentheses designate the peptide fragments of the A- and B-chains, respectively, indicated by the figures in parentheses, E^1, E^2, E^3 and E^4 are the same or different each representing glutamic acid or a neutral amino acid residue which can be coded for by nucleotide sequences, X represents an L-threonine, L-arginine or L-lysine residue, Y and Z are the same or different and each represent an amino acid residue wherein any side chain hydroxy group may be alkylated, and m and n are the same or different and each represent zero or one, and R represents an amido or ester residue which blocks the C-terminal carboxyl group of the B-chain, with the proviso that not all of E^1, E^2, E^3 and E^4 are glutamic acid residues when X is a threonine residue, or, when E^1, E^2, E^3 and E^4 each is a glutamic acid residue and X is a threonine residue, the group of formula $-Y_m-Z_n-R$ represents $-NH_2$, -Arg-NH_2, -Arg-Arg-NH_2, -Arg-Lys-NH_2, -Dab-Dab-NH_2, -Dap-Dap-NH_2, -Lys-NH_2, -Lys(Lau)-NH_2, -Lys-Arg-NH_2, -Lys-Lys-NH_2, -Orn-NH_2 or -Orn-Orn-NH_2, with the further proviso that the compound of formula I has at least one charge more than human insulin at a pH value of 7, said process being characterized in (a) transpeptidating porcine insulin or a compound of the general formula II:

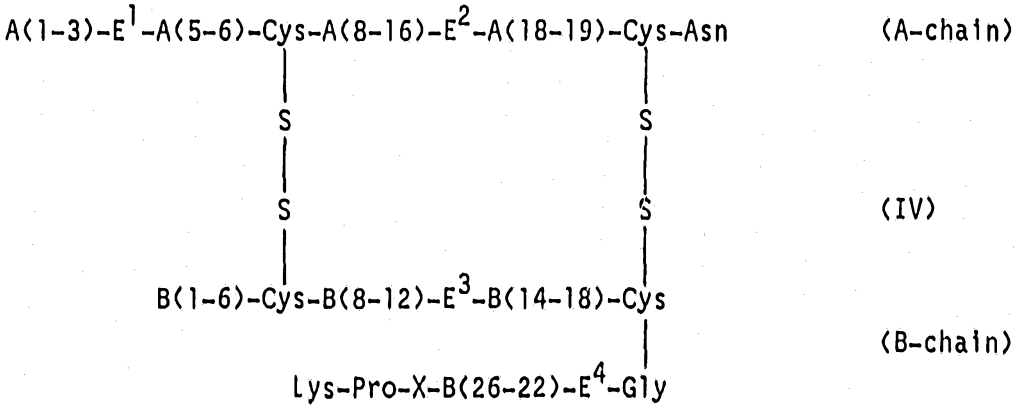


wherein A and B designate the fragments of the A- and B-chains indicated by numbers in parentheses, Q is a peptide chain with q amino acids, q is an integer from 0 to 33, R is Lys or Arg, and r is zero or one and E^1, E^2, E^3, E^4 and X each are as defined above, with a compound of the general formula III:

MS

$$H\text{-}Y_m\text{-}Z\text{-}_n\text{-}R \qquad \text{(III)}$$

wherein Y, Z, R, *m* and *n* each are as defined above, and wherein side chain amino groups and hydroxy groups in Y and Z optionally are blocked with amino and hydroxy protecting groups, using trypsin or a trypsin like enzyme as a catalyst, or b) coupling a compound of formula IV



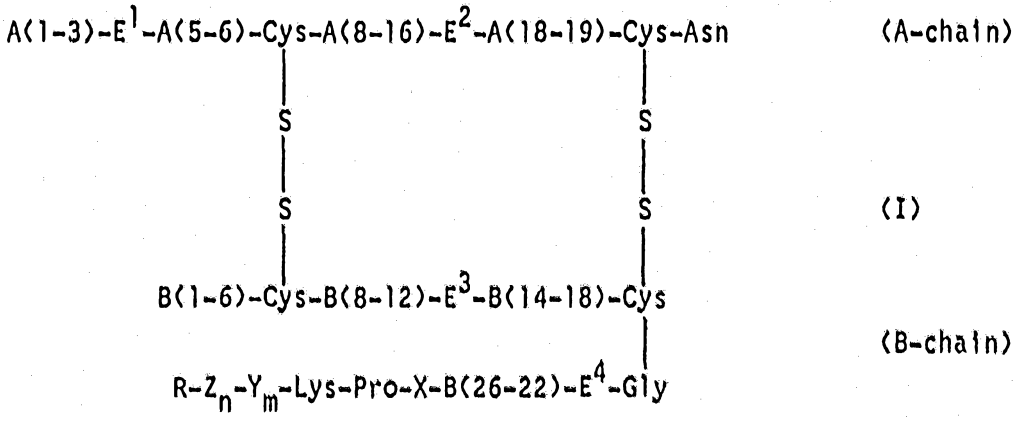
(A-chain)

(IV)

(B-chain)

wherein E^1, E^2, E^3, E^4 and X each are as defined above, with a compound of formula III by trypsin or a trypsin like enzyme.

According to a third embodiment of this invention, there is provided an injectable solution with prolonged insulin action, characterized in that it contains a compound of the general formula I



(A-chain)

(I)

(B-chain)

wherein the letters A and B followed by figures in parentheses designate the peptide fragments of the A- and B-chains, respectively, indicated by the figures in parentheses, E^1, E^2, E^3 and E^4 are the same or different each representing glutamic acid or a neutral amino acid residue

MTS

which can be coded for by nucleotide sequences, X represents an L-threonine, L-arginine or L-lysine residue, Y and Z are the same or different and each represent an amino acid residue wherein any side chain hydroxy group may be alkylated, and m and n are the same or different and each represent zero or one, and R represents an amido or ester residue which blocks the C-terminal carboxyl group of the B-chain, with the proviso that not all of E^1, E^2, E^3 and E^4 are glutamic acid residues when X is a threonine residue, or, when E^1, E^2, E^3 and E^4 each is a glutamic acid residue and X is a threonine residue, the group of formula $-Y_m-Z_n-R$ represents $-NH_2$, -Arg-NH_2, -Arg-Arg-NH_2, -Arg-Lys-NH_2, -Dab-Dab-NH_2, -Dap-Dap-NH_2, -Lys-NH_2, -Lys(Lau)-NH_2, -Lys-Arg-NH_2, -Lys-Lys-NH_2, -Orn-NH_2 or -Orn-Orn-NH_2, with the further proviso that the compound of formula I has at least one charge more than human insulin at a pH value of 7, together with a pharmaceutically acceptable diluent.

According to a fourth embodiment of this invention, there is provided an injectable solution with prolonged insulin action, characterized in that it contains a compound according to the first embodiment together with a pharmaceutically acceptable diluent.

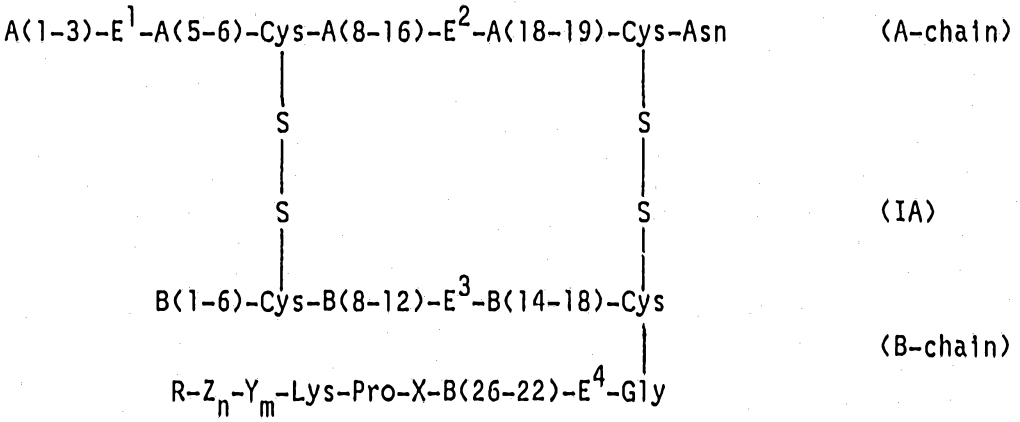
According to a fifth embodiment of this invention, there is provided a kit comprising a solution according to the third embodiment and a separately contained solution of zinc ions.

According to a sixth embodiment of this invention, there is provided a kit comprising a first solution according to the solution of the third embodiment and a separately contained second solution according to the solution of the third embodiment wherein the concentration of at least one component in said second solution is different from the concentration of the same component in said first solution.

According to a seventh embodiment of this invention, there is provided a method of treatment of diabetes mellitus in a patient requiring said treatment which method comprises administering to said patient an effective amount of either a compound according to the first embodiment or of a solution according to the third embodiment.

According to an eighth embodiment of this invention, there is provided a method of treatment of diabetes mellitus in a patient requiring said treatment which method comprises administering to said patient an effective amount of an injectable solution with prolonged insulin action, said solution characterized in that it contains a compound of the general formula

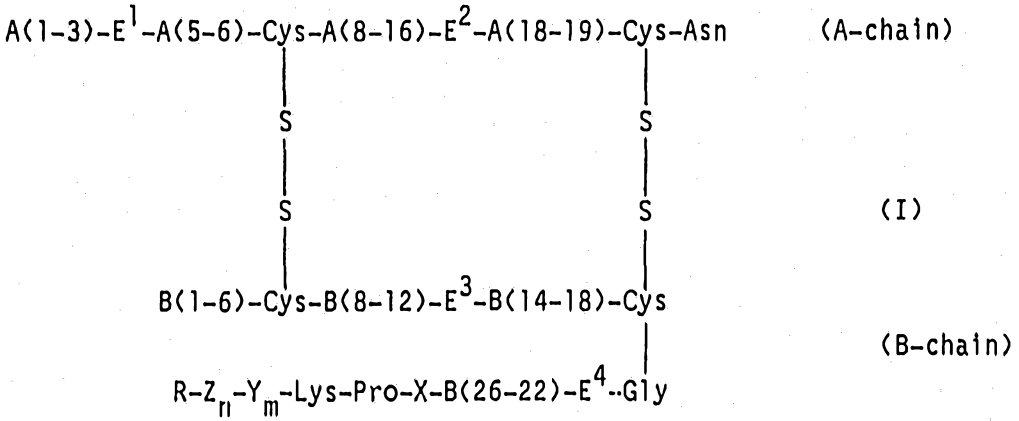
MS



wherein the letters A and B followed by figures in parentheses designate the peptide fragments of the A- and B-chains, respectively, indicated by the figures in parentheses, E^1, E^2, E^3 and E^4 are the same or different each representing glutamic acid or a neutral amino acid residue which can be coded for by nucleotide sequences, X represents an L-threonine, L-arginine or L-lysine residue, Y and Z are the same or different and each represent an amino acid residue wherein any side chain hydroxy group may be alkylated, and m and n are the same or different and each represent zero or one, and R represents an amido or ester residue which blocks the C-terminal carboxyl group of the B-chain, with the proviso that not all of E^1, E^2, E^3 and E^4 are glutamic acid residues when X is a threonine residue, or, when E^1, E^2, E^3 and E^4 each is a glutamic acid residue and X is a threonine residue, the group of formula $-Y_m-Z_n-R$ represents $-NH_2$, $-Arg-NH_2$, $-Arg-Arg-NH_2$, $-Arg-Lys-NH_2$, $-Dab-Dab-NH_2$, $-Dap-Dap-NH_2$, $-Lys-NH_2$, $-Lys(Lau)-NH_2$, $-Lys-Arg-NH_2$, $-Lys-Lys-NH_2$, $-Orn-NH_2$, $-Orn-Orn-NH_2$, $-Thr-NH_2$, $-Thr-OBu^t$ or $-Thr(Bu^t)-OBu^t$, with the further proviso that the compound of formula I has at least one charge more than human insulin at a pH value of 7, together with a pharmaceutically acceptable diluent.

