Formulating Insulin for Oral Administration: Preparation of Hyaluronan-Insulin Complex

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Purpose. To investigate the behaviour of peptides and hyaluronan in strong acid solutions containing electrolytes in the preparation of a new formulation of insulin, hyaluronan-insulin complex, and to evaluate the in vivo oral activity of the formulation.

Methods. Individual processing parameters in the preparation of the insulin complex were first refined, and two formulations were subsequently investigated. The chemical structure, particle size and hydrophilic/hydrophobic properties of the insulin complex in these formulations were studied using light scattering techniques, amino acid analysis, atomic force microscopy and cryo-transmission electron microscopy. The in vivo activity of oral hyaluronan-insulin complex was then evaluated by measuring the decrease in blood glucose concentrations in streptozotocin diabetic rats.

Results. Five of seven batches of the two insulin complex formulations fit the baseline criteria for approval of the new formulation. The formulation consists of a transparent aqua sol containing a solid hydrophobic phase as precipitate. Glucose-lowering activity was demonstrated after oral administration of the insulin complex to diabetic rats.

Conclusion. A new insulin formulation, a hyaluronan-insulin complex, has been developed and oral activity has been demonstrated.

KEY WORDS: hyaluronan-insulin complex; glucose-lowering activity; insulin; hyaluronan; insulin formulation.

INTRODUCTION

Currently, one of the main challenges in research associated with the medical use of proteins and peptides is to find a formulation suitable for oral administration. Orally administered proteins are normally rapidly destroyed in the alimentary canal by proteases, peptidases, and/or gastric acid. Moreover, the gut epithelium is an effective barrier, which prevents, both physically and as a result of the local digestive enzymes, the uptake of proteins into the systemic circulation. As a consequence, very few pharmaceuticals containing proteins intended for oral administration have been developed (1). Those available include oral vaccines containing microbial proteins (2). Obviously, any new formulation for oral administration of proteins and peptides, where systemic effects are required, has to be designed to protect the proteins against inactivation during transport to and uptake through the gut epithelium.

Studies using liposomes (3,4) or stabilized foams (5,6) as structural carriers have indicated that the fluidity of the lyotropic liquid crystalline phases makes these systems unstable in a biological environment. The mobility of these structures results in large variations in the exposure and release of encapsulated peptides, making them less suitable for medical use without further stabilisation.

In this study, we used hyaluronan (7), a polymer with a documented safety record (8–10), as a carrier for insulin, the model protein used in these investigations. In order to improve the oral bioavailability of insulin, it is necessary to reduce the size of the molecule and adjust the hydrophobic/lipophilic balance of the insulin.

The main aim of this study was to investigate and optimize a method of formulating insulin with hyaluronan for oral intake. We also show that the hyaluronan-insulin complex has oral glucose-lowering activity in hyperglycemic streptozotocin-diabetic rats. In a follow-up study, the biological properties of hyaluronan-insulin complex and native insulin, and their capacity to decrease blood glucose concentrations in streptozotocin-diabetic rats, will be compared (11).

MATERIALS AND METHODS

Materials

Insulin, human recombinant, lyophilized, sterilized, and with a specific activity of >26 IU/mg, was purchased from Roche Molecular Biochemicals (Mannheim, Germany). Hyaluronan with a relative molar mass of 7.7×10^6 Da was obtained from Pharmacia AB (Uppsala, Sweden). A specific fraction of the hyaluronan, with a relative molar mass of about 1.5×10^5 Da, was prepared by stepwise acid hydrolysis and subsequent dialysis. The molecular mass and intrinsic viscosity of each batch of hydrolyzed hyaluronan were determined using high-performance size-exclusion chromatography combined with refractive index, multiple angle laser light scattering, and viscometry detection (SEC-RI-MALLS-Visc) (12) using a concentration range from 8.5 to 25 mg/ml of water.

Human serum albumin (HSA, 200 mg/ml) was obtained from Pharmacia & Upjohn (Stockholm, Sweden). Streptozotocin was provided by Pharmacia Corp. (Kalamazoo, MI, USA). Other raw materials, of biotechnological performance standard (cell culture tested), were purchased from Sigma (St. Louis, MO, USA). All solutions used were filtered (0.22-μm diameter) or sterilized for 25 min at 125°C before use.

