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PROCESS OF PRODUCING INSULIN CRYSTALS OF SUBSTANTIALLY UNIFORM SIZE AND COMPOSITIONS THEREOF

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Injectable insulin preparations are known which have a protracted effect which is exclusively or mainly based on the presence of insulin crystals in aqueous suspension, vide for instance Science, 116, 394–398, 1952, and J. Am. Med. Assoc., 150, 1667–1671, 1952. Like other injectable insulin preparations, these preparations are marketed in ampoules having a closure entirely or partly consisting of rubber so that the cannula of an injection syringe can pierce through the rubber in order to take up the desired dose in the syringe. Ordinarily, an ampoule contains the aqueous insulin crystal suspension in an amount sufficient for several doses.

Whenever a dose is to be removed from the ampoule it is necessary first to shake the ampoule so that the settled insulin crystals are dispersed in order to form a suspension in which the insulin crystals are uniformly distributed. If it has not been ascertained that the insulin crystals are uniformly distributed in the aqueous suspension medium before the suspension is taken up into the injection syringe, each dose will not contain the same amount of insulin.

As the administration of insulin is usually carried out by the patients themselves it is important that a uniform suspension is obtainable without difficulties by a single shaking of an ampoule so that one is absolutely sure that each dose contains the same amount of insulin.

If the aqueous crystal suspension contains insulin crystals which, due to their size, are difficult to keep suspended, this security will not be present. Therefore it is of importance that the crystal suspension does not contain particularly big crystals, or, in other words, that the largest insulin crystals have a size which does not exceed a predetermined size which would make it impossible to be sure that, after the patient's shaking of the ampoule and until the desired dose has been taken up by the injection syringe, the same insulin concentration will be present throughout the suspension, and that no crystals will be present on the bottom of the ampoule.

According to the hitherto known processes of making crystalline insulin nothing has been done to adjust the size of the produced crystals. Such an adjustment would, besides, have had no pharmaceutical purpose because in the past insulin crystals have not been used as constituents of insulin preparations for clinical use.

The crystallization of insulin is well known, vide for instance Biochem. J. 28, 1592–1602, and l. c. 29, 1048–1054, and is generally carried out by causing the insulin to crystallize from an aqueous medium by changing the pH-value of the medium to a value in the neighborhood of the isoelectric point of the insulin. If the insulin in itself does not contain beforehand sufficient amounts 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) the crystallization medium is given the necessary metal content, which with regard to zinc is 0.4–0.52% of the weight of the insulin, 2 to 5 times this amount being, however, usually employed in practice, vide for instance the specification of U. S. Patent No. 2,143,590. Moreover, use is generally made of buffer substances to fix the pH-value during crystallization. Examples of such buffer substances are the well-known acetate buffer, borate buffer, citrate buffer, phosphate buffer, di-ethylbarbiturate buffer and maleate buffer. The crystalline insulin thus produced contains from 20 to 27 international units per mg. depending upon the purity of the crystals.

One of the purposes of the present invention is to provide a process of producing insulin crystals having a size which does not exceed a predetermined size, so that directly in the crystallization process a crystal charge will be obtained which may be used as such for clinical purposes and which need not be subjected to a fractionating sedimentation to separate too large crystals.

The process according to the invention belongs to the art in which the insulin is caused to crystallize from an aqueous medium having a pH-value between 5 and 7 in the presence of a crystallization-promoting metal, and the characteristic feature of the invention is that the crystallization takes place while seeding with insulin crystals in a predetermined manner.

By means of a seeding process in accordance with the invention it is possible to produce insulin crystals having a size which does not exceed a predetermined size, depending upon the quantity of seed crystals employed, the size of the seed crystals, and the insulin concentration during crystallization.

In the following description the size of the insulin crystals is to be understood as the length in μ of the longest diagonal of the crystal.

In order to determine the seed quantity when the produced insulin crystals are not to exceed a predetermined definite size, the seed crystals are used in an amount corresponding to the following equation:

$$P = I \times \frac{d_p^3}{d_i^3 - d_p^3}$$

where P indicates the quantity in grams of seed crystals to be used, I the weight in grams of insulin to be crystallized, i. e. the insulin in the crystallization medium, d_p the largest size of the seed crystals and d_i the desired upper limit of the size of the insulin crystals to be produced.

