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THE EFFECTS OF FREEZING ON COMMERCIAL INSULIN SUSPENSIONS

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SUMMARY

The effects of a single freezing and also the rate of thawing on the physical, chemical and biological parameters of several types of commercially available insulin suspensions were examined. The studies were made on injections of protamine zinc insulin, isophane insulin, insulin zinc suspension, both amorphous and crystalline, and included physical studies (particle size measurement, examination of crystal shape and sedimentation), biological studies described in the British Pharmacopoeia (1973) (mouse convulsion assay, insulin in solution test and prolongation of insulin effect in rabbits) and studies of chemical properties (electrophoretic mobility, pH and immunoreactivity).

The rate of sedimentation of each insulin suspension was at least three times faster after freezing than before, although the rate of thawing of the frozen insulin preparations had no marked effect on the sedimentation rate. Microscopic examination and particle size distribution studies showed that freezing and thawing produced clumping of insulin particles and some crystal damage. However, no loss of bioactivity was found with any of the insulin suspensions after they had been subjected to a single freezing and thawing. Similarly, no differences were found between the unfrozen and frozen-thawed insulin preparations in their immunoreactivity, electrophoretic mobility or pH.

INTRODUCTION

The 1973 British Pharmacopoeia (BP 1973) directs that insulin preparations should be stored between 2 and 10°C and should not be allowed to freeze. Mathews (1967) noted that insulin crystals in suspension are liable to increase in size if the suspension is frozen. He also commented that the biological potency was unaffected and that suspensions settled more rapidly after being stored frozen. Preliminary studies in this laboratory on frozen and thawed insulin suspensions showed crystal damage which suggested that the prolonged hypoglycemic action of previously frozen insulin suspensions may be impaired.

The present study examines the effects of a single freezing and the rate of thawing on physical, biological and chemical properties of commercially available insulin suspensions.

MATERIALS AND METHODS

The following insulin suspensions were obtained from commercial sources:
Protamine zinc insulin injection, 80 U/ml, 10 ml
Isophane insulin (N.P.H.) injection, 80 U/ml, 10 ml
Insulin zinc suspension (amorphous) injection, 80 U/ml, 10 ml
Insulin zinc suspension (crystalline) injection, 80 U/ml, 10 ml

Each insulin type was produced by a different manufacturer. Vials of each type were randomly divided into three treatment groups, A, B and C. Groups B and C were placed in a freezer at −17°C and each vial was separated from neighbouring vials to ensure equivalent storage conditions. After 45 h, vials in group B were thawed slowly at 21°C, while group C was thawed quickly in a water bath at 37°C. Groups B and C were then placed with group A, which was stored at 3–5°C.

Studies of physical properties

Sedimentation. The rate of sedimentation of insulin suspensions in sealed manufacturer's vials was measured. The product labels were removed and the vials were allowed to equilibrate to ambient temperature (18.5–22.0°C). Each vial was gently inverted 10 times to ensure all sediment was uniformly suspended and the vial was immediately placed on a horizontal surface in front of a vertical card divided into 100 horizontal lines, 1 mm apart. The initial reading was the height of the meniscus and subsequent readings measured the height of the falling sediment horizon.

Particle size distribution. At least 20 determinations of the sizes of the particles in each group were made on a HIAC 5 channel electronic particle counter after suitable dilution of the sample in particle-free water. The widths of the channels were:

Channel number	1	2	3	4	5
Channel width (μm)	5–10	10–20	20–40	40–60	60–120

Each determination was made on a separate 5.65 ml aliquot of the diluted sample and corrected for background counts. A quantitative error may have occurred since the counter was calibrated for spherical particles; however comparison between treatment groups for each insulin type was considered valid.

Microscopic examination. From each of three vials in each group a drop of suspension was examined microscopically. Four randomly selected fields were studied at X250 magnification and photographed.