DETAILED PRACTICE OF THIS INVENTION

It has surprisingly been found that injectable solutions with a combined short and prolonged insulin action can be made using, as the active ingredient, a single insulin derivative having the general formula I



wherein the letters A and B followed by figures in parentheses designate the peptide fragments of the A- and B-chains, respectively, indicated by the figures in parentheses, E^1, E^2, E^3 and E^4 are the same or different each representing glutamic acid or a neutral amino acid residue which can be coded for by nucleotide sequences, X represents an L-threonine, L-arginine or L-lysine residue, Y and Z are the same or different and each represent an amino acid residue wherein any side chain amino group may be acylated and wherein any side chain hydroxy group may be alkylated, and m and n are the same or different and each represent zero or one, and R represents an amido or ester residue which blocks the C-terminal carboxyl group of the B-chain, with the proviso that not all of E^1, E^2, E^3 and E^4 are glutamic acid residues, when X is a threonine residue or, when E^1, E^2, E^3 and E^4 each is a glutamic acid residue, and X is a threonine residue, the group of formula $-Y_m-Z_n-R$ represents $-NH_2$, $-Arg-NH_2$, $-Arg-Arg-NH_2$, $-Arg-Lys-NH_2$, $-Dab-Dab-NH_2$, $-Dap-Dap-NH_2$, $-Lys-NH_2$, $-Lys(Lau)-NH_2$, $-Lys-Arg-NH_2$, $-Lys-Lys-NH_2$, $-Orn-NH_2$, $-Orn-Orn-NH_2$, $-Thr-NH_2$, $-Thr-Obu^t$ or $-Thr(Bu^t)-OBu^t$. Lau designates lauroyl and Dab and Dap represents α,γ-diminobutyric acid and α,β-diaminopropioic acid, respectively.

A subgroup of compounds of formula I are novel compounds having the general formula I wherein the letters A and B followed by figures in parentheses designate the peptide fragments of the A- and B-chains, respectively, indicated by the figures in parentheses, E^1, E^2, E^3 and E^4 are the same or different each representing glutamic acid or a neutral amino acid residue which can be coded for by nucleotide sequences, X represents an L-threonine, L-arginine or L-lysine residue, Y and Z are the same or different and each represent an amino acid residue wherein any side

chain hydroxy group may be alkylated, and m and n are the same or different and each represent zero or one, and R represents an amido or ester residue which blocks the C-terminal carboxyl group of the B-chain, with the proviso that not all of E^1, E^2, E^3 and E^4 are glutamic acid residues, when X is a threonine residue, or, when E^1, E^2, E^3 and E^4 each is a glutamic acid residue, and X is a threonine residue, the group of formula $-Y_m-Z_n-R$ represents $-NH_2$, -Arg-NH_2, -Arg-Arg-NH_2, -Arg-Lys-NH_2, -Dab-Dab-NH_2, -Dap-Dap-NH_2, -Lys-NH_2, -Lys(Lau)-NH_2, -Lys-Arg-NH_2, -Lys-Lys-NH_2, -Orn-NH_2 or -Orn-Orn-NH_2, with the proviso that the compound of formula I has at lease one charge more than human insulin at a pH value of 7.

In compounds of formula I, the C-terminal carboxyl group of the B-chain is blocked by an ester group or amide group, thereby eliminating the negative charge of the carboxyl

group. The change in charge introduced by the ester or amide group in position B30 can be further increased by substituting threonine in the B27-position with arginine or lysine and/or by substituting any of the four glutamic acid residues in the A4-, A17-, B13-, and B21-position with a neutral amino acid, preferably with a glutamine residue. Furthermore, a positive charge may be introduced by a basic amino acid in the B30- and/or B31-position. Since compounds of formula I can be applied in the clinic as solutions having a prolonged action, a decline in immunogenicity as compared to the commonly used suspensions of porcine or human insulins may occur.

The degree of prolongation can be enhanced and controlled by the addition of zinc ions.

Major parameters that control the degree of prolongation of the insulin effect are the concentration of zinc and the choice of the compound of formula I. With some analogs, e.g. Arg^{B27},Thr^{B30}-NH_2 human insulin, very prolonged action is obtained with only 3 zinc atoms per hexamer unit of insulin analog corresponding to 8 µg zinc/ml in a preparation containing about 240 nmole/ml. With other analogs, e.g. Lys^{B30}-NH_2 human insulin, moderate prolongation of action is obtained with 30 zinc per hexamer of insulin analog corresponding to 80 µg zinc/ml in a preparation containing about 240 nmole/ml. The range for preferred zinc contrations extends from 0 to 2 mg/ml, preferably from 0 to 200 µg/ml zinc with substitution in the B13 and/or B27 position and preferably from 20 to 200 µg/ml with other analogs.

The prolonged action of solutions of compounds of formula I in the presence of zinc ions is ascribed to the low solubility of such compounds at neutral pH. Only solutions of insulin derivatives in which the C-terminal of the B-chain was blocked, showed a more prolonged action than Actrapid™ porcine insulin.

The pH of the injectable solution of this invention should preferably be below and so close to the physiological pH as possible, the upper limit being the pH where precipitation

occurs. Stable solutions containing about 240 nmole/ml of compounds of formula I have been obtained at pH 5.5. The upper limit depends upon the constituents of the solution, i.e. isotonikum, preservative and zinc concentration, and upon the choice of compound of formula I. There is no lower pH limit of the solutions, but since the chemical stability of insulins is poor in acid solutions due to deamidation reactions and formation of dimers as high a pH as possible with respect to the physical stability of the solution is preferred. The preferred pH range for the injectable solutions of this invention is from 2.5 to 8.5, more preferred from 4.5 to 8.

A further aspect of this invention is that it provides improved flexibility for the patients. With two aqueous solutions, one containing a compound of formula I and the other containing a zinc salt, the patient can obtain a desired degree of prolonged action and a desired profile by mixing the two solutions appropriately. Thus, the patient has, using two stock solutions, the possibility of choosing one action and profile for the morning injection and another action and profile for the evening injection. Preferably, the zinc solution contains between about 10 µg and 20 mg zinc per ml. Alternatively, both of the stock solutions may contain zinc, either in the same or different concentrations, and/or both the stock solutions may contain a compound of formula I, either the same or different compounds.

Preferably, the injectable solutions of this invention have a strength of between about 60 and 6000 nmole/ml of the compound of formula I.

The neutral amino acid (E^1 through E^4) is, for example, glycine, valine, isoleucine, leucine, phenylalanine, tyrosine, methionine or preferably asparagine, glutamine, alanine, serine or threonine.

Examples of R are ester moieties, for example, lower alkoxy, preferably methoxy, ethoxy and most preferred tertiary butoxy, and such groups are present in compounds which are useful in the synthesis of human insulin, see, for example,

U.S. Patent specification No. 4,343,898. Such esters are Thr^{B30}-OBu^t human insulin and $Thr^{B30}(Bu^t)$-OBu^t human insulin (Bu^t designates tertiary butyl).

Furthermore, R can be a group of the general formula $-NR^1R^2$ wherein R^1 and R^2 are the same or different and each represents hydrogen or lower alkyl. Hereinafter the term "lower" designates that the group in question contains less than 7 carbon atoms, preferably less than 5 carbon atoms. An example of such a group is found in Thr^{B30}-NH_2 human insulin which is known as an intermediate in a synthesis of human insulin (see Carlsberg Res.Commun. 49 (1984), 463). In a preferred embodiment of this invention, R is $-NH_2$. Furthermore, R may be a lactam residue which preferably contains less than 8 atoms in the lactam ring, for example a lactam of a diaminocarboxylic acid.

In a preferred embodiment of this invention, R is uncharged.

At neutral pH, the charge of $-X^{27}$-Pro^{28}-Lys^{29}-Y_m-Z_n-R is +1 in Thr^{B30}-NH_2 human insulin, Thr^{B30}-OBu^t human insulin, $Thr^{B30}(Bu^t)$-OBu^t human insulin and Lys^{B29}-NH_2,des-(B30) human insulin, +2 in Lys^{B30}-NH_2 human insulin, Arg^{B30}-NH_2 human insulin, Orn^{B30}-NH_2 human insulin, Lys^{B27},Thr^{B30}-NH_2 human insulin and Arg^{B27},Thr^{B30}-NH_2 human insulin, and +3 in Lys^{B27},Lys^{B30}-NH_2 human insulin, Lys^{B27},Arg^{B30}-NH_2 human insulin, Arg^{B27},Lys^{B30}-NH_2 human insulin, Arg^{B27},Arg^{B30}-NH_2 human insulin, Lys^{B30}-Lys^{B31}-NH_2 human insulin, Arg^{B30}-Lys^{B31}-NH_2 human insulin, Arg^{B30}-Arg^{B31}-NH_2 human insulin, Orn^{B30}-Orn^{B31}-NH_2 human insulin, Dab^{B30}-Dab^{B31}-NH_2 human insulin and Dap^{B30}-Dap^{B31}-NH_2 human insulin.

According to one preferred embodiment of this invention, the amino acid residues designated Y and Z are residues from L-amino acids which are coded for by nucleotide sequences.

Any side chain amino group in the amino acid residues designated Y and Z may be acylated by an acid containing from 2 to 18 carbon atoms, preferably a fatty acid containing from 6 to 18 carbon atoms, for example, lauric acid. Thus, $-Y_m-Z_n-R$ may be $-Lys(Lau)-NH_2$.

Examples of preferred alkylated hydroxy groups are methoxy, ethoxy and tertiary butoxy.

In one group of preferred compounds of formula I Y and/or Z is a basic amino acid residue wherein the side chain amino group optionally is acylated (m = 1).

In another group of preferred compounds of formula I n is zero and Y is a basic amino acid residue (m = 1).

In a further group of preferred compounds of formula I Y and Z are both basic amino acid residues (m = 1, n = 1).