If, for instance, it is desired that the insulin crystals do not exceed about 30μ, and it is desired to crystallize 100 grams of insulin, the seed crystals must be employed in an amount of about 30 mgs. if the seed crystals have a size of about 2μ and less, and in an amount of about 240 mgs. if the seed crystals have a size of about 4μ and less.

With injectable insulin preparations, the protracted effect of which is based on the presence of insulin crystals in suspension it is, moreover, important that the suspended insulin crystals have mainly the same size, that is to say that the main part of the insulin present has the form of crystals which do not or only slightly differ from each other in size. This will secure a constant and completely reproducible clinical effect.

A further purpose of the present invention consists in providing such conditions during the crystallization that the main part of the insulin will crystallize as crystals the size of which is in the neighborhood of the desired upper limit.

This may be obtained by preventing, as far as possible, the formation of insulin crystals due to spontaneous crystallization and by using seed crystals of mainly the same size. In this way it is possible to obtain the result that the main part of the insulin of a crystal charge will

be present in mainly the same crystal size. Under these circumstances d_p will indicate approximately the size of the seed crystals in μ and d_i the crystal size in μ in which the main part by weight of the insulin will crystallize. When it is desired, for example, that the main part of the insulin crystals shall have a size of approximately 30μ it will be possible according to the invention by suppression of spontaneous crystallization to obtain 80 to 90% of the insulin in the form of 28 to 36μ crystals.

The spontaneous crystallization may be suppressed by making the difference between d_i and d_p small. This would, however, require a series of crystallizations in order to arrive at the desired value of d_i which is undesired in practice.

On industrial scale, it is desired to obtain the required value of d_i in one crystallization step which involves that the difference between d_i and d_p is relatively large. In such cases the spontaneous crystallization may, according to the invention, be suppressed by using a buffer substance, the anion of which does not bind the crystallization-promoting metal at neutrality. Examples of such buffer substances are borate, diethylbarbiturate, maleate and acetate buffers, among which the last mentioned buffer is preferred, when the produced crystal suspensions are to be used directly in the production of injectable insulin preparations.

Use may also be made of the process disclosed in Danish Patent No. 78,069, vide also the copending application U. S. Serial No. 362,142, filed June 16, 1953. According to this process crystallization takes place in the presence of halogen (chlorine, bromine and iodine) in ionogenic form in a concentration above 0.2 mole per litre. It is preferable to provide this halogen ion concentration by adding a sufficient quantity of a halogen salt of an alkali metal, ammonia or one of the alkaline earth metals or a mixture of such salts. In order to obtain maximal crystal crops it is not expedient to use higher halogen ion concentrations than about 1 mole per litre.

If crystallization is carried out in accordance with the above mentioned process it is not necessary to use buffer substances the anion of which does not bind the crystallization-promoting metal at neutrality. Other buffers, such as citrate buffer, may also be used. Finally, it should be mentioned that vigorous agitation during the crystallization promotes spontaneous crystallization.

In carrying out the process according to the present invention the insulin-containing aqueous crystallization medium may be produced in known manner. It is most common to produce an acid aqueous insulin solution with the necessary content of one or more crystallization-promoting metals (zinc, cobalt, nickel, cadmium, copper, manganese and iron, among which, as above mentioned, use is almost always made of zinc), and, if desired, buffer substances, and to adjust the solution to the pH of crystallization, but it is also possible to precipitate the insulin amorphously in an aqueous medium without the necessary metal content and then to cause the insulin to change into crystalline form by subsequent addition of the necessary amount of metal, for instance in the form of an aqueous solution of a metal salt. Finally, it is also possible to approach the pH of the crystallization from the basic side by using basic insulin solutions.

The minimum satisfactory amount of the crystallization-promoting metal zinc is 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. The concentration of insulin in the crystallization is generally ¼% to 4%, preferably ½% to 2% by weight of the crystallization medium.

The seed crystals are added either in the form of an aqueous suspension, for instance suspended in the aqueous medium in which the seed crystals are produced, or in dry e. g. freeze-dried form. If the insulin is present in precipitated amorphous form without the necessary metal content in the crystallization medium, the seed crystals may be added together with the missing metal content, for example the aqueous suspension of the seed crystals may in itself have the necessary content.

