(10)

PATENT SPECIFICATION (11) 1285023

1285023

DRAWINGS ATTACHED

(21) Application No. 38081/68 (22) Filed 9 Aug. 1968

(23) Complete Specification filed 29 July 1969

(45) Complete Specification published 9 Aug. 1972

(51) International Classification A61K 17/02

(52) Index at acceptance

A5B 313 31Y 382 383 38Y 394 778

(72) The inventors of this invention in the sense of being the actual Devisers thereof within the meaning of Section 16 of The Patents Act 1949 are:— JORGEN SCHLICHTKRULL and KLAVS HOLGER JORGENSEN, both Danish subjects of 28B, Ved Bellahoj, 2700 Bronshoj and 41, Askevaenget, 2830 Virum, Denmark, respectively.

(54) IMPROVEMENTS IN OR RELATING TO INJECTABLE INSULIN PREPARATIONS

(71) We, NOVO TERAPEUTISK LABORATORIUM A/S, a Danish Body Corporate, of 115, Fuglebakkevej, 2200 Copenhagen N, Denmark, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to novel injectable insulin preparations.

Divided from this application is our copending Application No. 10157/72 (Serial No. 1285024) which describes and claims a process of purifying commercial or crude insulin by column chromatography, which process comprises applying a water-containing solution of the insulin to be purified to a column of an anion exchanger, the solution having a pH in the range from 6 to 10, eluting the column in fractions with a water-miscible organic solvent which contains water in an amount of from 30% to 70% by volume and which has a pH in the range from 6 to 10, collecting the eluate fraction(s) showing essentially a single component when analysed by polyacrylamide gel electrophoresis (DISC PAGE), and recovering the insulin thus purified from the collected fraction(s).

The present invention provides an injectable insulin preparation containing insulin recovered from pancreas glands and being dissolved or suspended in an injectable aqueous medium, said insulin being free of proteins of pancreatic origin with a molecular weight above 6000 and said insulin showing essentially a single component when a solution thereof is analysed by polyacrylamide gel electrophosesis (DISC PAGE).

Originally the general opinion was that properly recrystallized insulin in the form of sharp-edged rhombohedrons bounded by plane crystal faces constituted the pure protein, insulin, the configuration of which was determined by Sanger and co-worker, vide e.g. Biochemical Journal 60 (1955), 556. Later it was found, however, that this was not so. Scientific analytical investigations showed that the crystalline insulin referred to contains proteins of pancreatic origin with a molecular weight above that of the pure insulin (about 6000) and in addition thereto insulin-like substances of about the same molecular weight as that of the pure insulin.

Thus, it has been demonstrated that crystalline insulin dissolved in 1 M acetic acid may be fractionated into components (a), (b) and (c), respectively, by gel filtration on a column of Sephadex G-50 ("SEPHADEX" is a Registered Trademark) which is dextran cross-linked with epichlorohydrin to give an exclusive limit of penetration of about 10,000 (minimum mole weight of excluded substances), being in bead form (diameter 20 to 80 μ) and produced by PHARMACIA, Uppsala, Sweden. These three components may be isolated from their solution by conventional methods, e.g. salting out, precipitation with a zinc salt at neutral reaction, freeze drying etc. Both component (a) and component (b) contain proteins of pancreatic origin having a molecular weight above 6000.

The amount of component (a) may be 2 to 5 percent by weight of crystallized insulin and less than 1 percent by weight of recrystallized insulin. The amount of component (b) may in both cases be 4 to 8 percent.

The component (b) may be further resolved into a number of proteins with almost the same molecular size, but differing with respect to the charge. This has been demonstrated with the anion exchanger DEAE Sephadex A-50 and a solution of component (b) in 7 M urea buffered with TRIS (tris-(hydroxymethyl)aminomethane) at pH 7.7.