Animals

Outbred male insulin-naive Sprague-Dawley (SD) rats, with a body weight of 280–370 g, were purchased from B&K Universal AB (Sollentuna, Sweden). The streptozotocin diabetic rats were active and eating during the hours of darkness and had free access to water and pellet rat food throughout the study. In order to induce diabetes, the rats were injected with streptozotocin 4–7 days before the blood glucose characterizations and the start of the insulin experiments. Blood glucose concentrations were determined using blood glucose reagent strips (MediSense, Baxter Travenol, Deerfield, IL, USA). Blood samples were taken from an incision in the tip of the tail by carefully pressing blood along the tail vein. Streptozotocin-treated rats with an initial blood glucose level

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less than 15 mM were excluded from the investigation. The regional Animal Ethics Committee at Uppsala University approved all animal experiments, which were carried out under the supervision of a licensed veterinarian.

Dynamic Light Scattering

Changes in the hydrodynamic radius of hyaluronan during dissolution at pH 1.5 and 7.5 were followed using dynamic light scattering. The light source was a frequency-stabilized Coherent Innova Ar-ion laser emitting vertically polarised light at 488 nm. The detector consisted of a 4-μm monomodal fiber coupled to an ITT FW 130 photomultiplier. The autocorrelator was an ALV-5000 with 288 exponentially spaced channels, obtained from ALV-Laser Vertriebsgesellschaft GmbH (Langen, Germany). The scattering cell was immersed into a thermostatted bath with an index matching liquid (decaline). The measured intensity correlation function, $g_2(t)$, is related to the electric field correlation function, $g_1(t)$, by the Siegert relation

$$g_2(t) = 1 + \beta \cdot |g_1(t)|^2$$

where β is a nonideality factor (13). The electric field correlation function

$$g_1(t) = \int \tau \cdot A(\tau) \cdot e^{-t/\tau} d(\ln \tau)$$

was analyzed in terms of a continuous distribution of relaxation times using a constrained regulating algorithm, REPES (13). The diffusion coefficient, D, was then obtained from the relaxation rate (13,14). The diffusion coefficient was measured at three concentrations of hyaluronan, 10, 30, and 90 μg/ml, and the hydrodynamic radius was calculated from the extrapolated value of the diffusion coefficient at infinite dilution using the Stokes-Einstein relationship (15).

Measurements of pH, ς-Potential, Solubility, and Hydrophobic Properties of Hyaluronan

The ς-potential at different pH values was determined using the Doppler effect in the light scattering from particles moving in an electric field in a Zeta Sizer 4000, Master S version PCS: v 1.26 (Malvern Instruments, Palo Alto, CA, USA). The dissolution of the samples was detected by a decrease in light scattering (turbidity) or was followed by direct observation of the turbidity of the dispersions. The proton binding capacity was measured by acid-base titration (16).

Determination of Zinc Content

Native insulin and hyaluronan-insulin complex were treated with 2% nitric acid for 2 h at 120°C in sealed ampoules, filtered through a 00 H-filter from Whatman, and assayed by mass spectrometry in a SCIEX Elan 6100 ICP-MS (Perkin Elmer, Boston, MA, USA).

Formulation of Hyaluronan-Insulin Complex

All materials used in the development of the formulation were sterilized. Solutions of native insulin were filtered (pore diameter 0.22 μm). Mixing, dialyzing, pH adjustment, and dispensing in vials were performed in laminar flow units under aseptic conditions. Insulin and hyaluronan were dissolved in an acid environment and dialyzed in dialysis bags, Spectra Pore R MWCO 6-8000, from Spectrum (Rancho Dominquez, CA, USA), containing about 4 ml of the relevant preparations. The physical appearance during complexation, that is, transparency, colloidal state, gel or precipitate formation, was evaluated by direct observation or by light scattering. Ion concentration, pH, and ocular appearance were determined after dialysis.