As mentioned, crystallization takes place at a pH-value between 5 and 7, and within this range it is most advantageous to use a value of 5.3 to 6.5 depending on the composition of the crystallization medium.

If the difference between d_i and d_p is small, the amount of seed crystals in grams will be relatively large. By the way of example, $d_i=30\mu$ and $d_p=20\mu$ will make it necessary to use an amount in grams of seed crystals being about 40% of the amount of insulin to be crystallized. As it is inappropriate for economical and other reasons to use such big amounts of seed crystals, it is preferred that d_p is considerably smaller than d_i, so that the amount of seed crystals is only a small fraction of the amount of insulin to be crystallized. If, thus $d_i=30\mu$ and $d_p=10\mu$ the required amount of seed crystals will be only about 4% of the amount of insulin to be crystallized.

It is preferred to use small seed crystals for example of a size of about 1μ, but larger seed crystals may also be used in dependence of the size of the final insulin crystals. When it is desired to produce insulin crystals having a size of about 30μ good results will be obtained by using seed crystals having a size of about 1 to about 7μ, preferably about 1 to about 4μ.

The seed crystals may be produced by subjecting the insulin crystal crop obtained by the conventional crystallization methods, to a fractionating sedimentation and selecting the fraction containing crystals of the desired size. However, the seed crystals are advantageously produced as disclosed in our U. S. patent application Serial No. 468,542, filed November 12, 1954, based on our Danish patent application No. 3691/53, filed November 13, 1953. According to the method disclosed in these patent applications suspensions of small insulin crystals are produced, which crystals are separated from each other and form individual (free) crystal bodies having seeding properties by letting the insulin crystallize from an aqueous medium in the presence of freeze-dried insulin produced in known manner, e. g. as described in Earl W. Flosdorf: Freeze-Drying, New York, 1949. Otherwise, the crystallization process is carried out in known manner in the presence of a crystallization-promoting metal, preferably zinc. The insulin crystals produced in accordance with the method disclosed in the patent applications referred to are of substantially uniform size, preferably from about 1 to about 7μ.

To further illustrate the process according to the invention reference is made to the following examples:

Example 1

500 mgs. of crystalline insulin from ox pancreas are dissolved in 50 millilitres of water containing 0.6 millilitre of 1 N HCl. Then 50 millilitres of a buffer solution are added containing 7 grams of NaCl and 10 millilitres of an aqueous solution containing 10% of citric acid, 0.4% of zinc (as zinc chloride) and sufficient NaOH to give the solution a pH-value of about 6.3. When necessary, the pH is adjusted to about 6.0. Before spontaneous crystallization begins, 2 millilitres of a seed crystal suspension are added, said suspension having the following composition: 0.03% of insulin crystals of a size of 3μ and less, 2.5 mgs. per 100 millilitres of zinc (as zinc chloride), 2.5 mgs. per 100 millilitres of citric acid, 0.08% of methyl-p-oxybenzoate and NaOH to provide a pH of 7.2 to 7.4. After moderate stirring for 10 to 20 hours crystallization is complete, and the crystals have a size of about 4 to 25μ. Without the seeding process crystals having a size varying from about 5 to 50μ would be obtained. Thus, the seeding process has prevented formation of insulin crystals between 25 and 50μ.

Example 2

500 mgs. of amorphous insulin from pig pancreas are

dissolved in 50 millilitres of water containing 6 millilitres of 0.1 N HCl, 15 millilitres of acetone are added and then 10 millilitres of a buffer solution containing 10% of citric acid, 0.4% of Zn (as zinc chloride) and sufficient NaOH to give the buffer solution a pH-value of about 6.3. Then water is added to give a total volume of 100 millilitres. The pH is adjusted to about 6.2 with HCl. Before spontaneous crystallization begins 2 millilitres of a suspension of seed crystals (2 to 3μ) are added, said suspension having the same composition as in Example 1. After cautious stirring for 10 to 20 hours crystallization is complete, and the main part of the insulin has crystallized in the form of crystals having a size of 20 to 25μ.

