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PROCESS FOR CRYSTALLIZATION OF INSULIN USING FREEZE DRIED INSULIN AS SEEDING MATERIAL

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Injectable insulin preparations are known which have a protracted effect exclusively or mainly based on the presence of insulin crystals in aqueous suspension, and it is also known that the protracted effect of such aqueous insulin crystal suspensions is to a certain degree dependent upon the size of the suspended insulin crystals, vide Science, 116, 394–398, 1952, and J. Am. Med. Assoc. 150, 1667–1671, 1952.

In the hitherto known processes of making crystalline insulin nothing has been done to regulate the size of the produced crystals. Besides, there would have been no purpose in undertaking regulation of crystal size because the crystals themselves have not heretofore been used as constituents of insulin preparations for clinical use.

It is only upon the appearance of insulin preparations of practical clinical utility based on aqueous insulin crystal suspensions that there arises the problem of obtaining crystals of substantially the same size or a crystal mass in which the main part by weight consists of crystals of a size within certain predetermined limits.

The present invention aims at providing a solution of the above mentioned problem. Thus, one of the objects of the invention is to cause the insulin to crystallize during the crystallization process in the form of crystals of substantially the same size. A further object of the invention is to produce insulin crystals suitable for use as seed material for the production of larger crystals of substantially the same size.

The invention is based on the observation that the presence of freeze-dried insulin during the crystallization process influences the course of the crystallization as regards the size of the produced crystals and the quantity of crystals of substantially the same size.

The crystallization of insulin is well known and very often described in the insulin literature, vide for instance Biochem. J., 28, 1592–1602, and l. c., 29, 1048–1054. Though the various crystallization methods may differ somewhat they are, however, based on the same principle, i. e. to cause the insulin to crystallize from an aqueous medium by changing the pH-value of the medium to in the neighborhood of the isoelectric point of the insulin, viz, between 5 and 7.

Crystallization requires the presence of a crystallization-promoting metal (zinc, cobalt, nickel, cadmium, copper, manganese or iron), and of these metals zinc is employed in most cases, e. g. as zinc chloride. If the insulin itself does not contain such metal in a sufficient amount, the aqueous crystallization medium must be given the necessary content thereof. The minimum satisfactory amount of crystallization-promoting metal is with regard to zinc about 0.4% to 0.52% of the weight of the insulin. In commercial crystallization practice usually 2 to 5 times the minimum amount is employed, vide for instance the specification of U. S. Patent No. 2,143,590.

The crystallization pH-value ordinarily lies between 5.4 and 6.5, and in order to establish this value use is generally made of a buffer substance or mixtures of buffer substances. Examples of such buffer substances are the well-known acetate buffer, borate buffer, citrate buffer, phosphate buffer, di-ethylbarbiturate buffer and maleate buffer.

It is most common to produce an acid aqueous insulin solution with the necessary metal content, and if desired, a buffer substance, and to adjust this solution to the crystallization pH, but it is also possible to precipitate the insulin amorphously in an aqueous medium without the necessary metal content, and then to transform the amorphous insulin into crystalline form by adding the necessary amount of metal, for instance in the form of an aqueous solution of a salt. Finally, it is possible to approach the crystallization pH from the basic side of the isoelectric point by using basic insulin solutions.

The present invention relates to a process of the above mentioned kind, and, as above indicated, the characteristic feature of the invention is that crystallization takes place in the presence of freeze-dried insulin.

The principles of freeze-drying are well known and have been applied to various drugs, so that it will be obvious to experts within the insulin field how to freeze-dry insulin, vide for example Earl W. Flosdorf: Freeze-Drying, New York, 1949.

According to one embodiment of the invention the freeze-dried insulin is suspended in an aqueous medium suitable as insulin crystallization medium, whereafter an aqueous insulin solution is added and crystallization is effected. According to another embodiment of the invention the freeze-dried insulin is added to the insulin-containing crystallization medium after it has been adjusted to the pH-value for crystallization, but before the formation of the crystals has commenced. According to still another embodiment, the insulin-containing crystallization medium is produced by mixing an acid aqueous insulin solution and a basic solution containing, if desired, a crystallization-promoting metal and buffer substance to obtain the pH-value for crystallization, and adding the freeze-dried insulin to the basic solution before the mixing process.

A pH of 5 to 7 may be employed to effect crystallization of the insulin but according to the present invention preferably a pH of 6.2 to 6.5 is employed.