The procedure comprised the following steps: dissolution of hyaluronan and insulin, complexation of the insulin and stabilization of the hyaluronan/insulin complex (Fig. 1). It is essential that hyaluronan be kept at low pH for no longer than 12 h, to prevent uncontrolled breakdown of the molecule. In order to evaluate the preparation steps and process parameters, 11 different preparations of insulin, each with a unique set of process parameters, were made. The monitored process parameters included the relative proportions of hyaluronan and insulin, the time interval between the addition of hyaluronan and insulin, the pH at which hyaluronan and insulin were dissolved, the time at which the dialysis medium was exchanged, the ion strength of the replacement medium (0.5 mM Tris buffer, pH 6.5, with 0–0.9% sodium chloride), and the duration of the dialyzing procedure.

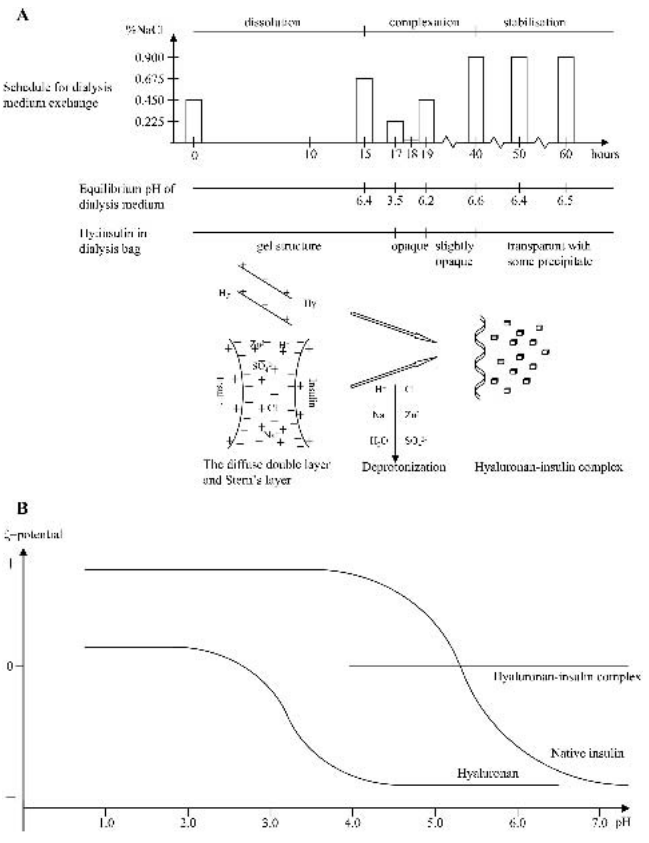

Fig. 1. (A) Schematic presentation of the proposed procedure comprising dissolution, compression, and stabilization of the formulation of hyaluronan-insulin complex. The upper part shows the exchanges of the dialysis solution with time along with the equilibration pH at the end of the dialysis intervals. The physical characteristics of the preparation inside the bags are specified. (B) The relative ζ-potentials of hyaluronan (based on the measured values at pH 1.5 and 7.5) and of insulin and hyaluronan-insulin complex at different pH values.

Preparation of Hyaluronan-Insulin Complex

Of the 11 formulations tested (trial and error procedure), two final preparation methods with slightly different proportions of insulin and hyaluronan were chosen, resulting in the best yield without any phase separation.

Preparation A was made with a molar ratio of insulin: hyaluronan = 12:1. Hyaluronan (92 mg) was slowly dissolved in 6.5 ml 1 M sodium sulphate to obtain a clear solution. Insulin (45 mg as a solid) was added to the solution, and a clear gel was formed at pH 1.5. The two-phase jelly-like mixture was stored for 12 h at 5–7°C. The transparent gel phase was then gently dispersed throughout the transparent solution phase, dispensed into a dialysis bag and dialyzed for 50 h at 5–7°C. Two batches of this preparation were made.