Biological studies

The following procedures were used:
(1) Biological assay by the mouse convulsion assay of the BP 1973.
(2) Insulin in solution test of the BP 1973.
(3) Prolongation of insulin effect in rabbits by the method of the BP 1973.

Prior to bioassay each insulin suspension was acidified to dissolve the precipitate. In

the prolongation test each suspension was gently shaken before injection to ensure the uniform distribution of the precipitate.

Studies of chemical properties

Electrophoresis. Ten microlitres of insulin suspension from a vial in each of the three groups were spotted at 1.5 cm intervals along the midline of a strip of Whatman No. 3 filter paper (30 cm × 11.5 cm). After the spots had dried, the paper was placed in an electrophoresis unit and the paper wetted with buffer as described in the BP 1973. The composition of the buffer, pH 2, was: formic acid, 5 ml; acetic acid, 15 ml; distilled water to 100 ml. Electrophoresis was carried out at 300 V for 2 h, after which the paper was removed and dried at 110°C for 15 min, sprayed with ninhydrin reagent and redried at 110°C.

Immunoreactivity. A CIS insulin radioimmunoassay kit was used to obtain antibody binding curves concurrently for groups A, B and C of each insulin type by measuring the displacement of [^{125}I]insulin from guinea-pig insulin antiserum by the test insulin. The test insulin was initially dissolved and diluted in distilled water and final dilutions of 0.59–6.67 μU/0.1 ml were made in phosphate buffer.

RESULTS

Studies of physical properties

The contents of the vials which had been frozen were visibly more coarsely particulate than the vials which had not been frozen. This observation was confirmed by the particle size distribution study, the results of which are given in Table 1. The study demonstrated formation of larger particles and a reduction in the total number of particles in the frozen preparations compared to the normally stored preparation. For example, groups B and C (slowly thawed and quickly thawed, respectively) of protamine zinc insulin showed an approximately 5-fold increase in the proportion of particles of 20–40 μm while the proportion of 5–20 μm particles decreased by approximately one-quarter when compared to group A (unfrozen). However, the rate of thawing produced no marked differences between the particle size distributions of groups B and C for each insulin type.

The results of the sedimentation study are presented in Table 2. The sedimentation rate for each insulin suspension was markedly increased by freezing and treatment groups B and C settled more rapidly than group A. The greatest differences in the time for 50% sedimentation (i.e. the time required for the sediment horizon to reach 50% of the meniscus height) occurred in the suspensions with the smaller particle sizes. The time required for 50% sedimentation of the insulin zinc suspension (amorphous), which is composed of particles <2 μm, was reduced from more than 120 min in the unfrozen preparation to 8–10 min for the suspensions which had been frozen and thawed. The unfrozen protamine zinc insulin (rod-shaped particles up to 70 μm in length) gave a time for 50% sedimentation of 9 min, whereas the frozen and quickly and slowly thawed suspensions gave 50% sedimentation times of 1 and 3 min, respectively. The rate of thawing produced minor differences; for each insulin type, group B tended to settle more quickly and have a thinner final layer of sediment than group C.

TABLE 1

PARTICLE SIZE DISTRIBUTION FOR INSULIN SUSPENSIONS MEASURED ON A HIAC 5 CHANNEL ELECTRONIC PARTICLE COUNTER

Treatment groups A, B and C represent unfrozen, frozen and slowly thawed, and frozen and quickly thawed vials of insulin suspension, respectively.

Insulin type	Group	Dilution factor	Average number of particles [a]	% of total particles/channel				
				1	2	3	4	5 [b]
Protamine zinc insulin	A	1 in 500	35879	45	53	4	–	–
	B	1 in 500	17639	34	40	23	3	–
	C	1 in 500	20229	37	40	21	2	–
Isophane insulin	A	1 in 500	37172	83	17	–	–	–
	B	1 in 500	23538	53	34	12	1	–
	C	1 in 500	26252	53	35	11	1	
Insulin zinc suspension (amorphous)	A	1 in 500	particles <5 μm and not detectable					
	B	1 in 500	3423	32	43	24	1	–
	C	1 in 500	3676	27	40	31	2	–
Insulin zinc suspension (crystalline)	A	1 in 250	21006	33	54	13	–	–
	B	1 in 250	20870	25	54	20	1	–
	C	1 in 250	20919	26	54	20	–	–

[a] Average number of particles per 5.65 ml aliquot for at least 20 aliquots.