and slip number 2

SPECIFICATION AMENDED - SEE ATTACHED SLIP and slip number 3

DEAE Sephadex A-50 is dextran cross-linked with epichlorohydrin to give an exclusive limit of penetration of about 10,000 (minimum molecular weight of excluded substances), carrying as functional groups diethylaminoethyl groups, being in bead form (diameter 40 to 120 μ) and produced by PHARMACIA, Uppsala, Sweden. The result of the separation process is shown in Figure 1. The peaks Nos. 4, 6 and 7 in the Figure correspond to the known substances; proinsulin and the intermediate and the non-convertible dimer. The other peaks have not yet been identified. They may all be isolated from their solution by known means. Proinsulin has been isolated from crystalline bovine insulin by Yip and Lin (Bio. Biophys. Res. Com., 29, 382—387 (1967)), and from porcine insulin by Chance and co-workers (Science, 161, 165—157 (1968)). The existence of the intermediate has been mentioned by Chance and co-workers (l.c.).

The component (c), which contains the proteins of pancreatic origin with a molecular weight of about 6000, may also be resolved by ion-exchange chromatography. It contains a main fraction, which is considered pure insulin, and some side-fractions of insulin-like substances, such as desamido insulins and an insulin-like substance containing more arginine than the amount present in the insulin molecule, and therefore being called "arginine insulin". The existence of desamido insulins has been mentioned by Harfenist & Craig (J. Am. Chem. Soc. 74, 3083 (1952)), by Thompson & O'Donnell (Austr. J. Biol., Sci., 393—400 (1960)) and by Chance and co-workers (Science, 161, 165—157 (1968)), who also mention an insulin-like component presumably being "arginine insulin".

The fraction of monodesamido insulin in component (c) is variable and dependent on the history of the insulin. Commonly it is 3 to 10 percent by weight of the component (c). The arginine insulin may constitute 2 to 3 percent by weight of the component (c).

The finding of impurities in even recrystallized insulin and the scientific fractionation of the impurities and identification of the main part thereof, did not lead to any attempts of changing the composition of the insulin preparations containing insulin recovered from pancreas glands and used in the diabetes therapy. Supersensitivity (allergy) against the insulin preparations available did appear almost completely after the general use of crystallized and recrystallized insulin. On the other hand, there existed and still exist a number of side-effects caused by the fact that after treatment for some months the diabetic patient's serum was found to contain immunoglobulins, which combine *in vitro* and *in vivo* with insulin, which means that the injection of the insulin preparations causes formation of insulin antibodies. Such formation was generally considered undesired for a number of reasons:

1. The production of insulin anibodies indicates a state of continuous antagonism of the body against the essential hormone, insulin.
2. This state was likely to be detrimental to the body cells, e.g. to the β-cells, which are destroyed in rabbits by immunization with insulin and adjuvant. It was suspected the the late diabetic complications such as angiopathy may become worse.
3. It was found that a high level of insulin antibodies sometimes leads to insulin resistance, i.e. a high dose requirement and a lack of metabolic control.
4. It was estimated that the actual dose requirement of the diabetic population exceeds the requirement that would be necessary, could the antibody formation be avoided.
5. The antibodies in some patients bind beef insulin more strongly than pork insulin, and hypoglycemic episodes were reported after a change in the species composition of the insulin preparations.
6. When the insulin antibodies become established in the patients there is a concomitant rise in the concentration of circulating exogenous serum insulin. Some of the serum insulin will be bound to the antibodies. The concentrations of the total and even of the free insulin may reach levels that are 10 to 100 times the normal physiological concentration. These high levels may be harmful to the tissues. These may promote atherosclerosis and they may damage the still existing β-cells. It was shown that high concentrations of insulin might have an inhibitory effect on the β-cell secretion.

The mechanism behind the development of the insulin antibodies and their properties have been studied with increasing concern during the last 5 to 10 years.

It was found that the species of insulin appeared to be the dominant factor in the development of the antibodies. Beef insulin which differs more (3 aminoacid positions) from the human insulin than does pork insulin (1 aminoacid position) is, as can be expected, more antigenic than pork insulin. On the other hand, pork insulin causes the formation of antibodies against pork insulin even in pigs. No explanation has yet been found for the latter observation though it has been suggested tentatively that the abnormally high concentrations of pork insulin at the site of injection may arouse the immunological apparatus in the pig to produce antibodies against its own insulin.