Preferred compounds of formula I are each of the following: $Gln^{A17},Arg^{B27},Thr^{B30}-NH_2$ human insulin, $Gln^{A17},Gln^{B13},Thr^{B30}-NH_2$ human insulin, $Gln^{A17},Lys^{B27},Thr^{B30}-NH_2$ human insulin, $Gln^{A17},Lys^{B30}-NH_2$ human insulin, $Gln^{A17},Thr^{B30}-NH_2$ human insulin, $Gln^{B13},Arg^{B27},Thr^{B30}-NH_2$ human insulin, $Gln^{B13},Lys^{B27},Thr^{B30}-NH_2$ human insulin, $Gln^{B13},Lys^{B30}-NH_2$ human insulin, $Gln^{B13},Thr^{B30}-NH_2$ human insulin, $Arg^{B27},Arg^{B30}-NH_2$ human insulin, $Arg^{B27},Lys^{B30}-NH_2$ human insulin, $Arg^{B27},Thr^{B30}-NH_2$ human insulin, $Lys^{B27},Arg^{B30}-NH_2$ human insulin, $Lys^{B27},Lys^{B30}-NH_2$ human insulin, $Lys^{B27},Thr^{B30}-NH_2$ human insulin, $Lys^{B29}-NH_2$,des-(B30) human insulin, $Thr^{B30}-NH_2$ human insulin, $Lys^{B30}-NH_2$ human insulin, $Lys^{B30}(Lau)-NH_2$ human insulin, $Lys^{B30}-Arg^{B31}-NH_2$ human insulin, $Lys^{B30}-Lys^{B31}-NH_2$ human insulin, $Arg^{B30}-NH_2$ human insulin, $Arg^{B30}-Arg^{B31}-NH_2$ human insulin or $Arg^{B30}-Lys^{B31}-NH_2$ human insulin.

Another preferred embodiment of this invention is preparations containing a compound of formula I wherein E^1, E^2, E^3 and/or E^4 is a glutamine residue, and/or X is Lys or Arg, and within this subclass of compounds of formula I, a further preferred embodiment is preparations containing a compound of formula I wherein the group $-Y_m-Z_n-R$ is $-Thr-NH_2$ or $-Lys-NH_2$.

Preferred compounds are Gln^{B13},Thr^{B30}-NH_2 human insulin, Gln^{A17},Thr^{B30}-NH_2 human insulin, Lys^{B27},Thr^{B30}-NH_2 human insulin and Arg^{B27},Thr^{B30}-NH_2 human insulin.

In one group of preferred compounds of formula I, E^1, E^3 and E^4 is each a glutamic acid residue.

In another group of preferred compounds of formula I, E^2 is a glutamine residue.

In a still further group of preferred compounds of formula I, X is an arginine or lysine residue.

As is well known in the art, not all of the amino acid residues in human insulin are essential for the insulin action.

Indeed, porcine insulin and bovine insulin which differs from human insulin in amino acid residues have been employed to treat diabetics. Considerable species to species variations in the insulin molecule exist. Thus, many peptide residues in the human insulin molecule may be changed without undue diminution in insulin activity, including some peptide residues important to the isoelectric point of the molecule.

It is obvious that the groups designated E^1, E^2, E^3, E^4, X, Y, Z and R are to be selected so that the resulting compound of formula I is pharmaceutically acceptable.

In the known biphasic insulin preparations, it is common to combine fast acting, soluble insulin with prolonged acting, crystalline insulin in the same injection. Using compounds of formula I of this invention, a similar combined short and prolonged action can be obtained with a solution of a single compound of formula I. The ratio between fast and long effect decreases as the concentration of zinc ions in the solution is increased.

Compounds of formula I may be prepared by a transpeptidation reaction in which porcine insulin or else a biosynthetic precursor compound having the correct insulin disulphide bridges and having the general formula II:

$$B(1\text{-}12)\text{-}E^3\text{-}B(14\text{-}20)\text{-}E^4\text{-}B(22\text{-}26)\text{-}X\text{-}B(28\text{-}29)\text{-}(Q_q\text{-}R)_r\text{-}A(1\text{-}3)\text{-}E^1\text{-}A(5\text{-}16)\text{-}E^2\text{-}A(18\text{-}21) \quad (II)$$

wherein the letters A and B followed by figures in parentheses designate the appropriate peptide fragments of the A- and B-chains, respectively, as indicated by the figures in parentheses, Q is a peptide chain with q amino acids, q is an integer from 0 to 33, R is Lys or Arg, and r is zero or one, and E^1, E^2, E^3, E^4 and X each are as defined above, is reacted with an amino compound of the general formula III:

$$H\text{-}Y_m\text{-}Z_n\text{-}R \quad (III)$$

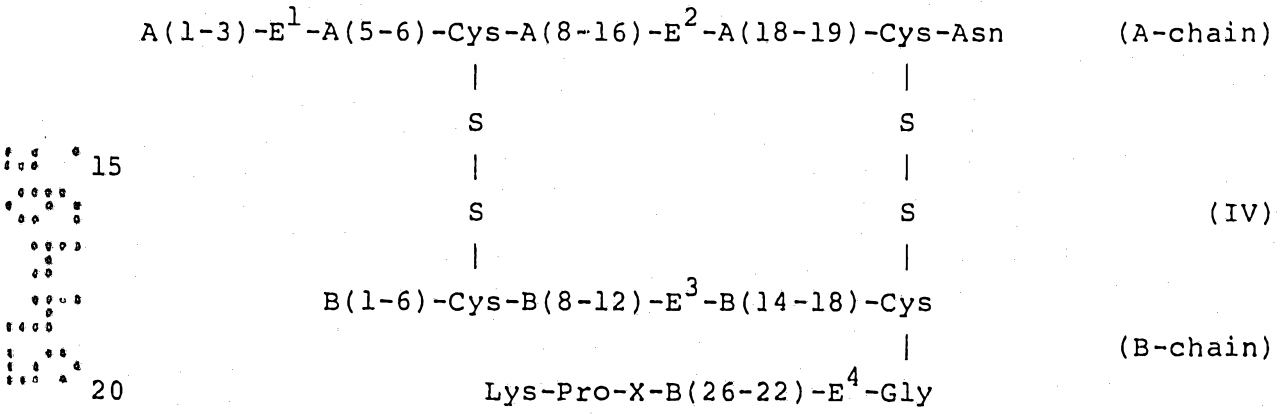
wherein Y, Z, R, m and n each are as defined above, and wherein side chain amino groups and hydroxy groups in Y and Z optionally are blocked with amino and hydroxy protecting groups, using trypsin or a trypsin like enzyme as a catalyst in a mixture of water and organic solvents as has been described in US Patent No. 4,343,898. Preferred compounds of formula III for use in this process are Thr-NH_2, Lys(Boc)-NH_2, Thr(Bu^t)-OBu^t, Thr-OBu^t, Ala-NH_2 and Arg(Boc)-NH_2. Amino groups may be derivatized by acylation with a fatty acid. Hydroxy groups may be protected by alkylation. If Y and Z contain groups which are reversibly blocked by amino protecting groups, these groups may be removed at a later stage, if such is desired, after the amino protected intermediate has been separated from the trypsin or trypsin like enzyme. Of the trypsin like enzymes, lysyl endopeptidase from Achromobacter lyticus is useful.

The compound of formula II may be expressed in a host organism such as yeast similar to the description in European patent application No. 163,529 using a gene having the correct codons for the amino acids in question. The gene encoding the novel insulin derivative is then inserted into a suitable expression vector which when transferred to yeast is capable of

expressing the desired compound. The product expressed is then isolated from the cells or the culture broth depending on whether it is secreted from the cells or not.

An example of a reversible amino protecting group is tertiary butoxycarbonyl and a reversible hydroxy protecting group is tertiary butyl. Such groups are removed under conditions which do not cause undesired alteration in the compound of formula I, for example, by trifluoroacetic acid.

Insulin compounds of formula I may also be prepared by a coupling reaction in which a compound of the general formula IV



wherein E^1, E^2, E^3, E^4 and X each are as defined above, is coupled to an amino compound of the above formula III by trypsin or a trypsin like enzyme under conditions similar to those described in European patent specification No. 17,938.

When insulin is manufactured by genetic engineering the additional one or two positive charges may appropriately be introduced internally in the insulin molecule, i.e. in the A4-, A17-, B13-, B21- or B27-position, leaving for trypsin catalyzed semisynthesis blocking of the C-terminal carboxyl group of the B-chain with an amino acid amide or an amino acid ester.

The advantage in introducing the additional positive charges within the frame of the 51 amino acids of the insulin molecule to form the novel compounds of formula I rather than by prolongation of the B-chain beyond the 30 residues of the

mammalian insulins relates to ease in preparation. In the semisynthetic transpeptidation a large molar excess of the amino acid amide or amino acid ester is employed. If a dipeptide amide or ester were to be used in the transpeptidation reaction, either price or solubility or both are prohibitive for use in large excess, and consequently the yield of the product becomes lower. Even when the same equimolar excess of, for example, Lys(Boc)-NH_2 and Lys(Boc)-Lys(Boc)-NH_2 is used in the transpeptidation reaction under similar conditions, the yield with the amino acid amide becomes substantially higher than with the dipeptide amide.

Insulin preparations of this invention are prepared by dissolving a compound of formula I in an aqueous medium at slightly acidic conditions, for example, in a concentration of 240 or 600 nmole/ml. The aqueous medium is made isotonic, for example, with sodium chloride or glycerol. Furthermore, the aqueous medium may contain zinc ions in a concentrations of up to about 20 µg of Zn^{++} per unit of insulin activity, buffers such as acetate and citrate and preservatives such as m-cresol or phenol. The pH value of the solution is adjusted towards neutrality without getting too close to the isoelectric point of the compound of formula I in order to avoid precipitation. The pH value of the final insulin preparation depends upon the number of charges that have been changed in the compound of formula I, the concentration of zinc ions, the concentration of the compound of formula I and the compound of formula I selected. The insulin preparation is made sterile by sterile filtration.

The insulin preparations of this invention are used similarly to the use of the known insulin preparations.

Any novel feature or combination of features described herein is considered essential to this invention.

Herein the abbreviations used for the amino acids are those stated in J.Biol.Chem. 243 (1968), 3558. The amino acids stated herein are in L configuration. In formula I and

elsewhere herein A(1-3) is Gly-Ile-Val, A(5-6) is Gln-Cys etc., cf. the amino acid sequence of human insulin. Unless otherwise indicated, the species of insulins stated herein is human.

Synthesis of the insulin compounds

The source of insulin was either porcine insulin or an insulin precursor expressed in yeast as described in the last-mentioned Danish patent application.

The insulin precursors were recovered from the fermentation broths by adsorption to LiChroprep™ RP-18 as described in Example 7 of the same Danish patent application. The precursors were eluted from the column with 0.2 M KCl, 0.001 M HCl in 33% (v/v) ethanol. The insulin precursors were crystallized from the pool by successive additions of water (1 volume per volume of pool), solid trisodium citrate to make 0.05 M and finally zinc acetate to make 0.006 M. The pH was adjusted to 6.8 and the mixture was left overnight at 4°C. The crystals were isolated by centrifugaton, washed with water and dried in vacuo.