[b] Increase in channel number corresponds to an increase in particle size (see Methods).

TABLE 2

THE EFFECTS OF FREEZING AND RATE OF THAWING ON THE SEDIMENTATION OF INSULIN SUSPENSIONS

Treatment groups A, B and C represent unfrozen, frozen and slowly thawed and frozen and quickly thawed vials of insulin suspension, respectively. Each value is the mean result from 3 vials.

Insulin type	Group	Sediment height (%) [a]	Time for 50% sedimentation (min)
Protamine zinc insulin	A	2.2	9
	B	2.2	1
	C	2.7	3
Isophane insulin	A	28	18
	B	8.1	3
	C	9.9	4
Insulin zinc suspension (amorphous)	A	95	>120
	B	7.3	8
	C	9.2	10
Insulin zinc suspension (crystalline)	A	67	70
	B	1	2
	C	2.5	2

[a] The height of the sediment is expressed as a percentage of the height of the meniscus of the insulin suspension at 60 min after resuspension.

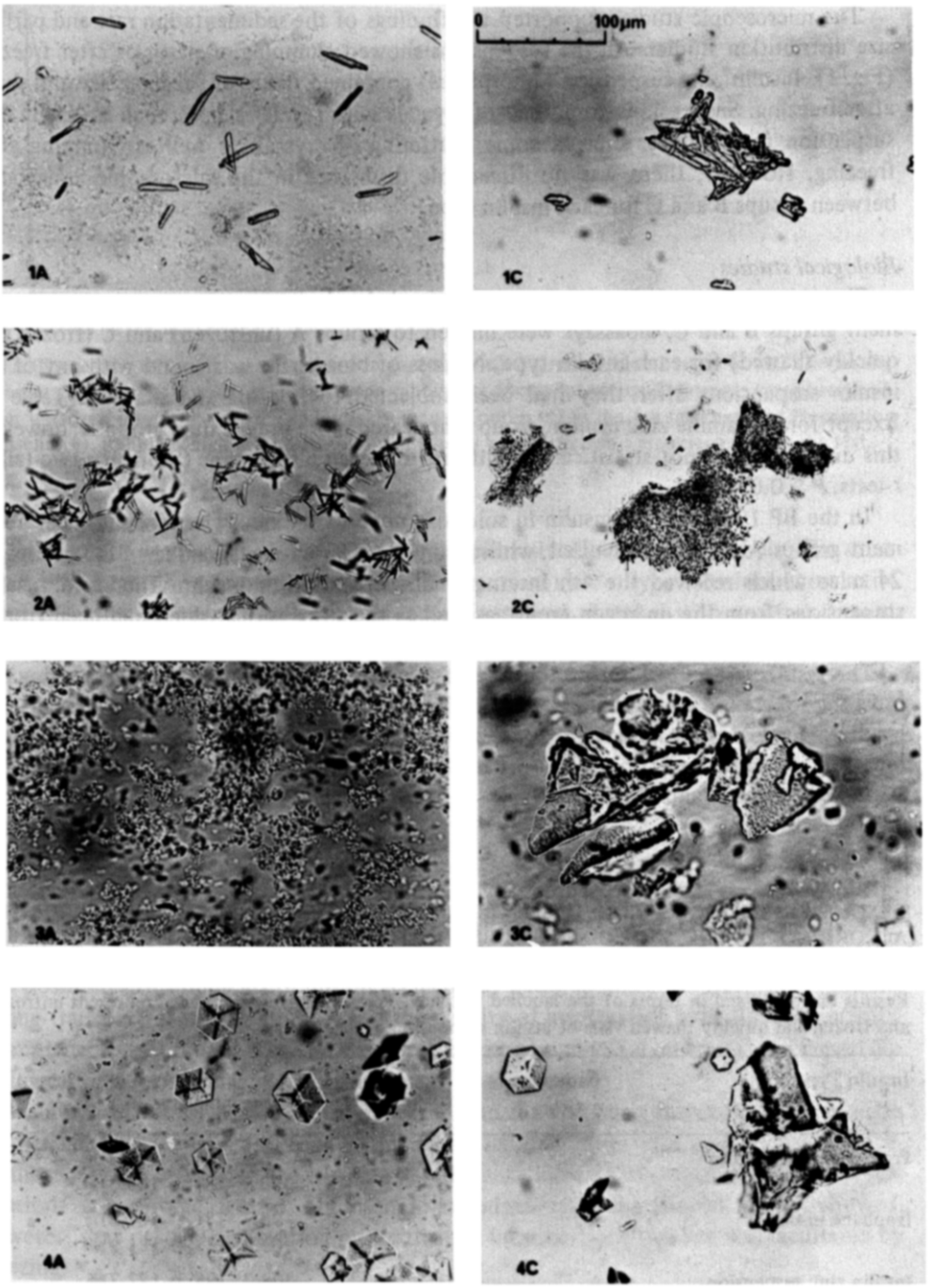

Fig. 1. Microscopic appearance at × 250 magnification of unfrozen (A) and frozen and quickly thawed (C) insulin suspensions. The insulin types are protamine zinc insulin (1), isophane insulin (2), insulin zinc suspension (amorphous) (3) and insulin zinc suspension (crystalline) (4). One scale division equals 20 µm.

The microscopic studies supported the findings of the sedimentation rate and particle size distribution studies. All the suspensions showed clumping of particles after freezing (Fig. 1). Insulin zinc suspension (amorphous) contained flakes of irregular size and shape after freezing. Suspensions composed of crystals with regular shapes, such as insulin zinc suspension (crystalline), showed some shattering of crystals as well as clumping after freezing. However, there was no discernible difference in the microscopic appearance between groups B and C for each insulin type.