The undesired formation of insulin antibodies referred to above was up to the conception of the present invention considered inevitable for the reason that it was considered a fact that pure insulin was an antigen. This was supported by Deckert in his thesis "Insulin Antibodies" (Copenhagen 1964), comprising fractionation and isolation of some of the impurities found in crystalline porcine insulin and biological and chemical investigations showing that the impurities give rise to the formation of antibodies which are different from insulin antibodies but that the formation of insulin antibodies might be facilitated when the insulin preparations are impure, vide pages 90 and 195.

The present invention is based on two novel and surprising observations breaking principally with the present knowledge in the art.

The first observation is that in immunological experiments carried out in animals insulin did not cause formation of insulin antibodies when the insulin was free of pancreatic proteins with a molecular weight above 6000 and showed essentially a single component when analysed by polyacrylamide gel electrophoresis (DISC PAGE).

The second observation is that the impurities isolated from crystalline insulin, viz. the components (a) and (b) referred to above and to some extent the insulin-like substances in component (c) are in principle responsible for the antigenic behavour of the known insulin preparations.

Rabbits were chosen for the immunologic experiments because they show the same type of serum antibody response as humans when treated with the known insulin preparations, i.e. similar characteristics of the antibody with respect to insulin binding capacity and affinity to insulin.

The rabbits were injected three times weekly with a constant dose of protein to be tested, 40 micro grams dissolved in 1 millilitre of a 0.04 M sodium phosphate buffer at pH 7.4. The proteins were component (a), fraction 3 of component (b), dimer, proinsulin, arginine insulin and monodesamido insulin, all derived from beef insulin. In addition thereto the rabbits were also injected three times weekly with corresponding solutions of recrystallized beef insulin and beef insulin free of component (a) and (b) and of arginine insulin and mainly free of monodesamido insulin, thus representing substantially pure insulin. The dose was about 0.5 international units per kilogram. The serum antibody levels were examined at regular intervals by conventional techniques. The level is expressed as the percentage of added ^{125}I-insulin (beef) bound to the antibodies in the sample. Some of the results are shown in Figure 2. Not shown in the Figure is the finding that the arginine insulin of component (c) produced a small but significant amount of antibody after 45 days, while it is questionable whether monodesamido insulin is antigenic.

The first two of the following four results are most surprising and contrary to expectations:—

1. The insulin preparation containing the pure insulin only, does not produce antibodies to insulin.
2. The component (a) which is not insulin causes an enormous production of antibody to insulin. The most likely explanation is that it contains something which is chemically related to insulin in some unknown way.
3. The conventional insulin of high purity, i.e. recrystallized insulin, raises antibodies as it does in humans.
4. Substances belonging to component (b), viz. No. 3 (see Figure 1), the dimer and the proinsulin, are antigenic, giving rise to the production of antibodies to insulin.

In the course of the immunological experiment there was a tendency in some of the rabbits to show elevated blood sugar values, viz. the rabbits injected with component (b) and especially those which received one of the fractions of component (b), namely the dimer. The dimer did not produce the highest level of antibodies, but, for unknown reasons, the blood sugar values in these animals were the highest. This effect of the component (b) and the dimer may be called diabetogenic or anti-insulin-like, when, in fact, it appears that the animals have come resistant to insulin as demonstrated in Figure 3.

After 14 weeks of treatment with the dimer (beef, 40 μg three times weekly) the rabbits were fasted for 16 hours and then given intravenous insulin (0.5 international units per kilogram). The control group of rabbits had been treated with the preparation containing pure insulin. It appears from the serum-glucose curves shown in Figure 3 that the control rabbits reacted to insulin in the expected way, whereas the rabbits treated with the dimer from component (b) were resistant to the insulin. The resistance may explain the elevation of the blood sugar values, but has not yet been explained itself. It appears to be similar to the resistance that develops in some patients in the course of treatment with the known insulin preparations. It is expected that the clinical resistance can be avoided by using insulin preparations free of component (b).

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Similar results have been obtained with the components of porcine insulin.

The surprising results of the experiments reported above show the importance of using insulin which is not only free of pancreatic proteins having a molecular weight above 6000 but also free of such insulin-like sub-

stances as arginine insulin and preferably also monodesamido insulin, in the preparation of injectable insulin preparations for clinical use.