Protected amino acids and protected peptides for enzymatic semisynthesis were either prepared by standard methods or purchased (custom synthesis) from either Nova Biochem or Bachem, both Switzerland.

The letters TM after a name indicates that it is a trade mark.

Example 1

Synthesis of Lys^{B30}-NH_2 human insulin

Solutions of 1 g of porcine insulin dissolved in 4 ml of 7.5 M acetic acid and 6.1 g of Lys(Boc)-HN_2,CH_3COOH

(N epsilon-Boc-L-lysine amide, hydroacetate salt) dissolved to 15 ml with N,N-dimethylacetamide were mixed and the mixture was cooled to 12°C. A solution of 0.1 g of trypsin in 2.08 ml of a 0.05 M solution of calcium acetate was added. After 96 hours at 12°C, the proteins were precipitated by the addition of 200 ml of acetone, and the precipitate was isolated by centrifugation. The precipitate was washed once with 100 ml of acetone, isolated by centrifugation and dried in vacuo.

The precipitate was dissolved in 50 ml of 0.01 N hydrochloric acid in ethanol/water (28/72 parts per volume) and the solution was applied to a 5 x 30 cm preparative high pressure liquid chromatography (hereinafter designated HPLC) column packed with silica particles substituted with octadecyldimethylsilyl (mean particle size 15 micron, pore size 100 Ångstrøm). The column was equilibrated with ethanol/0.2 M solution of ammonium sulphate adjusted to pH 3.5 with sulphuric acid, in a ratio of 38/62 (parts per volume). The proteins were eluted from the column with the same buffer at a rate of 2 litre/h. Lys(Boc)^{B30}-NH_2 human insulin was found in a peak eluting from the column between 60 and 75 minutes, after elution of unreacted porcine insulin. The ethanol was evaporated in vacuo and the evaporation was continued until the volume was reduced to about 125 ml. The Lys(Boc)^{B30}-NH_2 human insulin was isolated by successive additions of 25 ml of acetone, 100 mg of citric acid (monohydrate p.a.) and 9 mg of zinc chloride (p.a.). The pH was adjusted to 6.5 and after 1 h at room temperature the crystallisation was continued at 4°C for 24 h with gentle stirring. The crystals were spun down, washed once with 5 ml of ice-cold water, spun down and dried in vacuo. Yield: 456 mg of Lys(Boc)^{B30}-HN_2 human insulin.

The Lys(Boc)^{B30}-NH_2 human insulin (456 mg) was dissolved in 15 ml of trifluoroacetic acid and left for 3 h at room temperature. The trifluoroacetic acid was removed by lyophilization. The lyophilisate was dissolved in 50 ml of water, the pH adjusted to 2.5 and 10 g of sodium chloride was added. The salt cake of Lys^{B30}-NH_2 human insulin was isolated

by centrifugation. The salt cake was dissolved in 125 ml of water and the Lys^{B30}-NH_2 human insulin was crystallized by successive additions of 25 ml of acetone, 100 mg of citric acid (monohydrate p.a.) and 9 mg of zinc chloride (p.a.) and adjustment of the pH to 7.0. After 1 h at room temperature, the crystallisation was continued at 4°C for 24 h with gentle stirring. The crystals were spun down, washed once with 5 ml of ice-cold water, spun down again and dried. Yield: 387 mg of crude Lys^{B30}-NH_2 human insulin.

The crystals were dissolved in 50 ml of 0.005 N hydrochloric acid in ethanol/water (20/80, parts per volume) and the solution was applied to a preparative HPLC column as described above, this time equilibrated with ethanol/0.3 M solution of potassium chloride and 0.001 N hydrochloric acid, in a ratio of 35.5/64.5 (parts per volume). Elution with the same buffer at a rate of 2 litres/h resulted in a peak of Lys^{B30}-NH_2 emerging from the column between 55 and 90 min. The products were isolated from the pool as described for $Lys(Boc)^{B30}$-NH_2 human insulin above, except that the pH in the crystallization in the zinc containing citrate buffer was adjusted to 7.0 rather than 6.5. Yield: 267 mg of pure Lys^{B30}-NH_2 human insulin.

The amino acid composition was in agreement with the theory, alanine being 1 residue/molecule and lysine being 2 residues/molecule. The product was pure in DISC PAGE electrophoresis at pH 8.9, the rate of migration being 55% of that of porcine insulin corresponding to a difference in charges of about 2. For details of the DISC PAGE electrophoresis see Horm.Metab.Res.Supplement Series No. 5 (1974), 134.

Example 2

Synthesis of Arg^{B30}-NH_2 human insulin and Arg^{B30}-Arg^{B31}-NH_2 human insulin

Solutions of 1 g of porcine insulin in 3.32 ml of 8 M acetic acid and 3.9 g of Arg-NH_2,$(CH_3COOH)_2$ (L-arginine amide dihydroacetate salt) dissolved to 10 ml with N,N-dimethyl-formamid (hereinafter designated DMF) were mixed, and the mixture was cooled to 12°C. A solution of 0.1 g of trypsin in 1.2 ml of a 0.05 M solution of calcium acetate was added. After 1.4 hours at 12°C, the proteins were precipitated by the addition of 200 ml of acetone, and the precipitate was isolated by centrifugation. The precipitate was washed once with 100 ml of acetone, isolated by centrifugation and dried in vacuo.

The precipitate was dissolved in 50 ml of 0.01 N hydrochloric acid in ethanol/water (27/73 parts per volume) and the proteins were applied to a preparative column as described in Example 1. At first, an eluent composed of ethanol/0.3 M solution of potassium chloride and 0.001 N hydrochloric acid in a ratio of 35/65 (parts per volume), was pumped through at a rate of 2 litres/h for 4 hours. Three unresolved peaks very recorded, from about 60 to 120 minutes, from 120 to 150 and from 150 to 180 minutes. The proteins in the three pools very isolated as described for Lys^{B30}-NH_2 in Example 1. Yields: 414 mg, 142 mg and 107 mg for pools I, II and III, respectively.

Amino acid analysis combined with DISC PAGE electrophoresis showed that the insulin molecules of pool I had been coupled with from 2 to several arginine residues. The major component of pool II was insulin coupled to a single arginine amide residue, i.e. Arg^{B30}-NH_2 human insulin. Pool III was a mixture of porcine insulin, des(B30) human insulin, Arg^{B30} human insulin and Arg^{B30}-NH_2 human insulin.

Arg^{B30}-NH_2 human insulin

The proteins of pool II was dissolved in 10 ml of ethanol/water 3/2 (v/v) at pH 2. After addition of 12 mg of EDTA the pH was raised to 10 by a 0.1 N solution of sodium hydroxide. The solution was applied to a 2.5 x 25 cm column of QAE-Sephadex A-25 equilibrated with a buffer composed of 0.5 M NH_3, 0.05 N hydrochloric acid and 0.04 M solution of sodium chloride in 60% ethanol (v/v). The column was eluted with 30 ml/h with a linear gradient in sodium chloride from 0.04 M to 0.1 M using a total of 1 litre of eluent, while the pH was kept constant at about 10.0. Fractions of 10 ml were collected. Arg^{B30}-NH_2 human insulin emerged from the columns in fractions Nos. 58 - 74. The product was isolated from the pool by evaporation followed by crystallization at pH 7 in a zinc containing citrate buffer containing 15% acetone (v/v) as described for Lys^{B30}-NH_2 in Example 1. Yield: 53 mg. The product was homogeneous in DISC PAGE electrophoresis at pH 8.9, the rate of migration being 55% of that of insulin. The amino acid composition was in accordance with the theory for Arg^{B30}-NH_2 human insulin, showing 2 arginine residues and 1 residue of alanine per molecule of insulin.

Arg^{B30}-Arg^{B31}-NH_2 human insulin

The proteins of pool I were dissolved and subjected to ion exchange chromatography on QAE-Sephadex™ A-25 as described for the proteins in pool II. Arg^{B30}-Arg^{B31}-NH_2 human insulin emerged from the column in fractions Nos. 34 - 47. The product was isolated as described for Lys^{B30}-NH_2 human insulin in Example 1. Yield: 10 mg.

The product was homogeneous in DISC PAGE electrophoresis at pH 8.9, the rate of migration being 35% of that of insulin. The amino acid composition showed 3 arginine residues and 1 residue of alanine per molecule of insulin.

Example 3

Synthesis of Thr^{B30}-NH_2 human insulin

A solution of 1 g of porcine insulin in 5 ml of 10 M acetic acid and a suspension of 2.365 g of Thr-NH_2 (L-threonine amide, free base) suspended to make a volume of 13 ml with N,N-dimethylacetamide were mixed. After mixing, the Thr-NH_2 was dissolved. The mixture was cooled to 12°C and a solution of 0.1 g of trypsin in 2 ml of a 0.05 M solution of calcium acetate was added. After 72 hours at 12°C the proteins were precipitated by addition of 200 ml of acetone, and the precipitate was isolated by centrifugation. The precipitate was washed once with 100 ml of acetone, isolated by centrifugation and dried in vacuo.

The purification of Thr^{B30}-NH_2 human insulin from trypsin and unreacted porcine insulin was carried out as described for esters of human insulin (see Markussen: Methods in Diabetes Research, Vol. 1, Editors: Larner & Pohl (1984), 408). Yield 599 mg.

The product was homogeneous in DISC PAGE electrophoresis at pH 8.9, the rate of migration being 75% of that of insulin. The amino acid composition was in accordance with the theory, that is 3 residues of threonine and 1 residue of alanine per molecule of insulin.

In the starting material in Examples 4 through 10 $(Q_q\text{-}R)_r$ of formula II was chosen to Ala-Ala-Lys and constructed as described for yeast plasmid pMT610 in Example 10 in Danish patent application No. 582/85. Nucleotides coding for Gln^{B13}, Gln^{A17}, Arg^{B27} and Lys^{B27} were substituted in pMT610 by site specific mutagenisis using the procedure in Nucl.Acids.Res. 11 (1983), 5103 - 5112.

Example 4

Synthesis of Gln^{A17},Thr^{B30}-NH_2 human insulin

To a suspension of 3.12 g of Gln^{A17},B(1-29)-Ala-Ala-Lys-A(1-21) insulin precursor in 15 ml of acetic acid/DMF/water (11.4 ml acetic acid, 65.1 ml DMF, water to make 100 ml) 30 ml of 1 M Thr-NH_2 in DMF was added. The mixture was cooled to 12°C and 0.3 g of porcine trypsin dissolved in 7.5 ml of 0.05 M calcium acetate was added. Stirring was continued until the insulin precursor had dissolved. After 48 hours at 12°C the proteins were precipitated by addition of 400 ml of acetone. The proteins were isolated by centrifugation, washed once with 100 ml of acetone and dried *in vacuo*.