Example 3

1.12 grams of crystalline insulin from ox pancreas are dissolved in 50 millilitres of water containing 10 mgs. of Zn (as zinc chloride) and 2 millilitres of 1 N HCl. Then 50 millilitres of a solution are added which contains 7 grams of NaCl and 1.7 millilitres of 1 N NaOH. Immediately thereafter are added 2.5 millilitres of a seed crystal suspension having the same composition as in Example 1. The pH is adjusted to 5.4 to 5.6. After stirring for 15 to 20 hours crystallization is complete. The size of the crystals is less than 30μ, and the main part of the insulin is present as crystals of about this size.

Example 4

1.6 grams of crystalline insulin from ox pancreas are dissolved in 50 millilitres of water containing 10 mgs. of Zn (as zinc chloride) and 2 millilitres of 1 N HCl. Then 50 millilitres of a buffer solution are added which contains 1.36 grams of $CH_3COONa.3H_2O$, 7 grams of NaCl and 1 millilitre of 1 N NaOH. Immediately thereafter 3.5 millilitres of a seed crystal suspension as described in Example 2 are added. The pH is adjusted to 5.4 to 5.6. After stirring for 15 to 20 hours crystallization is complete, and the main part of the insulin has crystallized in the form of crystals of a size of about 30μ.

Example 5

1.12 grams of crystalline insulin from ox pancreas are dissolved in 50 millilitres of water containing 10 mgs. of Zn (as zinc chloride) and 2 millilitres of 1 N HCl. Then 50 millilitres of an aqueous solution are added which contains 1.36 grams of $CH_3COONa.3H_2O$, 11.9 grams of KBr and 1 millilitre of 1 N NaOH. Immediately thereafter are added 2.5 millilitres of a seed crystal suspension as described in Example 2. The pH is adjusted to from 5.4 to 5.6. After stirring for 15 to 20 hours crystallization is complete. The main part of the insulin has crystallized in the form of crystals of a size of about 30μ.

Example 6

1.6 grams of crystalline insulin are dissolved in 500 millilitres of water containing 100 mgs. of Zn (as zinc chloride) and 20 millilitres of 1 N HCl. Then 500 millilitres of a buffer solution are added which contains 13.6 grams of $CH_3COONa.3H_2O$, 70 grams of NaCl and 10 millilitres of 1 N NaOH. Then 1.8 millilitres of a seed crystal suspension as described in Example 2 are added. The pH is adjusted to 5.4 to 5.6. After stirring for 15 to 20 hours crystallization is complete. The main part of the insulin has crystallized in the form of crystals of a size of about 40μ.

Example 7

3.2 grams of crystalline insulin are dissolved in 50 millilitres of an aqueous solution containing 20 mgs. of Zn (as the zinc chloride) and 3 millilitres of 1 N HCl. Then 50 millilitres of an aqueous solution are added which contains 1.36 grams of $CH_3COONa.3H_2O$, 7 grams of NaCl and 2 millilitres of 1 N NaOH. Then 5 millilitres of a seed crystal suspension are added which is, as regards all constituents, 10 times as concentrated as that referred to in Example 2. After the course of about 20 hours crystallization is complete. The main part of the insulin has crystallized in the form of crystals of a size of about 15μ.

Example 8

Crystallization of insulin is carried out as described in Example 4, but instead of adding 3.5 millilitres of a suspension of seed crystals an equivalent amount in the form of a freeze-dried powder is added. By this procedure the same crystal size will be obtained as is obtained in Example 4.

The process according to the invention may be employed in connection with the processes for producing injectable insulin preparations with protracted effect referred to in copending application U. S. Serial No. 219,127 and also described in Belgian Patent No. 502,327. In this case the process according to the invention is carried out so as to obtain sterile suspensions having a predetermined insulin content per unit volume, preferably 20, 40 or 80 units per millilitre, a sufficient content of isotonics and a preserving agent, and, moreover, having a content of one or more crystallization-promoting metals above the lower limit referred to in application, Serial No. 219,127, viz. at least $11 \times A \times 10^{-3}$ milliequivalents per litre, "A" denoting the number of international units of insulin per millilitre of the suspension. Finally, as crystallization buffer use should not be made of substances the anions of which bind the crystallization-promoting metals at neutrality. The injectable insulin preparation obtained by this method comprises an aqueous medium having insulin crystals suspended therein, said medium and said crystals containing in non-toxic amounts at least one metal selected from the group consisting of zinc, cobalt, nickel, cadmium, copper, manganese and iron, the metal content of the suspended crystals being at least 0.25 milliequivalent per gram of crystals when said preparation has a pH of about 7, and the main part by weight of said crystals having substantially uniform size.