It is preferred to use crystalline insulin or insulin of a similar purity as starting material for the preparation of the freeze-dried insulin employed in accordance with the invention, and it is also preferred that the insulin is recovered from beef-pancreas glands. For instance, crystalline insulin dissolved in a dilute acid or a dilute base may be freeze-dried, the solution having a pH-value of, for example 3 and 7.5, respectively, or one may freeze-dry an insulin solution having a composition corresponding to that of the crystallization medium to which the freeze-dried insulin is to be added later-on. It is also possible to freeze-dry a solution of amorphous insulin (free of metals).

Usually clear solutions are freeze-dried, but it is not disadvantageous for a part of the insulin to be present in precipitated amorphous form before freeze-drying.

It has been found that the quantity of freeze-dried insulin added to the crystallization medium influences the course of the crystallization in such manner that under the same circumstances the crystals will be smaller the greater the quantity of freeze-dried insulin added. Therefore, it is expedient to add the freeze-dried insulin in an amount by weight which is a predetermined fraction of the amount by weight of the insulin to be crystallized. The freeze-dried insulin should be used in such an amount that the produced insulin crystals appear in form of in-

dividual crystal bodies of substantially the same size. The required amount of freeze-dried insulin will vary depending upon its composition and the crystallization conditions, e. g. the employed pH, the employed buffer, and the insulin concentration. The size of the insulin crystals is understood as the size in μ of the longest diagonal of the crystals.

The crystallization medium need not contain insulin beforehand, the desired insulin concentration in the crystallization medium being provided by the addition of the freeze-dried insulin.

The concentration of the insulin in the crystallization medium is not critical, but it is preferred to use the conventional concentration, i. e. between ¼% and 4% by weight of the medium, preferably between ½% and 2%.

Although the produced crystals or crystal suspensions may be used in insulin therapy they are, however, preferably used as seed material for the production of larger insulin crystals of uniform size. Their utility for this purpose is not only due to the fact that the crystals possess seed properties, but also that it is possible, when using the process according to the invention, to obtain crystals which are completely separated from each other and appear in the form of individual (free) crystal bodies of substantially the same size. It is preferred to give the crystals a size within the range of about 1μ to about 7μ.

Below is described more fully with reference to various specific examples how the freeze-dried insulin may be produced and how the crystallization process may be carried out by the addition of the freeze-dried insulin. In the following examples the crystalline insulin used as starting material contains about 0.4% of zinc based on the weight of the insulin although it will be understood that crystalline insulin containing up to 0.83 milliequivalent of the crystallization-promoting metals per gram of the crystals may be employed.

Example 1

500 mgs. of crystalline insulin from beef-pancreas glands are dissolved in 50 millilitres of water containing 4 milliliters of 0.1 N hydrochloric acid, and the resulting solution is freeze-dried in the usual way, for instance by immerging a glass flask containing the insulin solution in a freezing medium such as a mixture of ethanol and Dry Ice, to effect complete freezing of the solution, connecting the flask with a vacuum source to create an absolute pressure of about 0.05 mm. of Hg or less, and maintaining the vacuum over night without applying external heating.

The freeze-dried insulin is then added to an aqueous crystallization medium adjusted to pH 6.5 by means of NaOH and containing:

50 mgs. of citric acid (as sodium citrate) per 100 milliliters,
2 mgs. of Zn (as zinc chloride) per 100 milliliters,
½% of insulin,
0.1% of methyl-p-oxybenzoate,

in an amount corresponding to 20% of the quantity of insulin in the aqueous crystallization medium. The insulin crystallizes in the form of crystals which are uniform in size and form and have a size of about 5μ.

Example 2

500 mgs. of crystalline insulin are dissolved in 50 millilitres of water containing 4.3 millilitres of 0.1 N hydrochloric acid. This solution is mixed with 50 millilitres of a buffer solution containing:

50 mgs. of citric acid,
10 millilitres of 0.1 N sodium hydroxide,
2 mgs. of Zn (as zinc chloride),
0.16% of methyl-p-oxybenzoate,

and the pH-value of the mixture is adjusted to 6.3 which

makes the solution turbid due to precipitation of amorphous insulin. The turbid mixture thus produced is freeze-dried in the usual way under the same pressure as in Example 1.

When the freeze-dried insulin is added to the same crystallization medium as in Example 1 and in the same amount the insulin will crystallize in the form of crystals having a size of 5 to 7μ.

Example 3

The procedure is the same as in Example 2, with the exception that the insulin solution which is to be freeze-dried is adjusted to pH 6.6 instead of to 6.3, whereby the solution remains clear as no amorphous insulin is precipitated.