The precipitate was dissolved in 70 ml of 0.04 N HCl, the pH adjusted to 2.5 and the derivative was purified by HPLC as described in Example 1, except that an eluent composed of 35 parts of ethanol and 65 parts of 0.3 M KCl, 0.001 N HCl was used for elution. The derivative emerged from the column after about 3 column volums, and it was isolated by successive additions of 1 volume of water, solid trisodium citrate to make 0.05 M and solid zinc acetate to make 0.006 M. After adjustment of pH to 6.5 and stirring overnight at 4°C, crystals were harvested by centrifugation, washed with water and dried. Yield 1.64 g = 53%. Further purifications by anion exchange chromatography as described for human insulin esters (see Markussen, *ibid*, 410). Final yield: 1.15 g = 37%. The product was near homogeneous in DISC PAGE electrophoresis at pH 8.9, the rate of migration being 55% of that of insulin. In analytical reverse-phase HPLC (see Markussen *ibid*, 410) the product elutes at about the same rate as porcine insulin. The purity found to about 95%. Amino acid composition analysis showed identity to the suggested formula.

Example 5

Synthesis of Gln^{A17},Lys^{B30}-NH_2 human insulin

To a suspension of 3.15 g of Gln^{A17},B(1-29)-Ala-Ala-Lys-A(1-21) insulin precursor in 15 ml of acetic acid/DMF/water (4.57 ml acetic acid, 71.9 ml of DMF, water to make 100 ml) 30 ml of 0.4 M Lys(Boc)-NH_2 in DMF was added. The mixture was cooled to 12°C and 0.3 g of trypsin dissolved in 7.5 ml of 0.05 M calcium acetate was added. Stirring was continued until the insulin precursor had dissolved. After 48 hours at 12°C the proteins were isolated as described in Example 4.

The precipitate was dissolved in 70 ml of 0.04 N HCl, the pH adjusted to 2.5 and Gln^{A17},$Lys(Boc)^{B30}$-NH_2 human insulin was purified by HPLC as described in Example 1, except that elution was performed first with 2.3 l of an eluent composed of 37 parts ethanol and 63 parts of 0.3 M KCl, 0.001 N HCl, followed by an eluent composed of 39 parts of ethanol and 61 parts of aqueous 0.3 M KCl, 0.001 N HCl. The derivate emerged 25 minutes after the change of eluent, and it was isolated as described for Gln^{A17},Thr^{B30}-NH_2 human insulin in Example 4. Yield of Gln^{A17},$Lys(Boc)^{B30}$-NH_2 human insulin 906 mg = 29%.

Gln^{A17},$Lys(Boc)^{B30}$-NH_2 human insulin (1.55 g) was dissolved in 30 ml of trifluoroacetic acid (TFA) and left at room temperature for 2 hours. The TFA was removed by lyophilization. The residue was dissolved in 15 ml of water, the pH adjusted to 3 with 1 N NaOH and 22 ml of ethanol was added. The solution was applied to a 2.5 x 20 cm column of SP-Sephadex™ C-25 equilibrated with an ethanol/water 3/2 (v/v) buffer comprising 0.01 M citric acid, 0.03 M NaCl, pH adjusted to 4.5 with NaOH. The column was eluted with the same buffer, using a linear gradient in NaCl from 0.03 M to 0.4 M in a total of 1.6 l of eluent. The derivative eluted in 440 ml when the gradient reached 0.2 M NaCl. It was crystallized by addition of 1100 ml of water, solid trisodium citrate to make 0.05 M and

solid zinc acetate to make 0.006 M. Eventually the pH was adjusted to 6.8. After stirring overnight at 4°C the crystals were isolated by centrifugation, washed once with water and dried in vacuo. Yield: 1.00 g corresponding to 65% over last step and 19% from the Gln^{A17},B(1-29)-Ala-Ala-Lys-A(1-21) insulin precursor. The product was near homogeneous in DISC PAGE electrophoresis at pH 8.9, the rate of migration being 35% of that of insulin. In analytical HPLC the product emerges before porcine insulin, the purity being about 97%. Amino acid composition analysis showed 2 lysine residues per molecule, and otherwise identity to human insulin.

Example 6

Synthesis of Arg^{B27},Thr^{B30}-NH_2 human insulin

To a suspension of 3.8 g of Arg^{B27},B(1-29)-Ala-Ala-Lys-A(1-21) insulin precursor in 18 ml of acetic acid/water/DMF (11.4 ml acetic acid, 35 ml water, DMF to make 100 ml) 36 ml of 1 M Thr-NH_2 in DMF was added. The mixture was cooled to 12°C and 0.38 g of porcine trypsin in 6.84 ml of 0.05 M calcium acetate was added. After 48 hours at 12°C the proteins were precipitated with acetone as described in Example 4.

The derivative was purified by HPLC as described in Example 1 using first 1800 ml eluent composed of 35 parts of ethanol and 65 parts of aqueous 0.3 M KCl, 0.001 N HCl, followed by an eluent composed of 37 parts of ethanol and 63 parts of the aqueous solution. The derivative emerged 10 minutes after shift in eluent and it was isolated as described for Gln^{A17},Thr^{B30}-NH_2 insulin in Example 4. Finally it was purified on a column of SP-Sephadex™ C-25 as described for Gln^{A17},Lys^{B30}-NH_2 human insulin in Example 5.

The yield of Arg^{B27},Thr^{B30}-NH_2 human insulin was 1.63 g corresponding to 43%. Essentially one band was seen in DISC PAGE electrophoresis, the rate of migrating being 55% of that of insulin. In analytical HPLC the product emerge before porcine insulin, the purity being about 96%. Amino acid composition analysis shows 2 arginine residues per molecule, and otherwise identity to human insulin.

Example 7

Synthesis of Arg^{B27},Lys^{B30}-NH_2 human insulin

The compound was synthesized from 3.61 g of Arg^{B27},B(1-29)-Ala-Ala-Lys-A(1-21) insulin precursor using the methods described in Example 5. Yield of Arg^{B27},Lys^{B30}-NH_2 human insulin 0.78 g = 22%. One major band in DISC PAGE electrophoresis migrating 35% of the distance of insulin migration. Two minor bands visible. Purity in analytical HPLC 92%; the product emerge before porcine insulin. Amino acids composition analysis shows 2 arginine and 2 lysine residues per molecule and otherwise identity to human insulin.

Example 8

Synthesis of Lys^{B27},Thr^{B30}-NH_2 human insulin

The compound was synthesized from 7.0 g of Lys^{B27},B(1-29)-Ala-Ala-Lys-A(1-21) insulin precursor using the methods described in Example 6. Yield of Lys^{B27},Thr^{B30}-NH_2 human insulin was 3.15 g corresponding to 45%. DISC PAGE electrophoresis showed one major band and two minor bands, the main band migrating 55% of the distance of the insulin reference band. The purity in analytical HPLC was 96%. The

compound emerge earlier than porcine insulin in reverse phase HPLC. Amino acid composition analysis shows 2 lysine residues per molecule and otherwise identity to human insulin.

Example 9

Synthesis of Lys^{B27},Lys^{B30}-NH_2 human insulin

The compound was synthesized from 7.0 g of Lys^{B27},B(1-29)-Ala-Ala-Lys-A(1-21) insulin precursor using the methods described in Example 5. Yield of Lys^{B27},Lys^{B30}-NH_2 human insulin was 1.57 g corresponding to 22%. DISC PAGE electrophoresis showed one major band migrating to a distance of 35% of that of porcine insulin. One minor impurity is visible. Purity in analytical HPLC was 94%, the compound eluting well ahead of porcine insulin. Amino acid composition analysis showed 3 lysine residues per molecule and otherwise identity to human insulin.

Example 10

Synthesis of Gln^{B13},Thr^{B30}-NH_2 human insulin

The compound was synthesized from 3.05 g of Gln^{B13},B(1-29)-Ala-Ala-Lys-A(1-21) insulin precursor using the methods described in Example 6. Yield of final product was 0.88 g corresponding to 29%. DISC PAGE electrophoresis showed one major band, migrating 55% of the distance porcine insulin migrates. Purity by HPLC was 95%, the compound eluting later than porcine insulin. Amino acid composition analysis showed identity to that of human insulin.

Example 11

Preparation of injectable solutions of compounds of formula I

Sterile injectable solutions of the compounds of formula I for testing of the degree of prolonged action were made using 0.9% (w/v) sodium chloride as the isotonicum and 0.15% (v/v) m-cresol as the preservative. Injectable solutions were also made using 1.6% (w/v) glycerol as the isotonicum, using 0.3% (w/v) m-cresol as the preservative, and being buffered with 0.01 M sodium acetate. The concentration of zinc ions was varied from 0 to 160 µg/ml. The pH values of the solutions were adjusted sufficiently off the isoelectric point of the compounds of formula I to keep the solutions clear upon storage at 4°C. The solutions contained 240 nmole/ml of the compounds of formula I. The concentration of 240 nmole/ml was established by measurement of the absorbance at 276 nm of a more concentrated stock solution devoid of m-cresol, using the molar extinction coefficient for porcine insulin of 6100 for these derivatives (see Handbuch der Inneren Medizin, Vol. 7/Part 2A, Editor: Oberdisse, 1975, 113) and using the established potency for monocomponent porcine insulin of 28.5 U/mg dry substance (see Diabetes Care, Vol. 6/Supplement 1 (1983), 4). 1 U corresponds to 5.95 nmole.

Injectable solutions containing 240 nmole/ml of the compounds of formula I stated in Table 1 and having the pH values and content of zinc stated in the table were made.

Test for prolongation of insulin effect

The prolongation of the hypoglycemic effect produced by the injectable solutions of insulin was tested according to British Pharmacopoeia 1980, A 142, in fasted rabbits. Each test solution was administered subcutaneously in a dosis of 15.5 nmole per rabbit in 6 or 12 animals weighing 3 - 4 kg, and the course of the hypoglycemia was followed for 6 hours. For

comparison the fast acting preparation, Actrapid™ porcine insulin, was included in the tests. The results of the tests are shown in Table 1 and 2.

The result of tests for prolonged effect of certain compounds in rabbits is stated in Table 1, below. The glucose value is the mean, from 6 rabbits, of the glucose value in per cent of the initial value. Solutions were made isotonic with 0.9% NaCl, using 0.15% (v/v) m-cresol as the preservative.