Among the metals referred to above zinc is the preferred metal, and the suspended insulin crystals contain preferably about 1 to about 2.7% of zinc when the preparation has a pH of about 7.

The following example illustrates how the process according to the invention may be carried out:

Example 9

1.6 grams of crystalline insulin are dissolved in 50 millilitres of water containing 10 mgs. of zinc (as zinc chloride) and 2 millilitres of 1 N HCl. The solution is sterile-filtrated, and the filtered material is washed with 25 millilitres of water. To the 75 millilitres of filtrate are added 25 millilitres of a buffer solution prepared under aseptic conditions and containing 1.36 grams of

$CH_3COONa.3H_2O$

7 grams of NaCl and 1 millilitre of 1 N NaOH. Then 3.5 millilitres of a seed crystal suspension prepared under aseptic conditions and having the same composition as in Example 2 are added. The pH is adjusted to 5.5. After stirring for 15 to 20 hours crystallization is complete. The main part of the insulin has crystallized in the form of crystals of a size of about 30μ.

The crystal suspension is transferred into an injectable preparation, ready for use, by diluting it with 900 millilitres of a solution, prepared under aseptic conditions and containing 70 mgs. of zinc (as zinc chloride), 0.11% methyl-p-oxybenzoate and 2.7 millilitres of 1 N NaOH. The pH should be about 7.3 and the suspension adjusted to this value by means of 0.1 N NaOH or HCl.

The final preparation contains 40 international units of insulin per millilitre, and the suspended insulin crystals contain about 2.6% of zinc.

By the process according to the invention it is possible to produce insulin crystals of substantially uniform size within a broad range. However, as the main object of

the invention is to produce insulin crystals particularly suitable as constituents of therapeutical preparations comprising aqueous insulin crystal suspensions, and as this involves that the crystals should not settle so quickly that the insulin doses taken with interval from an ampoule after shaking do not contain the prescribed amount of insulin per millilitre due to sedimentation of some of the crystals, it is inconvenient to make the crystals bigger than about 40μ. On the other hand it seems that very small insulin crystals result in a decreased protracted effect. It is, therefore, not appropriate to use crystals smaller than about 10μ. In view thereof it is preferred that the main part by weight of the insulin crystals has a substantially uniform size within the range of 10μ to 40μ when the crystals are to be used in injectable aqueous insulin crystal suspensions.

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 of predetermined substantially uniform size from an insulin-containing aqueous medium having a pH-value between 5 and 7 in the presence of a crystallization promoting metal, the step which comprises seeding said medium with insulin crystals of substantially uniform size in an amount corresponding to the equation:

$$P=I\times\frac{d_p^3}{d_i^3-d_p^3}$$

wherein P is the quantity in grams of the seed crystals added, I is the amount in grams of insulin to be crystallized, d_p is the predetermined substantially uniform size in μ of the seed crystals and d_i is the size in μ in which the major proportion by weight of the insulin crystallizes from the medium.

2. In a process of producing insulin crystals of predetermined substantially uniform size from an insulin-containing aqueous medium having a pH-value between 5 and 7 in the presence of a crystallization-promoting metal, the step which comprises seeding said medium with insulin crystals of substantially uniform size not exceeding 10μ in an amount corresponding to the equation:

$$P=I\times\frac{d_p^3}{d_i^3-d_p^3}$$

wherein P is the quantity in grams of the seed crystals added, I is the amount in grams of insulin to be crystallized, d_p is the size in μ of the seed crystals and d_i is the predetermined substantially uniform size in μ in which the major proportion of the insulin crystals crystallizes from the medium, d_i lying within the range of about 10μ to about 40μ.