For analytical purposes use has previously been made of discontinuous polyacrylamide gel electrophoresis (DISC PAGE), also in connection with solutions of crystalline insulin. This specific gel electrophoresis was developed by B. J. Davis and L. Ornstein and is described in Ann. N. Y. Acad. Sci., 121, pages 321—439 and 404—427 (1964).

When the insulin just referred to is subjected to DISC PAGE and shows a single component it can be considered pure insulin. When the pure insulin is accompanied by minor amounts of monodesamido insulin, the insulin is considered substantially pure.

In accordance with the above explanations the novel insulin preparations according to the invention are characterized by containing insulin recovered from pancreas glands (natural insulin) and having the purity referred to, said insulin being dissolved or suspended in amorphous and/or crystalline form in an injectable aqueous medium. Said medium shows preferably a pH of approximately 7. At this pH-value the formation of monodesamido insulin is minimized during storage of the preparations.

The insulin to be used in the novel insulin preparations according to the invention may be produced by subjecting commercial insulin to gel filtration followed by ion exchange chromatography and collection of the fractions corresponding to the central major part of the insulin peak (the largest peak) and recovery of the insulin contained in the pooled fractions. By gel filtration the proteins of pancreatic origin having a molecular weight above about 6000 are removed and the arginine insulin and partly or completely the monodesamido insulin are removed by ion exchange chromatography. It is known to subject crystalline insulin to gel filtration, vide for instant J. Clin. Invest., Vol. 42, No. 12, 1869—1871 (1963) and Biochem. 2, 461—464 (1963), but the gel filtration was not followed by ion exchange chromatography.

A better and simpler purification process is to subject recrystallized insulin to column chromatography on a perferably strongly basic anion exchanger, using as eluent a water-containing, water-miscible organic solvent, collecting the fractions corresponding to the central major part of the insulin peak and recovering the insulin contained in the pooled fractions.

For the purpose of recovering insulin it is known to subject acid or neutralized alcoholic extracts from pancreas to column chromatography on a cation exchanger, such as carboxymethyl cellulose (British Patent Specification No. 1106076), sulphonated resins synthesized from the telomer of styrene and divinyl benzene (British Patent Specification No. 1054523) or on an anion exchanger such as diethylaminoethyl cellulose (British Patent Specification No. 931954). However the insulin obtained by these processes contains impurities as does the crystalline insulin obtained by the conventional recovery processes including precipitation several times by salting out of the crude insulin solutions with sodium chloride.

It is also known from British Patent Specification No. 881855 to subject unspecified zinc insulin to column chromatography on the cation exchanger "Dowex 50—X1" ("Dowex" is a Registered Trade Mark), which is a sulphonated styrene-divinylbenzene copolymer in bead form produced by The Dow Chemical Company, Midland, U.S.A., using aqueous ethanol as eluent, but even when recrystallized insulin is used as starting maerial, the recovered insulin will show mainly the same impurities as does the starting material.

Furthermore it is known from British Patent Specification No. 913042 to purify crystalline insulin mixed with amorphous or microcrystalline foreign materials by washing the impure crystalline with an aqueous liquid of which the pH is held at from 3.8 to 4.3 with the aid of a weak acid or a buffer mixture. However, this purification step does not remove such impurities as proinsulin, the dimer or the intermediate.

Fnially, it is known from British Patent Specification No. 871341 to subject 6 times crystallized insulin and insulin purified as described by Lens (Biochem. et Biophys. Acta (1948), 2, 76) to column chromatography on a styrene-divinyl benzene copolymer cation exchanger deposited on an inert extender of large superficial area, but the insulin will after this chromatography still contain impurities such as proinsulin, the dimer and the intermediate together with arginine insulin and monodesamido insulin since the presence of these impurities will not prevent the chromatogram from looking as if the material eluted from the column were essentially homogeneous.