Table 1

Compound of formula I	Zn^{++}, µg/ml	pH	Glucose in percent of initial 1/2 h	1 h	2 h	4 h	6 h
Lys^{B30}-NH_2 insulin	0	4.5	53	54	49	79	100
Lys^{B30}-NH_2 insulin	80	4.5	63	62	63	73	80
Lys^{B30}-NH_2 insulin	160	4.5	87	80	68	90	93
Arg^{B30}-NH_2 insulin	0	4.5	57	53	51	65	81
Arg^{B30}-NH_2 insulin	80	4.5	76	68	63	73	78
Thr^{B30}-NH_2 insulin	0	4.2	46	46	39	64	91
Thr^{B30}-NH_2 insulin	160	4.2	64	58	50	70	69
Thr^{B30}-OBu^t insulin	0	4.0	59	65	59	79	100
Thr^{B30}-OBu^t insulin	160	4.0	81	75	62	66	88
$Thr^{B30}(Bu^t)$-OBu^t insulin	0	4.0	64	60	54	71	82
Arg^{B30}-Arg^{B31}-NH_2 insulin	0	4.5	72	70	68	67	68
Arg^{B30}-Arg^{B31}-NH_2 insulin	160	4.5	91	87	78	73	68
Actrapid™ porcine insulin	15	7	53	48	42	70	98

The result of tests for prolonged effect of certain compounds in rabbits is stated in table 2, below. The glucose value is the mean, from 12 rabbits, of the glucose value in percent of the initial value. Test solutions were made isotonic with 1.6% (w/v) glycerol, using 0.3% (w/v) m-cresol as the preservative, and being buffered by 0.01 M sodium acetate.

Table 2

Compound of formula I	Zn^{++}, µg/ml	pH	Glucose in percent of initial 1 h	2 h	4 h	6 h
Gln^{A17},Thr^{B30}-NH_2 insulin	80	4.5	65	63	68	83
Gln^{A17},Lys^{B30}-NH_2 insulin	80	4.5	60	56	73	86
Gln^{B13},Thr^{B30}-NH_2 insulin	6.7	4.5	91	92	92	90
Arg^{B27},Thr^{B30}-NH_2 insulin	80	4.5	88	86	85	81
Arg^{B27},Thr^{B30}-NH_2 insulin	8.5	4.5	62	64	66	67
Arg^{B27},Lys^{B30}-NH_2 insulin	80	4.5	85	83	81	79
Arg^{B27},Lys^{B30}-NH_2 insulin	10.9	4.5	78	73	69	67
Lys^{B27},Thr^{B30}-NH_2 insulin	7.4	4.5	56	55	62	61
Lys^{B27},Lys^{B30}-NH_2 insulin	9.5	4.5	72	65	65	60
Lys^{B30}-NH_2 insulin	80	4.5	74	83	80	82
Reference insulin						
Actrapid™ porcine insulin	15	7	58	56	87	100

The potencies of insulin compounds were assessed in the mouse blood sugar depletion test (British Pharmacopoeia 1980, A 141 - A 142). In order to minimize the problem of estimating potency of insulins having a timing different from the standard, insulin solutions for potency determinations were made up without additions of zinc. Solutions were made up to contain 240 nmole/ml based on the absorbance at 276 nm. The zinc content of solutions were 8 - 10 µg/ml, arizing from the crystalline derivatives. The estimated potencies of some insulin compounds are shown in Table 3, below.

Table 3

	Potency relative to insulin, %	Confidence limits (P = 0.05), %
Gln^{A17},Thr^{B30}-NH_2 insulin	67	58 - 75
Gln^{A17},Lys^{B30}-NH_2 insulin	62	51 - 72
Arg^{B27},Thr^{B30}-NH_2 insulin	122	102 - 145
Arg^{B27},Lys^{B30}-NH_2 insulin	84	74 - 94

Example 12

Preparation of Gln^{B21},Thr^{B30}-NH_2 human insulin.

1800 mg of single-chain Gln^{21},des(30-65)-human proinsulin prepared by one of the biosynthetic methods described above were added to 350 ml of a suspension of 70 ml (sedimented volume) of matrix bound trypsin (Trypsin-Sepharose® Fast Flow, 0.8 mg of enzyme per ml of gel) in a 0.05 M tris(hydroxymethyl)aminomethane solution, 20% (by volume) ethanol, pH 8.1 (hydrochloric acid) and the mixture was stirred gently for 6 hours at 4°C. The gel was filtered off and the filtrate containing Gln^{B21},des-Thr^{B30} human insulin was adjusted to a pH value of 6.3. The resulting protein precipitate was isolated by centrifugation and freeze-dried. The protein was redissolved in a mixture of 4 g of threonine amide, 20 ml of ethanol and 8 ml of water. The pH value was adjusted to 6.3 with acetic acid and 32 ml of Trypsin-Sepharose® were added. After standing for 2 hours at 20°C with gentle stirring, the gel was filtered off and the protein was precipitated by addition of 10 volumes of 2-propanol. The air-dried precipitate was redissolved in a 0.02 M tris(hydroxymethyl)aminomethane solution, 60% (by volume) ethanol, pH 9 (hydrochloric acid), applied to a 5 x 20 cm Q-Sepharose® CL-6B Fast Flow column equilibrated with the same buffer and was then eluated with a linear gradient of from 0 to 0.1 M sodium chloride solution in the same buffer for 15 hours with a flow of 500 ml per hour.

The ethanol was removed in vacuo from the fraction containing Gln^{B21},Thr^{B30}-NH_2 human insulin, and the protein was precipitated by adjusting the pH value to 6.5. After centrifugation and freeze-drying of the precipitate, 200 mg of Gln^{B21},Thr^{B30}-NH_2 human insulin were obtained.

The identity of the product was confirmed by amino acid analysis and by decomposition with S. Aureus protease.

Example 13

Preparation and biological effect of preparations containing human insulin diamides.

15 mg of Gln^{B21},Thr^{B30}-NH_2 human insulin were dissolved in 4 ml of a 0.3% m-cresol solution by adjusting the pH value to 9.8 with a 1 M sodium hydroxide solution. After addition of 13 µl of a 0.1 M zink acetate solution, the volume was stirred gently until the solution was clear, whereupon it was sterilized by filtration. Then 5 ml of solution sterilized by filtration and containing 25 mM of disodium phosphate, 1.4% sodium chloride and 0.3% m-cresol were added, and the mixture was left for 30 minutes with gentle stirring now and then. Finally, the pH value was adjusted to 7.3 with sterile hydrochloric acid, and the volume was increased to 10 ml with a 0.3% m-cresol solution sterilized by filtration.

A preparation containing Gln^{A4},Thr^{B30}-NH_2 human insulin and a preparation containing Gln^{A17},Thr^{B30}-NH_2 human insulin were prepared analogously as described above.

By subcutaneous injection of the first mentioned preparation into pigs in an amount of 36 µl per kg, the maximum hypoglycemic effect is obtained much later than that of a usual rapid acting insulin preparation Velosulin® containing human insulin.

A graph of the effect of the preparation versus the time is shown in the accompanying figure.

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Example 14

Preparation of Gln^{A4},Gln^{B13},Thr^{B30}-NH_2 human insulin.

900 mg of single-chain Gln^{13},Gln^{69},des(30-65)-human proinsulin prepared by one of the biosynthetic methods described above were added to 175 ml of a suspension of 35 ml (sedimented volume) of matrix bound trypsin (Trypsin-Sepharose® Fast Flow, 0.8 mg of enzyme per ml of gel) in a 0.05 M tris-(hydroxymethyl)aminomethane solution, 20% (by volume) ethanol, pH 10 (hydrochloric acid) and the mixture was stirred gently for 5 hours at 20°C. The gel was filtered off and the filtrate containing Gln^{A4},Gln^{B13}des-Thr^{B30}des(B30)-human insulin was adjusted to a pH value of 6.5. The resulting protein precipitate was isolated by centrifugation and freeze-dried. The protein was redissolved in a mixture of 2 g of threonine amide, 20 ml of dimethyl formamide and 10 ml of water. The pH value was adjusted to 6.5 with acetic acid and 16 ml of Trypsin-Sepharose® was added. After standing for 2 hours at 20°C with gentle stirring, the gel was filtered off and the protein was precipitated by addition of 10 volumes of 2-propanol. The air-dried precipitate was redissolved in a 0.02 M triethylamine solution, 60% (by volume) ethanol, pH 9.5 (hydrochloric acid), applied to a 5 x 20 cm Q-Sehparose® CL-6B Fast Flow column equilibrated with the same buffer and was then eluated with a linear gradient of from 0 to 0.1 M sodium chloride solution in the same buffer for 15 hours with a flow of 500 ml per hour.

The ethanol was removed in vacuo from the fraction containing Gln^{A4},Gln^{B13},Thr^{B30}-NH_2 human insulin, and the protein was precipitated by adjusting the pH value to 6.5. After centrifugation and freeze-drying of the precipitate, 100 mg of Gln^{A4},Gln^{B13},Thr^{B30}-NH_2 human insulin were obtained.

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The identity of the product was confirmed by amino acid analysis and by decomposition with S. Aureus protease followed by multi-stage Edman decomposition of the fractions.

Example 15

Preparation of Gln^{A4},Thr^{B30}-NH_2 human insulin.

200 mg of human proinsulin wherein the glutamic acid of position A4 has been replaced by glutamine, introduced by one of the biotechnological methods described in the introductory part, were dissolved in a mixture containing 1.60 ml of N,N-dimethyl formamide, 0.40 ml of water and 500 mg of L-threonine amide. The pH value of the solution was then adjusted to 6.5 with acetic acid. 10 mg of porcine trypsin, dissolved in 0.40 ml of a 0.001 M calcium acetate solution, was added and the reaction mixture was left at 12°C. After 48 hours, the reaction was stopped by addition of 40 ml of 2-propanol. The resulting protein precipitate was isolated by centrifugation and subsequently redissolved in 10 ml of 1 M acetic acid in a 7 M urea solution. After filtration, the solution was applied to a 2.6 x 90 cm column of Sephadex® G50 SF previously equilibrated with 1 M acetic acid and was then eluted with the equilibration buffer at a rate of 25 ml per hour. The eluate was detected for UV-absorption and the proteinaceous main fraction was collected and lyophilized.

The isolated protein was redissolved in 20 ml of a 60% (by volume) ethanol solution by adjusting the pH value to 8.25 with a 1 M tris(hydroxymethyl)aminomethane solution (TRIS). After filtration, the solution was applied to a 1.5 x 20 cm column of Q-Sepharose® CL-6B FF previously equilibrated with 0.02 M TRIS/hydrochloric acid in 60% (by volume) ethanol adjusted to a pH value of 8.25. The column was eluted at 20°C at a rate of 50 ml per hour with equilibration buffer for 1.5 hour and then for the next 16 hours with a linear gradient from 0 to 0.1 M sodium chloride solution in the same buffer.

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The eluate was detected for UV-absorption and the proteinaceous main fraction eluting at a sodium chloride concentration of approximately 0.05 M was collected. The ethanol was removed in vacuo and the pH value in the aqueous residue was adjusted to 6.1. After 4 hours at 4°C, the precipitation was isolated by centrifugation and lyophilized to provide 50 mg of Gln^{A4},Thr^{B30}-NH_2 human insulin.

Example 16

Formulation and biological effect of an insulin suspension preparation containing Gln^{A4},Thr^{B30}-NH_2 human insulin.