3. A process of producing insulin crystals of predetermined substantially uniform size which comprises establishing an aqueous medium containing insulin dissolved therein and containing a halogen ion selected from the group consisting of chloride, bromide and iodide in a concentration of more than 0.2 mole per litre, and adjusting the pH of said medium to a value between 5 and 7 while seeding said medium with insulin crystals in an amount corresponding to the equation:

$$P=I\times\frac{d_p^3}{d_i^3-d_p^3}$$

wherein P is the quantity in grams of the seed crystals added, I is the amount in grams of insulin to be crystallized, d_p is the size in μ of the seed crystals and d_i is the predetermined substantially uniform size in μ in which the major proportion of the insulin crystals crystallizes from the medium.

4. An injectable insulin preparation comprising an aqueous medium having insulin crystals suspended therein, said medium and said crystals containing in non-toxic amounts at least one metal selected from the group consisting of zinc, cobalt, nickel, cadmium, copper, manganese and iron, the metal content of the suspended

crystals being at least 0.25 milliequivalent per gram of crystals when said preparation has a pH of about 7, and the major proportion by weight of said crystals having a substantially uniform size, said crystals being formed by seeding an insulin-containing aqueous medium having a pH value between 5 and 7 and containing a crystallization-promoting metal with insulin crystals of substantially uniform size in an amount corresponding to the equation:

$$P=I\times\frac{d_p^3}{d_i^3-d_p^3}$$

wherein P is the quantity in grams of the seed crystals added, I is the amount in grams of insulin to be crystallized, d_p is the size in μ of the seed crystals and d_i is the substantially uniform size in μ in which the major portion by weight of the insulin crystallizes from said last-named medium.

5. An injectable insulin preparation comprising an aqueous medium having insulin crystals suspended therein, said crystals and said medium containing zinc in non-toxic amounts, the zinc content of the suspended crystals being about 1 to about 2.7% by weight when said preparation has a pH of about 7, and the major portion by weight of said crystals having a substantially uniform size, said crystals being formed by seeding an insulin-containing aqueous medium having a pH value between 5 and 7 and containing a crystallization-promoting metal with insulin crystals of substantially uniform size in an amount corresponding to the equation:

$$P=I\times\frac{d_p^3}{d_i^3-d_p^3}$$

wherein P is the quantity in grams of the seed crystals added, I is the amount in grams of insulin to be crystallized, d_p is the size in μ of the seed crystals and d_i is the substantially uniform size in μ in which the major portion by weight of the insulin crystallizes from said last-named medium.

6. An injectable insulin preparation as defined in claim 4, wherein the major proportion by weight of the insulin crystals has substantially the same size within the range of 10μ to 40μ, said crystals being formed by seeding an insulin-containing aqueous medium having a pH value between 5 and 7 and containing a crystallization-promoting metal with insulin crystals of substantially uniform size in an amount corresponding to the equation:

$$P=I\times\frac{d_p^3}{d_i^3-d_p^3}$$

wherein P is the quantity in grams of the seed crystals added, I is the amount in grams of insulin to be crystallized, d_p is the size in μ of the seed crystals and d_i is the substantially uniform size in μ in which the major portion by weight of the insulin crystallizes from said last-named medium.

7. A process of producing an aqueous suspension of insulin crystals of predetermined substantially uniform size, which comprises dissolving under aseptic conditions crystalline insulin in an aqueous acidified, zinc-containing medium having a pH-value below 5, adding thereto a sterile buffer solution containing sodium acetate and sodium chloride, the sodium chloride being added in an amount such that the chlorine ion concentration is more than 0.2 mole per liter, adjusting the pH of the mixture to a value of 5.3 to 6.5, and adding to said mixture, before crystallization starts and under aseptic conditions insulin crystals of substantially uniform size not exceeding 10μ and in an amount corresponding to the equation:

$$P=I\times\frac{d_p^3}{d_i^3-d_p^3}$$

wherein P is the quantity in grams of the seed crystals added, I is the amount in grams of insulin to be crystallized, d_p is the size in μ of the seed crystals and d_i is the predetermined substantially uniform size in μ in which

the major portion proportion of the insulin crystallizes from the medium d_p not exceeding 10μ and d_i being about 30μ.

8. A process as defined in claim 1, wherein said insulin is derived from pork pancreas glands.

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