Preparation B, with a molar ratio of insulin:hyaluronan = 15:1, was made from 78.9 mg hyaluronan in 7.0 ml 1 M sodium sulfate. Hyaluronan was titrated by adding 1.0 ml of 1 M hydrochloric acid to result in a pH of 1.6. Insulin, 45.1 mg in 0.2 ml 1 M hydrochloric acid and 0.8 ml 1 M sodium sulfate, was added to the hyaluronan preparation to form a cloudy solution (pH 1.8). The solution was dialyzed for 50 h. Five batches of this preparation were made.

Quality Control of Hyaluronan-Insulin Complex

Each of the seven batches of the insulin complex was tested for compliance with the following test criteria:

1. No oxidation: no cysteic acid, originating from the cleavage and oxidation of S-S- bridges, detected during amino-acid analysis.
2. Molar ratio insulin:hyaluronan = 12 to 30:1.
3. Osmolality: 290–320 mmol/kg.
4. pH: 5–6.
5. Appearance: colloidal state with a solid phase as a precipitate.
6. Blood glucose decrease in streptozotocin-diabetic rats 1 h after oral administration: statistically greater than the average blood glucose decrease in 24 untreated diabetic rats (11).

Evaluation of Hyaluronan-Insulin Complex

Insulin concentrations were determined by amino-acid analysis at the Biomedical Center, Uppsala University. Values for threonine and serine were corrected for destruction using the standard recoveries of 0.96 and 0.90, respectively, during 24 h of hydrolysis.

Hyaluronan concentrations were calculated from the corrected recovery of glucosamine during amino-acid hydrolysis (from the standard recovery of 0.5) using the residual weight 379 of the dimeric repeating subunit of hyaluronan.

Osmolality was calculated from the vapour pressure measured with VAPRO, Vapor Osmometer 5520 (Wescor Inc., Logan, Utah, USA).

Atomic force microscopy (AFM): the the insulin complex in two of the approved batches was examined at the Center for Surface Biotechnology, Uppsala University, by Dr. Magnus Bergkvist using AFM on hydrophobic silica (DDS) surfaces, according to methods described by Bergkvist (17). Intermittent contact AFM was performed using a Digital Instrument NANOSCOPE IIIa multimode system (micro masch series NSC12/SC12; the resonance frequency of the tips was ~10 kHz and the force constant was 0.08 N/M). Hydrophobic DDS surfaces are used for single macromolecular surface interactions to analyze structures down to nanometer scale in size (MicroMasch, Tallinn, Estonia).

Cryotransmission electron microscopy (Cryo TEM; Zeiss 902A, Overkochen, Germany) was used to identify the insulin complex from the batches used in the AFM study, using the technique for of viewing small structures described by Almgren *et al.* (18).

Evaluation of Insulin Activity

The *in vivo* oral activity of the hyaluronan-insulin complex in batches fullfilling the quality control criteria (1 to 5) was evaluated in streptozotocin-diabetic rats. As a control, the *in vivo* oral activity also of noncomplexed mixure between native insulin and hyaluronan in a molar ratio of 15:1 was evaluated in streptozotocin-diabetic rats. The insulin preparations, 0.5–1.5 ml, were administered through a feeding tube. Each formulation was diluted with isotonic sodium chloride to an appropriate concentration. The blood glucose concentration was measured using blood glucose reagent strips 1 h after administration.

The change in glucose concentration, $\Delta G_{or}(t_i)$, was calculated according to Eq. 1 in each treated rat

$$\Delta G_{or}(t_i) = G_{or}(t_i) - G_{or}(t_0) \quad (1)$$

where $G_{or}(t_0)$ is the blood glucose concentration at t_0 = 07.00 h, and t_i is the time of day at which the first sample after administration was taken. The change in glucose concentration was compared with the average change in glucose concentration, taken at the same time of day, in 24 nontreated streptozotocin-diabetic control rats as described in Jederström *et al.* (11). Briefly, the changes in glucose concentration, $\Delta G_c(t)$, in these control rats were determined every hour for a day. The distribution of these blood glucose values did not significantly deviate from the normal distribution at any of the time points. The analysis of the statistical significance of $\Delta G_{or}(t_i)$ was based on comparisons with the 0.05-quantile of the change in glucose concentration in the control rats at the same time of day $[\Delta G_{c,\alpha=0.05}(t_i)]$. The decrease in glucose concentration in the test rats, $\Delta G_{or}(t_i)$, was considered as statistically significant if it was greater than $\Delta G_{c,\alpha=0.05}(t_i)$.