As mentioned in the foregoing the preferred process of making of the insulin to be used in the preparations according to the invention is to subject recrystallized insulin to column chromatography on a preferably strongly basic anion exchanger and to elute with a water-containing, water-miscible organic solvent. Suitable anion exchangers are well-known in the art. An example thereof is DOWEX 1x2 which is a copolymer of styrene and 2% divinyl benzene, carrying benzyltrimethylammonium groups as functional groups, being in bead form, and produced by the The Dow Chemical Company of Midland, U.S.A. Another example is QAE-Sephadex A-25 which is dextran cross-linked with epichlorohydrin, to give an ex-

clusive limit of penetration of about 5000 (minimum molecular weight of excluded substances), carrying as functional groups diethyl - (2 - hydroxy - propyl) - amino - ethyl groups, being in bead form (diameter 40 to 120 μ) and produced by PHARMACIA, Uppsala, Sweden. Examples of preferred eluents are methanol, ethanol and propanols, which can be used in a concentration of 30 to 80 percent (v/v), preferably 50 to 70 percent (v/v). The pH-value should preferably be kept within the range of 6 to 9.

The following example illustrates how the insulin to be used in the preparations according to the invention may be produced:

EXAMPLE A

A buffer of the following composition is prepared:

1.25 kilograms of tris(hydroxymethyl)-aminoethane
725 millilitres of 12.3 N HCl
62.4 litres of 96 percent (v/v) ethanol
Water to make a total volume of 100 litres
The pH is 7.3 at 25°C.

1.3 kilograms of the anion exchanger QAE-Sephadex A-25 are swollen in the buffer and the finest particles are removed by decantation. This material is used for packing of a column with a diameter of 15 centimeters and a height of 25 centimeters. The column is equilibrated with the buffer.

18 grams of recrystallized pork or beef insulin containing about 0.4 percent Zn are dissolved in a mixture of 0.72 grams of ethylene diamine tetraacetic acid disodium salt, 10.2 grams of tris(hydroxymethyl)aminoethane and 720 millilitres of 60 percent (v/v) ethanol. The insoluble material is removed by centrifugation. 10.7 millilitres of 6 N HCl are added to the clear solution which is then applicated on the column. The elution is carried out with the buffer at a temperature of 25°C and at a rate of 1.2 litres per hour. Fractions of 0.5 litres are collected.

The extinctions at 276 nm (E_{276}) are measured and plotted against the volumes of eluate. The fractions corresponding to the central major part of the insulin peak (the largest peak) are pooled, and the insulin is precipitated from the pool by adding one pool volume of 0.02 M zinc acetate. The precipitate is isolated by centrifugation and crystallized from an acetone-containing citrate buffer, in a known manner.

The yield is 8.2 grams of pure insulin.

The insulin is recrystallized from a buffer of the following composition:

2.0 percent of insulin
0.8 percent of Zn^{++} (as chloride) calculated on the weight of insulin
0.1 M sodium acetate
7.0 percent of sodium chloride
HCl to pH 5.45 to 5.55.

The insulin is dissolved in water containing HCl and the $ZnCl_2$, whereupon a solution of the proper quantities of sodium acetate and sodium chloride in water is added. The crystallization is carried out at room temperature with mechanical stirring. The crystallization is completed within one or two days. The insulin is isolated by filtration, washed with water and dried in vacuo. The yield is 8.0 grams.

EXAMPLE B

A buffer of the following composition is prepared:

0.1 M NH_4Cl
0.02 M NH_3
60 per cent (v/v) ethanol
The pH is 8.3 at 25°C.

15 grams of Dowex 1x2 (mesh: 50/100 are swollen in the buffer and the finest particles are removed by decantation. The material is used for the packing of a column with a diameter of 1.6 cm and a height of 25 c. The column is equilibrated with the buffer.

500 mg of insulin crystallized once from a citrate buffer are dissolved in a mixture of 20 mg of ethylene diamine tetra acetic acid disodium salt, 5 ml of the buffer, 5 ml of 60 per cent (v/v) ethanol and 0.04 ml of 13.3 M NH_3 (final pH 8.5). The insoluble material is removed by centrifugation and the clear solution is applicated on the column. The elution is carried out with the buffer at a temperature of 25°C and a rate of 7.5 ml/h. Fractions of 5 ml are collected.