50 mg of Gln^{A4},Thr^{B30}-NH_2 human insulin were dissolved in 8 ml of water containing 70 mg of sodium chloride and 33 µl of m-cresol by adjusting the pH value to 10.3 with a 1 M sodium hydroxide solution and the pH value was then adjusted to 9.8 with 1 M hydrochloric acid. 30 µl of a 0.1 M zinc acetate solution was added and the volume was adjusted to 10 ml with water.

23 mg of sodium dihydrogen phosphate, monohydrate were dissolved in 2 ml of water containing 17.5 mg sodium chloride and 8 µl of m-cresol. The pH value was then adjusted to 5.2 with a 5 M sodium hydroxide solution and the volume was adjusted to 2.5 ml with water.

The buffer solution was added to the insulin solution and the pH value was adjusted to 7.3. The resulting suspension was then left at room temperature over night for complete precipitation.

Subcutaneous injection of 40 µl of this suspension preparation into streptozotocin-diabetic rats resulted in a more protracted hypoglycemic reaction than the corresponding reaction induced by a standard NPH insulin preparation (Insulartard®).

Example 17

Preparation of Gln^{A4},Gln^{B13} human insulin B30 hexyl amide.

400 mg of human proinsulin, wherein the glutamic acids of positions A4 and B13 have been replaced by glutamine, introduced by one of the biotechnological methods described in the introductory part, were dissolved in a mixture containing 3.20 ml of N,N-dimethyl formamide, 0.80 ml of water and 1.20 g of L-threonine hexyl amide. The pH value of the solution was then adjusted to 6.5 with acetic acid. 20 mg of porcine trypsin, dissolved in 0.80 ml of a 0.001 M calcium acetate solution, was added and the reaction mixture was left af 12°C. After 48 hours, the reaction was stopped by addition of 80 ml of 2-propanol. The resulting protein precipitate was isolated by centrifugation and subsequently fractionated by gelfiltration and ion exchange chromatography as described in example 15 to provide 60 mg of Gln^{A4},Gln^{B13} human insulin B30 hexyl amide.

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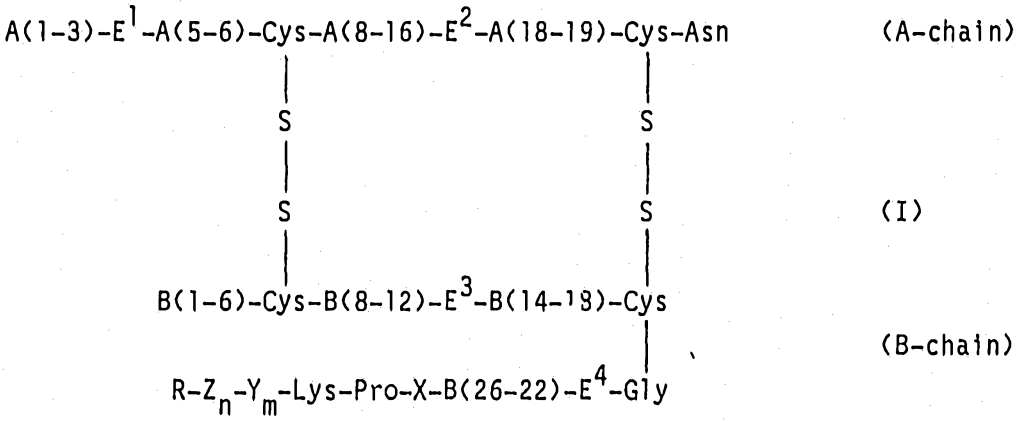
The claims defining the invention are as follows:

1. A compound of the general formula I



wherein the letters A and B followed by figures in parentheses designate the peptide fragments of the A- and B-chains, respectively, indicated by the figures in parentheses, E^1, E^2, E^3 and E^4 are the same or different each representing glutamic acid or a neutral amino acid residue which can be coded for by nucleotide sequences, X represents an L-threonine, L-arginine or L-lysine residue, Y and Z are the same or different and each represent an amino acid residue wherein any side chain hydroxy group may be alkylated, and m and n are the same or different and each represent zero or one, and R represents an amido or ester residue which blocks the C-terminal carboxyl group of the B-chain, with the proviso that not all of E^1, E^2, E^3 and E^4 are glutamic acid residues when X is a threonine residue, or, when E^1, E^2, E^3 and E^4 each is a glutamic acid residue and X is a threonine residue, the group of formula $-Y_m-Z_n-R$ represents $-NH_2$, -Arg-NH_2, -Arg-Arg-NH_2, -Arg-Lys-NH_2, -Dab-Dab-NH_2, -Dap-Dap-NH_2, Lys-NH_2, -Lys(Lau)-NH_2, -Lys-Arg-NH_2, -Lys-Lys-NH_2, -Orn-NH_2 or -Orn-Orn-NH_2, with the further proviso that the compound of formula I has at least one charge more than human insulin at a pH value of 7.

2. A compound according to Claim 1, characterized in that E^1, E^3 and E^4 each is a glutamic acid residue.

3. A compound according to any one of the preceding claims, characterized in that E^2 is a glutamine residue.

4. A compound according to any one of the preceding claims, characterized in that Y and/or Z is a basic amino acid residue (m = 1 or 0, n = 1 or 0).

5. A compound according to any one of the preceding claims, characterized in that n is zero, and Y is a basic amino acid residue (m = 1).

6. A compound according to any one of the preceding claims, characterized in that Y and Z are both basic amino acid residues (m = 1, n = 1).

7. A compound according to any one of the preceding claims, characterized in that R is a group of the general formula $-NR^1R^2$ wherein R^1 and R^2 are the same or different and each represent hydrogen or lower alkyl, and preferably R is $-NH_2$.

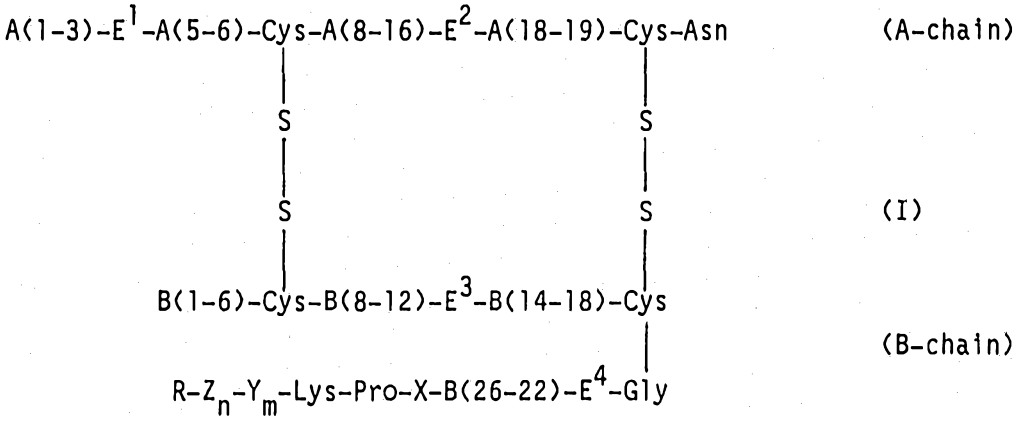
8. A compound according to any one of the Claims 1 through 6, characterized in that R is lower alkoxy, preferably tertiary butyloxy.

9. A compound according to any one of the Claims 1 through 6, characterized in that R is a residue of a lactam which preferably contains less than 8 atoms in the lactam ring.

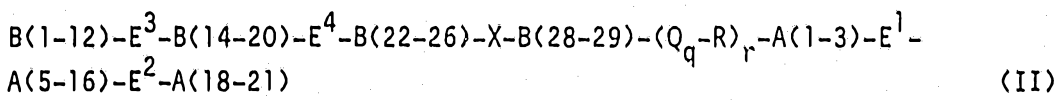
10. A compound according to Claim 1, characterized in that it is Gln^{A17},Arg^{B27},Thr^{B30}-NH_2 human insulin, Gln^{A17},Gln^{B13},Thr^{B30}- NH_2 human insulin, Gln^{A17},Lys^{B27},Thr^{B30}-NH_2 human insulin, Gln^{A17},Lys^{B30}-NH_2 human insulin, Gln^{A17},Thr^{B30}-NH_2 human insulin, Gln^{B13},Arg^{B27},Thr^{B30}-NH_2 human insulin, Gln^{B13},Lys^{B27}, Thr^{B30}-NH_2 human insulin, Gln^{B13},Lys^{B30}-NH_2 human insulin, Gln^{B13},Thr^{B30}-NH_2 human insulin, Arg^{B27},Arg^{B30}-NH_2 human insulin, Arg^{B27},Lys^{B30}-NH_2 human insulin, Arg^{B27},Thr^{B30}-NH_2 human insulin, Lys^{B27},Arg^{B30}-NH_2 human insulin, Lys^{B27},Lys^{B30}-NH_2 human insulin, Lys^{B27},Thr^{B30}-NH_2 human insulin, Lys^{B29}-NH_2,des-(B30) human insulin, Lys^{B30}-NH_2 human insulin, Lys^{B30}(Lau)-NH_2 human insulin, Lys^{B30}-Arg^{B31}-NH_2 human insulin, Lys^{B30}-Lys^{B31}-NH_2 human insulin, Arg^{B30}-NH_2 human insulin, Arg^{B30}-Arg^{B31}-NH_2 human insulin or Arg^{B30}-Lys^{B31}-NH_2 human insulin.

11. An insulin derivative having a charge which is positive, substantially as hereinbefore described with reference to any one of Examples 1, 2, 4 to 10, 12, 14, 15 or 17.