RESULTS AND DISCUSSION

Physicochemical Evaluation of Hyaluronan

Molar Mass and Viscosity of Hyaluronan

More than 80% of the hyaluronan used had a relative molecular mass ranging between 1.24×10^5 and 1.86×10^5 Da. The 1.50×10^5 to 3.00×10^5 Da hyaluronan was significantly less viscous than the 900,000 Da hyaluronan. The high viscosity of the higher molar mass fraction made it difficult to obtain a homogeneous mixture during preparation of the hyaluronan-insulin complex and was therefore not used.

The Size of Hyaluronan

In the light scattering measurements, the relaxation time distributions were essentially single-modal under all condi-

tions. The intensities of the undiluted and water-diluted hyaluronan samples were similar, whereas that of the sample diluted with acid was about 2-fold greater. This correlates nicely with the hydrodynamic radii obtained from extrapolation of the diffusion coefficients to zero concentration. For water-diluted hyaluronan, the hydrodynamic radius was 65 nm, and the corresponding value for the acid-diluted samples was 105 nm (Table I). The diffusion coefficients of the samples diluted in water and acid at the concentrations used in the extrapolation were linear functions of concentration.

These results are consistent with an increase in hyaluronan particle dimensions in an acidic environment and suggest that the hyaluronan molecule unfurls from a curled cylinder to a straight chain on the addition of strong acid. This expansion of the molecule when the pH is lowered to 1.5 is clearly linked with the protonisation of the glucuronic acid and *N*-acetylglucosamine residues of the molecule, as indicated by the ζ-potential.

The ς-Potential of Hyaluronan

The ζ-potential of hyaluronan changed from a highly negative charge (–59 mV) to a positive charge (3.1 to 16.9 mV) when the pH was lowered from 6 to 2–3 (Table I). This straightening of hyaluronan and the change in charge are necessary to allow insulin to form a stable complex with the hyaluronan molecule. When the environment is subsequently returned from acidic to neutral, the hyaluronan can reform around the insulin to form various complexes.

Physicochemical Evaluation of the Hyaluronan-Insulin Complex

AFM

The following samples of hyaluronan and insulin in a mixture and of the hyaluronan-insulin complex, preparation A, were examined on silica surfaces as described in (17):

Sample A, native insulin 1 mg/ml and hyaluronan 1mg/ml, in a molar ratio of 25:1, dissolved in isotonic sodium chloride, containing Tris buffer 0.5 mM, pH 6.5, did not lead to any detectable complex formation, as illustrated in Fig. 2, Sample A. The lack of hydrophobic sites on the hyaluronan and native insulin molecules prevented attachment to the hydrophobic surface of the scanned site.

Sample B, hyaluronan-insulin complex, a precipitate in the clear solution from dialysis, assayed to contain 2.4 mg insulin/ml with a molar ratio of insulin:hyaluronan = 19:1, was seen as a mixture of threads and solid spots (<10 nm), Fig. 2, Sample B. The molecules obviously had hydrophobic sites, which were able to bind to the hydrophobic surface of the scanned DDS site.

Sample C, the clear supernatant, the solution from dialysis centrifuged at 11,000 rpm (20,000 × *g*) at 10°C for 15 min, assayed to contain 1.6 mg/ml of insulin:hyaluronan in a molar ratio of 15:1, contained a solid phase (particles) of the hydrophobic hyaluronan-insulin complex visible under AFM. The spots appeared to be about 2 nm in diameter, Fig. 2, Sample C.

Sample D, the sediment after centrifugation mixed with 100 μl of the clear solution from the dialysis procedure, assayed to contain 5.2 mg insulin per ml with a molar ratio of insulin:hyaluronan of 54:1, was seen as distinct spots and parallel threads, with some wider strips, Fig. 2, Sample D. The largest spots appeared to be about 2 nm in diameter and the strips were 1 nm in width. This suggests that the insulin complex was hydrophobic (i.e., it was adsorbed to the hydrophobic surface of the DDS site) and that the precipitate contained threads of insulin that were associated with a helical hyaluronan structure in a mixture with solid small particles. Both structures contained hyaluronan-insulin complex and hyaluronan.