The extinctions at 276 nm are measured and plotted against the fraction numbers. The fractions corresponding to the central major part of the insulin peak (the largest peak) are pooled and the insulin precipitated by adding 100 ml of water, 1.3 ml of 1 N HCl and 2 ml of 1 M zinc acetate per 100 ml of the pool. The precipitate is isolated by centrifugation and crystallized from an acetone-containing citrate buffer, in a known manner. The yield of pure insulin is 200 mg.

EXAMPLE C

A buffer of the following composition is prepared:

0.06 M tris(hydroxymethyl)aminoethane
0.02 N HCl
0.075 M NaCl
60 per cent (v/v) ethanol
The pH is 8.3 at 25°C.

80 grams of QAE-Sephadex A-25 are swollen in the buffer and the finest particles

are removed by decantation. The material is used for the packing of a column with a diameter of 2.5 cm and a height of 50 cm. The column is equilibrated with the buffer.

2.5 grams of insulin crystallized once from a citrate buffer are dissolved at 0°C in a mixture of 450 mg of tris(hydroxymethyl)-aminomethane, 100 mg of ethylene diamine tetra acetic acid tetrasodium salt, 25 ml of buffer, 25 ml of 60 per cent (v/v) ethanol and 0.08 ml of 4 N HCl (final pH at 25°C: 8.4). The insoluble material is removed by centrifugation at 2 to 4°C and the clear supernatant is applicated on the column. The elution is carried out with the buffer at a temperature of 4°C and at a rate of 26 ml/h. Fractions of 20 ml are collected.

The extinctions at 276 nm are measured and plotted against the fraction numbers. The fractions corresponding to the central major part of the insulin peak (the largest peak) are pooled and the insulin precipitated by adding one pool volume of 0.02 M zinc acetate, 0.03 N HCl. The precipitate is isolated by centrifugation and crystallized from an acetone-containing citrate buffer, in a known manner.

The yield is 1.3 grams of pure insulin.

The following examples illustrate how to make the insulin preparations according to the invention, use being made of the insulin crystals mentioned above being free of proteins of pancreatic origin having a molecular weight above 6000 showing essentially a single component when solutions thereof are subjected to DISC PAGE.

Example 1

Neutral Insulin Injection 40 i.u./ml

160 mg of pork insulin crystals produced as described in Example C are dissolved in 20 ml of 0.01 N hydorchloric acid. The solution is sterilised by filtration, and the filtrate mixed aseptically with 60 ml of a sterile aqueous solution containing 0.1 g of methyl - p - hydroxybenzoate. Finally, 20 ml of a sterile solution containing 136 mg of sodium acetate, 0.7 g of sodium chloride, and 10 ml of sodium hydoxride are added. The final preparation has a pH of about 7.3. It is filled aseptically in sterilised vials.

Example 2

Biphasic Insulin Injection 40 i.u./ml

1.6 grams of beef insulin crystals produced as described in Example A are dissolved in 75 ml of 0.04 N hydrochloric acid containing zinc chloride equivalent to 1.1 mg of zinc. The solution is sterilised by filtration, and the filtrate mixed aseptically with constant stirring with 25 ml of a sterile solution containing 1.36 grams of sodium acetate, 7 grams of sodium chloride and sufficient sodium hydroxide to produce a pH of 5.4 to 5.5 in the mixture. Stirring is continued for about twenty hours or until the insulin precipitated is converted into rhombohedral crystals. The suspension is then added aseptically to 800 ml of a sterile aqueous solution containing 1 gram of methyl - p - hydroxybenzoate and the mixture diluted with 100 ml of a solution of sufficient sodium hydroxide to produce a preparation having a pH of about 7. To this preparation is added a sterile neutral solution of insulin containing 1.6 mg of pure insulin per ml until a quarter of the insulin is present in soluble form. The suspension is filled aseptically in sterilised containers.