The product of crystallization is insulin crystals of a size of about 2μ.

Example 4

The same procedure is followed as in Example 2, with the exception that the insulin solution which is to be freeze-dried is adjusted to pH 7.0. In this case the product of the crystallization will be insulin crystals of a size of about 2μ.

Example 5

The procedure is as in Example 2 with the exception that the insulin solution which is to be freeze-dried is adjusted to pH 7.5. In this case the product of crystallization is crystals of a size of 1 to 1.5μ.

Example 6

500 milligrams of highly purified amorphous insulin free of crystallization-promoting metals are dissolved in 50 millilitres of water containing 4.3 millilitres of 0.1 N hydrochloric acid. The solution thus produced is mixed with 50 millilitres of an aqueous solution containing 50 mgs. of citric acid, 10 millilitres of 0.1 N sodium hydroxide and 0.16% of methyl-p-oxybenzoate, and the pH-value of the mixture is adjusted to 6.7. The mixture thus produced is freeze-dried in the same manner as in Example 1.

When the freeze-dried insulin is added to the same crystallization medium as in Example 1 and in the same amount the insulin crystallizes in the form of crystals 5μ in size.

Example 7

500 mgs. of crystalline insulin are dissolved in 50 millilitres of water containing 4.3 millilitres of 0.1 N hydrochloric acid. The solution thus produced is mixed with 50 millilitres of a solution containing 50 mgs. of citric acid, 2 mgs. of zinc (as zinc chloride), 10 millilitres of 0.1 N sodium hydroxide and 0.16% of methyl-p-oxybenzoate. The pH value of the mixture is adjusted to about 5.0, whereafter 100 mgs. of insulin freeze-dried as in Example 3 are added and the mixture is agitated. The insulin crystallizes in the form of crystals of 2.5μ size, which are, however, inclined to adhere to each other, which make them less appropriate for use as seed crystals.

Example 8

The procedure of Example 7 is followed with the exception that crystallization takes place at pH 5.5 instead of 5.0. The crystals thus produced will have a size of about 2μ and will adhere less to each other than those produced in Example 7.

Example 9

The procedure of Example 7 is followed with the exception that crystallization takes place at pH 6.0, by which procedure crystals of 2μ size which are completely separated from each other are obtained.

Example 10

The procedure of Example 7 is followed, except that crystallization takes place at pH 6.3. The crystals pro-

duced have the same characteristics and size as those obtained in Example 9.

Example 11

The procedure is the same as in Example 7, except that crystallization takes place at pH 7.0. By this procedure insulin crystals of a size of 1 to 2μ will be obtained, but only a part of the insulin will crystallizze due to the relatively high solubility of the insulin under the crystallization conditions.

Example 12

500 mgs. of crystalline insulin are dissolved in 50 millilitres of water containing 4.3 millilitres of 0.1 N hydrochloric acid, and the solution thus produced is mixed with 50 millilitres of an aqueous solution containing 178 mgs. of $Na_2HPO_4.2H_2O$, 2 mgs. of zinc (as zinc chloride), 3.8 millilitres of 0.1 N hydrochloric acid and 0.16% of methyl-p-oxybenzoate. The pH-value of the mixture is adjusted to 6.3, whereafter 100 mgs. of freeze-dried insulin produced as described in Example 3 are added, and the mixture is agitated until crystallization is complete. Insulin crystals of a size of about 2μ are obtained.

Example 13

500 mgs. of crystalline insulin are dissolved in 50 millilitres of water containing 4.3 millilitres of 0.1 N hydrochloric acid, and the solution is mixed with 50 millilitres of an equeous solution containing 136 mgs. of $CH_3COONa.3H_2O$, 2 mgs. of zinc (as zinc chloride), 4.5 millilitres of 0.1 N sodium hydroxide, and 0.16% of methyl-p-oxybenzoate. The pH-value of the mixture is adjusted to about 6.3, whereafter 100 mgs. of freeze-dried insulin prepared as described in Example 3 are added, and the mixture is agitated. The insulin crystals thus produced have a size of about 2μ.

Example 14

To 50 millilitres of an aqueous solution containing 50 milligrams of citric acid, 4.5 mgs. of nickel (as nickel chloride), 10 millilitres of 0.1 N sodium hydroxide and 0.16% of methyl-p-oxybenzoate, there are added 50 millilitres of an insulin solution having the same composition as the insulin solution which in Example 6 is subjected to freeze-drying, whereafter 100 mgs. of insulin freeze-dried as described in Example 3 are added immediately. The pH-value of the mixture is adjusted to about 6.2. Upon crystallization, insulin crystals having a size of about 2μ are obtained.