Cryo-Transmission Electron Microscopy

Cryo-TEM was used to demonstrate the presence of the hyaluronan-insulin complex in the test suspensions, preparation A, (Fig. 3).

Sample C, the clear supernatant from centrifugation of the dialyzed preparation, contained a number of aggregates sized between 20 and 200 nm in diameter. The lower picture in Fig. 3 is of a large aggregate consisting of small spots. Single spots outside and spots within the aggregate were less than 10 nm in diameter. We suggest that the spots comprised hyaluronan-insulin complexes, containing 1.6 mg/ml, assayed to contain a molar ratio of insulin:hyaluronan of 15:1. These spots may be the same as the solid phase of hydrophobic hyaluronan-insulin complexes found in the AFM study, which was composed of particles about 2 nm in diameter (Fig. 2, Sample C). Cryo-TEM analyses of Sample B, the clear solution obtained directly from the dialysis procedure, and Sample D did not differ to any great extent. However, more

Table I. Physicochemical Properties of Hyaluronan (1.5×10^5 Da) in Solutions of Varying Concentration and Acidity

Hy (μg/ml)	HCl 1 M (ml)	Distilled water (ml)	Zeta sizer			Hydrodynamic radius, R_n (nm)
			KCP[a]	pH	ς-potential (mV)	
10	—	1	2844	6.5	–59	65
10	1	1	—	2.4	3.1	—
5	1	1	2542	—	16.9	105
30	—	1	—	—	—	65
15	1	1	—	2.5	—	105
90	—	1	—	—	—	65
45	1	1	—	—	—	105

[a] Number of particles counted.