Biological studies

Since the studies of physical properties revealed only minor differences between treatment groups B and C, bioassays were limited to groups A (unfrozen) and C (frozen and quickly thawed) for each insulin type. No loss of bioactivity was found with any of the insulin suspensions after they had been subjected to freezing and thawing (Table 3). Except for protamine zinc insulin, group C appeared more potent than group A; however, this difference was not statistically significant for each insulin type (Student's two-tailed t-tests, $P > 0.05$).

In the BP 1973 test for insulin in solution, none of the mice given insulin from treatment groups A, B or C convulsed, whilst from 7 to 15 mice responded in the groups of 24 mice which received the 4th International Preparation of Insulin. Therefore, insulin suspensions from the unfrozen group, as well as the suspensions which had been frozen and thawed, complied with the BP 1973 requirements for insulin in solution.

The results of the test for prolongation for groups A and C indicated that freezing did not affect the time course of the insulin effect in rabbits. Fig. 2 presents the results for insulin zinc suspension (amorphous) in which group C had an almost identical effect on rabbit blood sugar levels to group A. Each other insulin type gave similar hypoglycemic responses for the A and C groups.

TABLE 3

RESULTS OF THE MOUSE CONVULSION BIOASSAY OF INSULIN SUSPENSIONS PERFORMED ACCORDING TO THE METHOD DESCRIBED IN THE B.P. 1973, USING THE 4th INTERNATIONAL PREPARATION OF INSULIN AS THE REFERENCE STANDARD

Results are expressed in terms of the labelled potency. Treatment groups A and C represent unfrozen and frozen and quickly thawed vials of insulin suspension, respectively.