Example 15

To 100 millilitres of an aqueous solution containing 50 mgs. of citric acid, 2 mgs. of zinc (as zinc chloride) and 0.08% of methyl-p-oxybenzoate, and adjusted to pH 6.5 by means of sodium hydroxide, are added while stirring 600 mgs. of insulin which has been freeze-dried as described in Example 3. After stirring for 5 to 10 hours the added insulin has crystallized in the form of crystals having a size of about 2μ.

Example 16

To 50 millilitres of an aqueous solution containing 50 mgs. of citric acid, 2 mgs. of zinc (as zinc chloride), 10 millilitres of 0.1 N sodium hydroxide and 0.16% of methyl-p-oxybenzoate, there are added while stirring 100 mgs. of insulin freeze-dried as described in Example 3, and immediately thereafter there are added 500 mgs. of crystalline insulin dissolved in 50 millilitres of water containing 4.3 millilitres of 0.1 N hydrochloric acid. The pH-value of the mixture is then adjusted to about 6.0. Crystallization occurs while the mixture is stirred and the resulting insulin crystals have a size of about 2μ.

Example 17

To 50 millilitres of an aqueous solution containing 50 mgs. of citric acid, 2 mgs. of zinc (as zinc chloride) and about 9 millilitres of 0.1 N sodium hydroxide (to provide a pH of about 11.8) are added 100 mgs. of freeze-dried insulin produced as described in Example 3.

The freeze-dried insulin appears to go into solution, and after a short period of time up to one hour, preferably a few minutes, there are added 50 millilitres of an insulin solution containing 500 mgs. of crystalline insulin, 4.3 millilitres of 0.1 N hydrochloric acid and 0.16% of methyl-p-oxybenzoate and the pH is adjusted to about 6.3. After the course of 5 to 10 hours crystallization is complete. The size of the crystals is about 2 to 3μ.

While the freeze-dried insulin may be added to an insulin-containing crystallization medium in the form of a dry substance, vide Examples 1 to 15, or in the form of an aqueous suspension, vide Example 16, the freeze-dried insulin may also be added to an insulin-containing solution in partly dissolved or apparently dissolved form, vide Example 17, provided the apparently dissolved freeze-dried insulin has only been stored a relatively short time, so that the freeze-dried insulin is still able to act as seeding material during the insulin crystallization. In view thereof the expression "in the presence of freeze-dried insulin" as used in the claims intends to cover the use of freeze-dried insulin in solid form, in aqueous suspension and/or in apparently dissolved form.

The process of the invention also includes the modification which comprises adding to an aqueous suspension of amorphous insulin freeze-dried insulin together with a sufficient amount of one or more crystallization-promoting metals to effect crystallization. Aqueous suspensions of amorphous insulin able to crystallize always contain some dissolved insulin which presumably is able to initiate the desired crystallization in the presence of the freeze-dried insulin.

If the insulin crystals produced according to the above examples are to be used as seed material for industrial production of injectable insulin crystal suspensions containing insulin crystals of uniform size, it will be appropriate to ensure that no change of the seed crystals in the suspension medium takes place during storage. For this purpose a quantity of a crystallization-promoting metal may be added to the suspension medium of the seed crystals such that the suspension is stable at about pH 7, whereafter the mixture is adjusted to this pH-value. Thus, each of the suspensions of insulin crystals produced according to the examples may be diluted in the ratio: 1:1 with an aqueous solution containing 50 mgs. of Zn (as zinc chloride) per 100 millilitres and 0.1% of methyl-p-oxybenzoate while adding sufficient sodium hydroxide to obtain a pH-value of 7 to 7.5. By this procedure the seed crystal suspension is stabilized so that the crystals do not grow together and form twins or bigger conglomerates.

A stabilization of the seed crystal suspension may also be obtained by freezing it down, for instance by means of a mixture of ethanol and Dry Ice or another freezing medium, if desired after dilution with an equal volume of water, and storing the frozen suspension e. g. at −10° C.

Finally, it should be noted that in the practical industrial application of the process the crystallization is usually carried out under sterile conditions so that sterile crystal suspensions are obtained either for direct therapeutical use or for employment in making sterile suspensions of larger insulin crystals for direct therapeutical use.