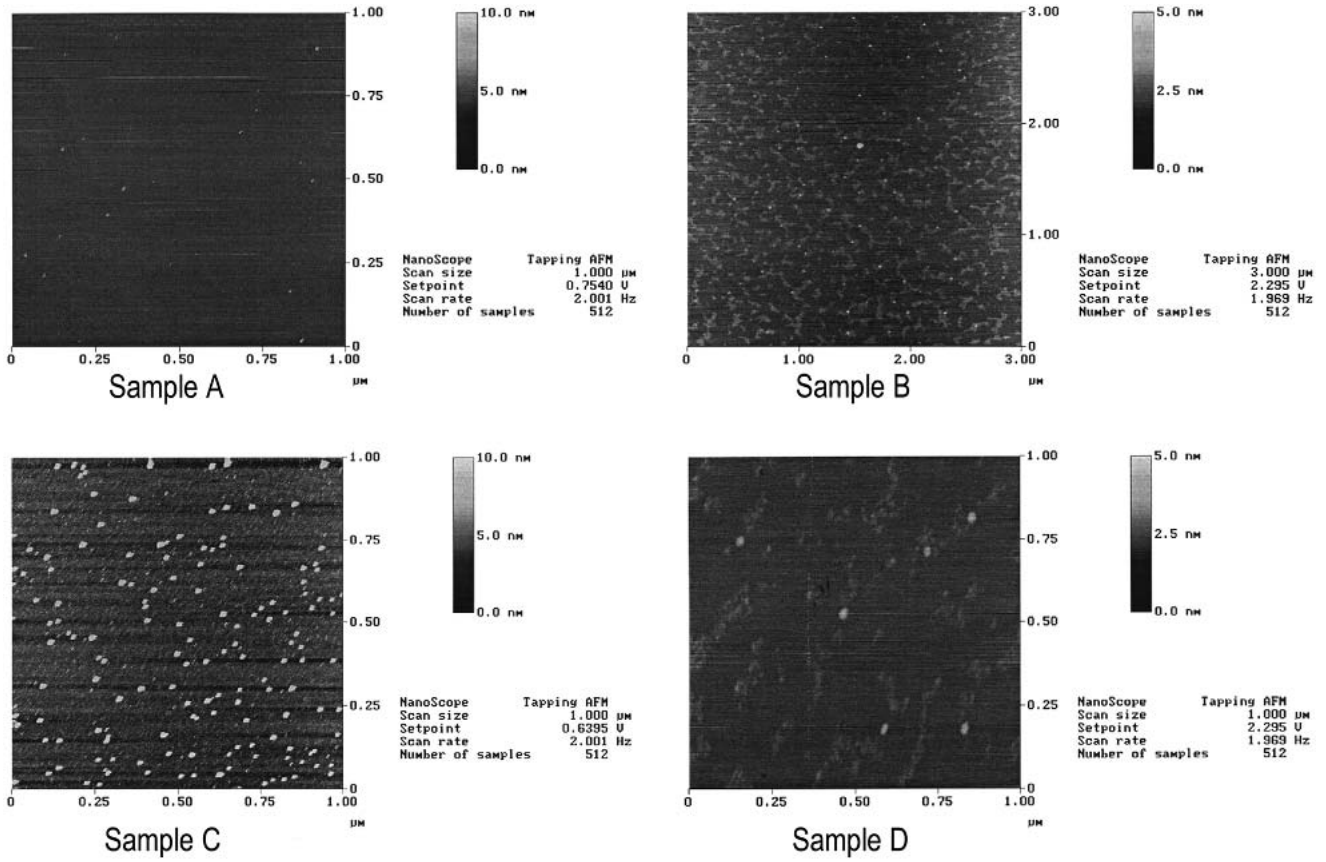

Fig. 2. Atomic force microscopy of samples where the size of the objects shown is related to the light intensity and the calibration scales to the right of the pictures.

distinct and larger aggregates were detected in Sample D, in which the sediment obtained after centrifugation was suspended in 100 µl of the clear solution from the dialysis procedure. The spots were more compact, but no helical structures were identified.

Zinc Analyses

The results of the mass spectrometric analysis of zinc are summarized in Table II. The molar ratio of insulin:Zn changed during the preparation of the hyaluronan-insulin complex . The native, rDNA-derived insulin had a ratio of about 3, which is in line with the ratios of crystallized insulin, containing two hexameric forms, of 6:2 and 6:4 (19). The analysis confirmed the presence of zinc in native insulin and the loss of 80% of this during the preparation of the insulin complex. It has been reported that removal of zinc from insulin results in a reversible state of structural flexibility and also that the presence of zinc in the molecule may be an important factor in its hypoglycemic activity, which is more prolonged for 4-zinc insulin than for 2-zinc insulin (19).

Table II. Mass Spectrometric Analysis of Zinc in Insulin Preparations

Preparation of insulin	Zinc content ppb	Molar ratio Insulin:Zn
Native insulin, 25.2 mg[a]	9733	2.96
Hyaluronan-insulin complex, 24.6 mg[a] (molar ratio insulin:hyaluronan = 22.6)	1747	15.8
Native pig 4-zinc insulin (hexameric) (19)	—	1.5
Native pig 2-zinc insulin (hexameric) (19)	—	3.0

[a] In 10 ml Tris buffer 10 mM, pH 6.5, saline.

Native human insulin is stored in the β-cells as a Zn^{2+}-stabilized hexamer. The cell parameters of crystallised 2Zn-insulin and 4Zn-insulin hexamers are a_h = 82.5 Å, b_h = 82.5 Å, c_h = 34.0 Å, γ = 120°, and a_h = 80.7 Å, b_h = 80.7 Å, c_h = 37.6 Å, γ = 120°, respectively (21). The hexamer crystals, organized as three dimmers, could schematically be described as a disk (or a cylinder) with diameter 16.5 nm and height 3.4 nm. The biologically active form of insulin is the monomer that interacts with the insulin receptor, but the monomer self-associates into dimers and higher oligomers in the bloodstream. The secondary structure of insulin monomer at neutral pH in solution is considered being similar to that of the crystallographically identified T-state of the hexamer (20). The complex containing insulin:hyaluronan in a molar ratio of 15:1 that is described in this paper was 2–10 nm in size, indicating a considerable reduction from that of native insulin during the complexation procedure.