Insulin Type	Group	No. of assays	Combination results (95% fiducial limits)
Protamine zinc insulin	A	3	96.3 (85.0–109.3)%
	C	3	92.9 (81.8–105.6)%
Isophane insulin	A	2	93.7 (81.7–107.8)%
	C	3	103.2 (87.4–121.9)%
Insulin zinc suspension (amorphous)	A	3	108.2 (95.3–122.7)%
	C	4	120.8 (104.0–139.6)%
Insulin zinc suspension (crystalline)	A	3	97.6 (85.1–112.4)%
	C	3	108.3 (97.1–120.1)%

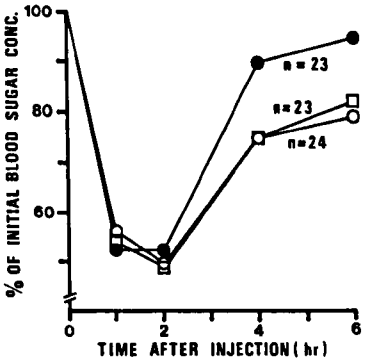

Fig. 2. The effect of insulin zinc suspension (amorphous) on rabbit blood sugar levels. The results represent the mean of 3 tests, each test using rabbits per treatment group. Treatment groups received either unfrozen insulin (○), frozen and quickly thawed insulin (□) or the 4th International Preparation of Insulin (●). Rabbits which convulsed following insulin administration were rejected from the test.

Studies of the chemical properties

A single spot was produced with paper electrophoresis of each insulin sample. The same distance of migration was obtained for each group of an insulin type. The immunoreactivity of the three groups for each type of insulin appeared identical with no displacement or change in shape of the insulin antibody binding curves, obtained by plotting % of radioactivity bound to the antibody versus the concentration of unlabelled insulin.

Groups A, B and C of insulin zinc suspension (crystalline and amorphous) complied with the zinc in solution specification of the BP 1973, and for each insulin type all groups complied with the relevant BP 1973 specification for pH.

DISCUSSION

Several studies have examined the stability of insulin preparations at elevated temperatures (see Pingel and Volund, 1972). However, there appear to be few published reports dealing with the effects of freezing on the biological properties of insulin suspensions. The possibility of shipments of insulin accidentally being subjected to freezing conditions during transport or during storage, and the paucity of information available on this topic prompted this examination of the effects of freezing on physical, chemical and especially biological properties of insulin suspensions.

Insulin suspensions which had been frozen and thawed once, showed marked changes in their physical properties, such as increased sedimentation rate and crystal damage. Mathews (1967) attributed the increase in sedimentation rate to crystal growth, after suspensions were stored frozen. Microscopic examination in the present studies showed, however, that particle aggregation rather than an increase in crystal size was facilitated by freezing.

The damage to insulin crystal structure was examined further because of the potential for loss of biological activity and changes in chemical properties. However, in this study crystal damage was not associated with a detectable increase in soluble insulin or with changes in the time course of insulin induced hypoglycemia, when tested by the BP 1973

prolongation test. It would be expected that the prolongation test, where the insulin suspension is injected subcutaneously into rabbits, should parallel the clinical situation, although species differences may exist (Schlichtkrull et al., 1975).

Changes in the crystal shape, unless accompanied by denaturation of the protein, would not be expected to affect the biological potency because the suspensions are dissolved in acidic solutions prior to bioassay. However, since no loss of potency was observed in the insulin suspensions which had been frozen and thawed when compared with an unfrozen suspension of the same type, and similar immunoreactivity was obtained, it was concluded that no denaturation of the insulin suspensions occurred.

Although a single freezing and thawing did not appear to alter the biological or chemical properties of the insulin suspensions which were studied, the marked increase in the rate of sedimentation and the aggregated crystals which may not readily pass through a syringe needle suggest that it may be difficult to obtain reproducible doses of insulin suspensions which have been frozen.

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