Having thus fully described our invention we claim as new and desire to secure by Letters Patent:

1. In a process of producing insulin crystals from an aqueous insulin-containing medium by adjusting the pH of said medium to a value between 5 and 7, said medium containing at least one metal selected from the group consisting of zinc, cobalt, nickel, cadmium, copper, manganese and iron in bivalent form, the step which comprises adding freeze-dried insulin to said medium be-

fore the crystals begin to be formed, whereby the insulin crystals appear in the form of individual crystal bodies of substantially the same size.

2. In a process of producing insulin crystals from an aqueous medium containing insulin in non-crystalline form and at least enough of a metal selected from the group consisting of zinc, cobalt, nickel, cadmium, copper, manganese and iron in bivalent form to equal 0.5% of the dry weight of the insulin, by adjusting the pH of said medium to a value between 5 and 7, the step of adding freeze-dried insulin to said medium before the crystals begin to be formed, whereby the insulin crystals appear in the form of individual crystal bodies of substantially the same size.

3. A process of crystallizing insulin from an aqueous medium containing said insulin in solution and also containing at least one metal selected from the group consisting of zinc, cobalt, nickel, cadmium, copper, manganese and iron in bivalent form in an amount of at least 0.5% of the dry weight of the insulin, which comprises changing the pH of the medium to a value approximating the isoelectric point of the insulin, and, before the crystals begin to be formed, adding freeze-dried insulin to said medium, whereby the insulin crystals appear in the form of individual crystal bodies of substantially the same size.

4. A process of crystallizing insulin which comprises establishing an acidic aqueous medium containing the insulin to be crystallized in solution, said medium having a pH below 5 and containing at least one metal selected from the group consisting of zinc, cobalt, nickel, cadmium, copper, manganese and iron in bivalent form in an amount of at least 0.5% of the dry weight of the insulin, establishing an alkaline aqueous medium containing freeze-dried insulin, and mixing the two media in amounts to produce a third medium having a pH between 5 and 7, whereby the insulin crystals appear in the form of individual crystal bodies of substantially the same size.

5. In a process of producing insulin crystals wherein the insulin is crystallized from an aqueous medium containing said insulin and adjusted to a pH of 5.4 to 6.5 and containing at least one metal selected from the group consisting of zinc, cobalt, nickel and cadmium in a total amount of at least 0.5% of the dry weight of the insulin, the step which comprises adding freeze-dried insulin to the said medium before the crystals begin to be formed, whereby insulin crystals appear in the form of individual crystal bodies having substantially the same size, the amount of said freeze-dried insulin added being a predetermined fraction of the amount by weight of the insulin in the medium to provide crystals of a size not exceeding about 7μ.

6. A process of crystallizing insulin which comprises establishing an aqueous insulin-containing medium, freeze-drying said medium to produce freeze-dried insulin, establishing an aqueous medium containing insulin in non-crystalline form and at least one metal selected from the group consisting of zinc, cobalt, nickel and cadmium in a total amount of at least 0.5% of the dry weight of the insulin, adjusting the pH of the said medium to a value between 5 and 7, and adding said freeze-dried insulin to said medium before the insulin crystals begin to be formed, whereby the crystals appear in the form of individual crystal bodies of substantially the same size.

7. A process of crystallizing insulin which comprises establishing two aqueous solutions of crystalline insulin, freeze-drying one of said solutions to produce freeze-dried insulin, adding said freeze-dried insulin to the other solution and adjusting the pH of the mixture to a value between 5 and 7, whereby the insulin crystallizes in the form of individual crystal bodies of substantially the same size.

8. A process of producing insulin crystals in the form of individual crystal bodies of substantially the same size, which comprises adding freeze-dried insulin to an aqueous medium containing at least one metal selected from the group consisting of zinc, cobalt, nickel, cadmium, copper, manganese and iron in bivalent form in an amount of at least 0.5% of the dry weight of the insulin added and adjusting the pH of the mixture to a value between 5 and 7.

9. A sterile aqueous suspension of insulin crystals in the form of individual crystal bodies of substantially the same size produced by adjusting to a value between 5 and 7 the pH of an aqueous insulin containing medium also containing at least one metal selected from the group consisting of zinc, cobalt, nickel, cadmium, copper, manganese and iron in bivalent form, and adding freeze-dried insulin to said medium before the crystals begin to be formed.

References Cited in the file of this patent

UNITED STATES PATENTS

2,143,590	Scott	Jan. 10, 1939
2,648,622	Waugh	Aug. 11, 1953

OTHER REFERENCES

Haurowitz: Chem. and Biol. of Proteins, 1950, Academic Press, NYC., pp. 6 and 7.

Greenberg: Amino Acids and Proteins, 1951, Chas. Thomas, Springfield, Ill., pp. 286 and 287.