12. A process for preparing a compound of the general formula I



wherein the letters A and B followed by figures in parentheses designate the peptide fragments of the A- and B-chains, respectively, indicated by the figures in parentheses, E^1, E^2, E^3 and E^4 are the same or different each representing glutamic acid or a neutral amino acid residue which can be coded for by nucleotide sequences, X represents an L-threonine, L-arginine or L-lysine residue, Y and Z are the same or different and each represent an amino acid residue wherein any side chain hydroxy group may be alkylated, and m and n are the same or different and each represent zero or one, and R represents an amido or ester residue which blocks the C-terminal carboxyl group of the B-chain, with the proviso that not all of E^1, E^2, E^3 and E^4 are glutamic acid residues when X is a threonine residue, or, when E^1, E^2, E^3 and E^4 each is a glutamic acid residue and X is a threonine residue, the group of formula $-Y_m-Z_n-R$ represents $-NH_2$, $-Arg-NH_2$, $-Arg-Arg-NH_2$, $-Arg-Lys-NH_2$, $-Dab-Dab-NH_2$, $-Dap-Dap-NH_2$, $-Lys-NH_2$, $-Lys(Lau)-NH_2$, $-Lys-Arg-NH_2$, $-Lys-Lys-NH_2$, $-Orn-NH_2$ or $-Orn-Orn-NH_2$, with the further proviso that the compound of formula I has at least one charge more than human insulin at a pH value of 7, said process being characterized in (a) transpeptidating porcine insulin or a compound of the general formula II:



wherein A and B designate the fragments of the A- and B-chains indicated by numbers in parentheses, Q is a peptide chain with q amino acids, q is an integer from 0 to 33, R is Lys or Arg, and r is zero or one and E^1, E^2, E^3, E^4 and X each are as defined above, with a compound of the general formula III:

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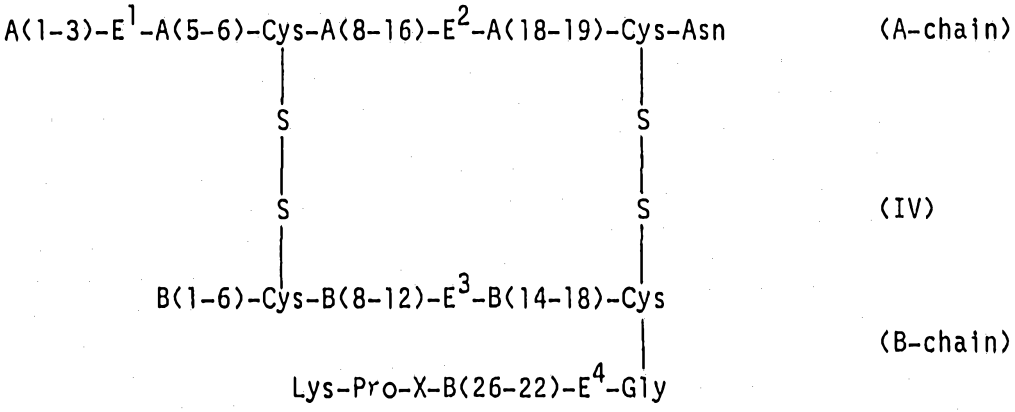
$$H\text{-}Y_m\text{-}Z\text{-}_n\text{-}R \qquad \text{(III)}$$

wherein Y, Z, R, m and n each are as defined above, and wherein side chain amino groups and hydroxy groups in Y and Z optionally are blocked with amino and hydroxy protecting groups, using trypsin or a trypsin like enzyme as a catalyst, or b) coupling a compound of formula IV

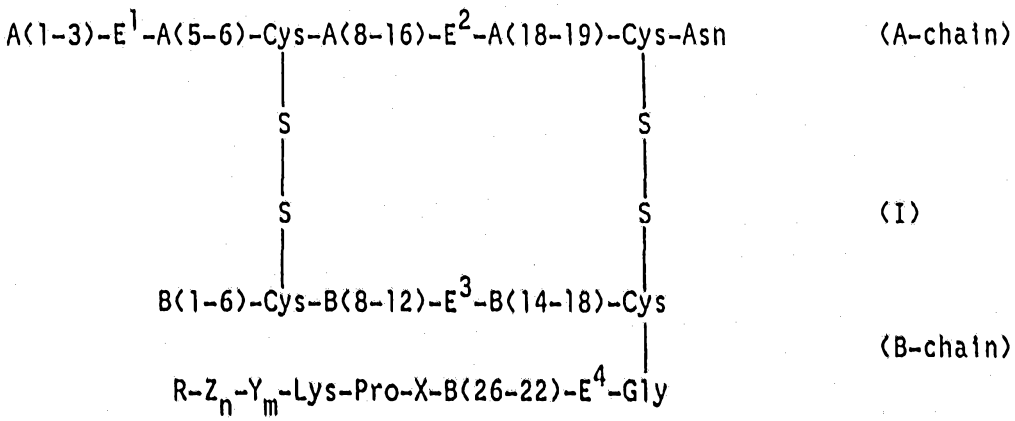


wherein E^1, E^2, E^3, E^4 and X each are as defined above, with a compound of formula III by trypsin or a trypsin like enzyme.

13. A process for preparing an insulin derivative having a charge which is positive, substantially as hereinbefore described with reference to any one of Examples 1, 2, 4 to 10, 12, 14, 15 or 17.

14. The product of the process of Claim 12 or Claim 13.

15. An injectable solution with prolonged insulin action, characterized in that it contains a compound of the general formula I



wherein the letters A and B followed by figures in parentheses designate the peptide fragments of the A- and B-chains, respectively, indicated by the figures in parentheses, E^1, E^2, E^3 and E^4 are the same or different each representing glutamic acid or a neutral amino acid residue which can be coded for by nucleotide sequences, X represents an L-threonine, L-arginine or L-lysine residue, Y and Z are the same or different and each represent an amino acid residue wherein any side chain hydroxy group may be alkylated, and m and n are the same or different and each represent zero or one, and R represents an amido or ester residue which blocks the C-terminal carboxyl group of the B-chain, with the proviso that not all of E^1, E^2, E^3 and E^4 are glutamic acid residues when X is a threonine residue, or, when E^1, E^2, E^3 and E^4 each is a glutamic acid residue and X is a threonine residue, the group of formula $-Y_m-Z_n-R$ represents $-NH_2$, -Arg-NH_2, -Arg-Arg-NH_2, -Arg-Lys-NH_2, -Dab-Dab-NH_2, -Dap-Dap-NH_2, Lys-NH_2, -Lys(Lau)-NH_2, -Lys-Arg-NH_2, -Lys-Lys-NH_2, -Orn-NH_2 or -Orn-Orn-NH_2, with the further proviso that the compound of formula I has at least one charge more than human insulin at a pH value of 7, together with a pharmaceutically acceptable diluent.

16. An injectable solution with prolonged insulin action, characterized in that it contains a compound according to any one of Claims 1 to 11 or 14 together with a pharmaceutically acceptable diluent.

17. A solution according to Claims 15 or 16, further characterized in that it contains zinc ions.

18. A solution according to Claim 17, wherein the zinc ions are present in an amount from 2µg to 2mg zinc per ml.

19. A solution according to Claims 17 or 18 wherein the zinc ions are present in an amount from 5µg to 200µg zinc per ml.

20. An injectable solution with prolonged insulin action, substantially as hereinbefore described with reference to any one of Examples 11, 13 or 16.

21. A kit comprising a solution according to any one of Claims 15 to 17 and a separately contained solution of zinc ions.

22. A kit according to Claim 21 wherein the zinc ions are present in said solution of zinc ions in an amount from 10µg to 20mg zinc per ml.

23. A kit comprising a first solution according to the solution of any one of Claims 15 to 17 and a separately contained second solution according to the solution of any one of Claims 15 to 17 wherein the

concentration of at least one component in said second solution is different from the concentration of the same component in said first solution.

24. A method of preparing a more prolonged acting solution according to any one of Claims 15 to 20 by mixing together the solutions contained in any one of the kits according to any one of Claims 21 to 23.

25. A method of preparing a solution according to any one of Claims 15 to 20, characterized in that a solution of a compound of formula I as defined in Claim 15, is mixed with a zinc solution resulting in a solution with a zinc concentration of up to 2mg/ml.

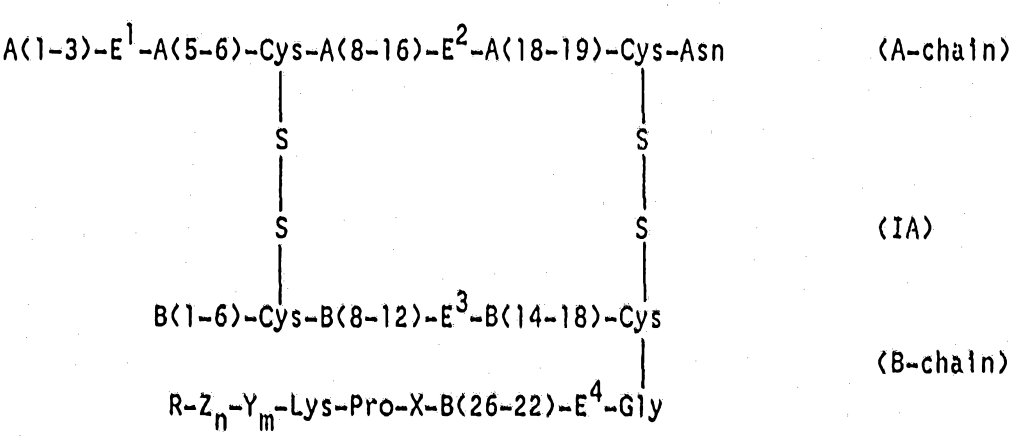
26. The method of Claim 25, wherein said solution of a compound of formula I contains zinc.

27. The method of Claim 25 or Claim 26, wherein said zinc solution contains a compound of formula I as defined in Claim 15.

28. The method according to Claim 26 or Claim 27, wherein the concentration of zinc in each of said solution of a compound of formula I and said zinc solution is less than about 4mg/ml.

29. A method of treatment of diabetes mellitus in a patient requiring said treatment which method comprises administering to said patient an effective amount of either a compound according to any one of Claims 1 to 11 or 14 or of a solution according to Claim 20.

30. A method of treatment of diabetes mellitus in a patient requiring said treatment which method comprises administering to said patient an effective amount of an injectable solution with prolonged insulin action, said solution characterized in that it contains a compound of the general formula



wherein the letters A and B followed by figures in parentheses designate the peptide fragments of the A- and B-chains, respectively, indicated by the figures in parentheses, E^1, E^2, E^3 and E^4 are the same or different each representing glutamic acid or a neutral amino acid residue which can be coded for by nucleotide sequences, X represents an L-threonine, L-arginine or L-lysine residue, Y and Z are the same or different and each represent an amino acid residue wherein any side chain hydroxy group may be alkylated, and m and n are the same or different and each represent zero or one, and R represents an amido or ester residue which blocks the C-terminal carboxyl group of the B-chain, with the proviso that not all of E^1, E^2, E^3 and E^4 are glutamic acid residues when X is a threonine residue, or, when E^1, E^2, E^3 and E^4 each is a glutamic acid residue and X is a threonine residue, the group of formula $-Y_m-Z_n-R$ represents $-NH_2$, $-Arg-NH_2$, $-Arg-Arg-NH_2$, $-Arg-Lys-NH_2$, $-Dab-Dab-NH_2$, $-Dap-Dap-NH_2$, $-Lys-NH_2$, $-Lys(Lau)-NH_2$, $-Lys-Arg-NH_2$, $-Lys-Lys-NH_2$, $-Orn-NH_2$, $-Orn-Orn-NH_2$, $-Thr-NH_2$, $-Thr-OBu^t$ or $-Thr(Bu^t)-OBu^t$, with the further proviso that the compound of formula I has at least one charge more than human insulin at a pH value of 7, together with a pharmaceutically acceptable diluent.

31. The method according to claim 30, wherein said solution is further characterized in that it contains zinc ions.

32. The method according to Claim 31, wherein said zinc ions in said solution are present in an amount from 2µg to 2mg zinc per ml.

33. The method according to Claim 31 or Claim 32, wherein said zinc ions in said solution are present in an amount from 5µg to 200µg zinc per ml.

DATED this TWENTY-NINTH day of APRIL 1991
Novo Nordisk A/S

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SPRUSON & FERGUSON

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