The sterile solution of pure insulin is prepared by the following method: 0.4 gram of pure insulin (pork) prepared according to Example A is dissolved in 25 ml of 0.02 N hydrochloric acid containing zinc chloride equivalent to 0.25 mg of zinc. The solution is sterilised by filtration and the filtrate diluted aseptically with 200 ml of a sterile aqueous solution containing 0.25 gram of methyl - p - hydroxybenzoate and 25 ml of a sterile solution containing 0.34 grams of sodium acetate, 1.75 grams of sodium chloride and sufficient sodium hydroxide to produce a pH of about 7 in the final solution.

Example 3

Insulin Zinc Suspension 40 i.u./ml

First the crystalline fraction of the preparation is prepared by the following method: 1.6 grams of pork insulin crystals prepared according to Example B are dissolved in 75 ml of 0.02 N hydrochloric acid containing zinc chloride equivalent to 10 mg of zinc. The solution is sterilised by filtration and the filtrate mixed aseptically while stirring with 25 ml of a sterile solution containing 13.6 grams of sodium acetate, 7 grams of sodium chloride, and sufficient sodium hydroxide to produce a pH of 5.4 to 5.5 in the mixture. Stirring is continued for about twenty hours or until the pure insulin precipiated is converted into rhombohedral crystals. The suspension is then added aseptically with continuous stirring to 900 ml of a sterile solution containing zinc chloride equivalent to 70 mg of zinc, 1 gram of methyl - p - hydroxybenzoate, and sufficient sodium hydroxide to produce a pH of about 7.3 in the final crystalline fraction.

The amorphous fraction is prepared by the following method: 1.6 grams of pure insulin prepared according to Example A are dissolved in 100 ml of 0.02 N hydrochloric acid containing zinc chloride equivalent to 10 mg of zinc, the solution is sterilised by filtration and the filtrate diluted aseptically with 800 ml of a sterile solution containing zinc chloride equivalent to 70 mg of zinc and 1 gram of methyl - p - hydroxybenzoate. 100 ml of a sterile solution containing 1.36 grams of sodium acetate, 7 grams of sodium chloride and sufficient sodium hydroxide to produce

a pH of about 7.3 in the final amorphous fraction are then added aseptically with continuous stirring.

The insulin zinc suspension is prepared by mixing aseptically 7 volume of the crystalline suspension and 3 volumes of the amorphous suspension. The mixture is filled aseptically in sterilsed vials.

WHAT WE CLAIM IS:—

1. An injectable insulin preparation containing insulin recovered from pancreas glands and being dissolved or suspended in an injectable aqueous medium, said insulin being free of proteins of pancreatic origin with a molecular weight above 6000 and said insulin showing essentially a single component when a solution thereof is analysed by polyacrylamide gel electrophosesis (DISC PAGE).

2. An injectable insulin preparation as claimed in claim 1, in which the injectable aqueous medium has a pH of approximately 7.

3. An insulin preparation as claimed in claim 1 or claim 2 whenever produced by a process described in any one of Examples 1 to 3.

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Printed for Her Majesty's Stationery Office, by the Courier Press, Leamington Spa, 1972.
Published by The Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.

PATENT RULES 1968

SPECIFICATION NO 1285023

The following amendment was made under Rule 94(3) on 11 June 1975

Page 1, Heading *delete* all reference to entry No (72) *insert*

The inventor of this invention in the sense of being the actual Deviser thereof within the meaning of Section 16 of the Patents Act 1949 is:-

Jorgen Schlichtkrull a Danish subject of 28B, Ved Bellahoj, 2700 Bronshoj Denmark

THE PATENT OFFICE
12 November 1976

Bas 32311/1

PATENTS ACT 1949

SPECIFICATION NO 1285023
SLIP NO 2

The following corrections were allowed under Section 76 on 30 September 1976:—

Page 2, line 56, *after* able *delete* did appear *insert* disappeared.

THE PATENT OFFICE
17 December 1976

Bas 32404/1

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2. An injectable insulin preparation as claimed in claim 1, in which the injectable aqueous medium has a pH of approximately 7.

3. An insulin preparation as claimed in claim 1 or claim 2 whenever produced by a process described in any one of Examples 1 to 3.

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Printed for Her Majesty's Stationery Office, by the Courier Press, Leamington Spa, 1972.
Published by The Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.

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THE PATENT OFFICE
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Bas 32404/1

* C L M S *