Proposed Mechanism for the Formation of Hyaluronan-Insulin Complex

Figure 1 summarises the procedure used to prepare the insulin complex.

When hyaluronan is dissolved in a solution with a pH

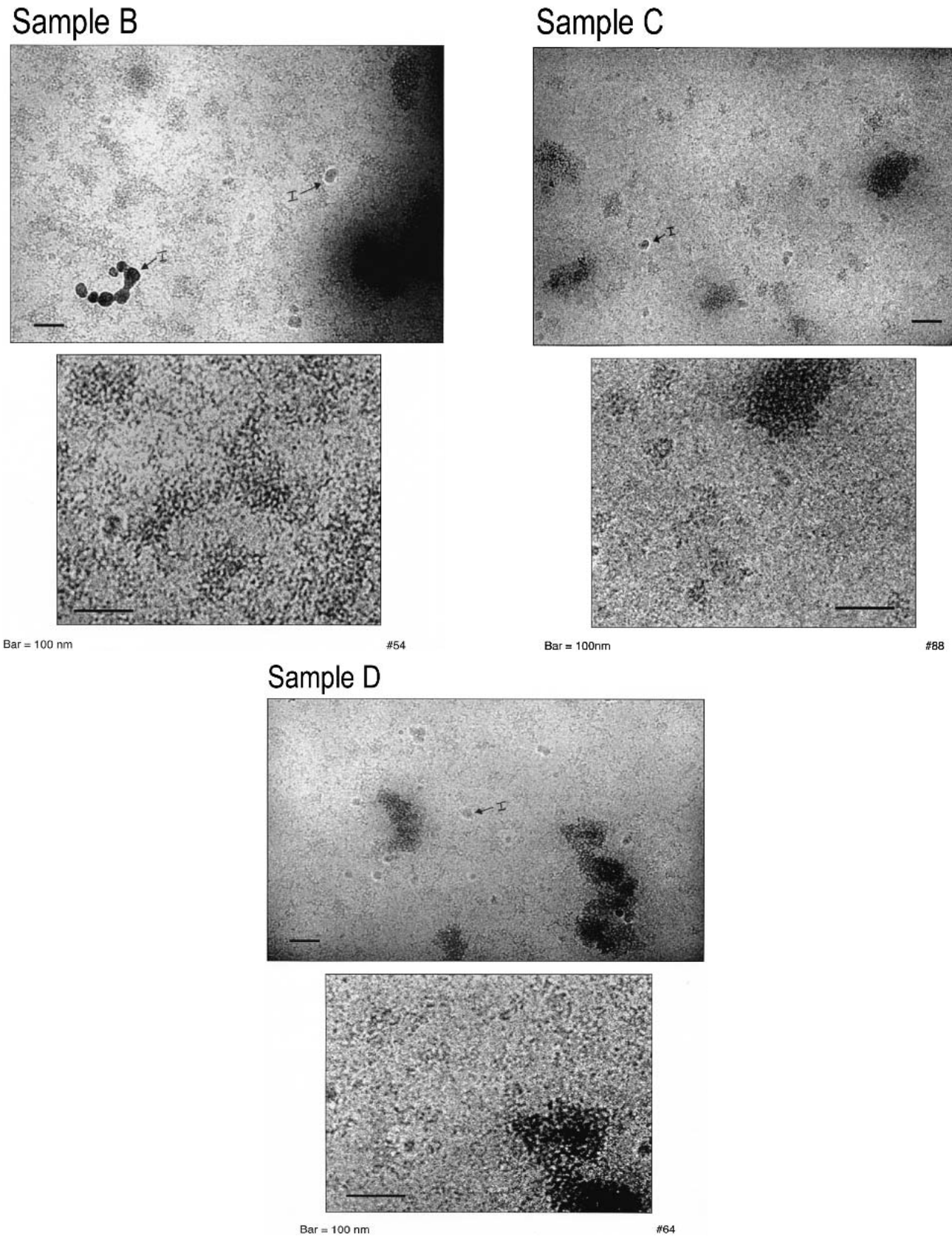

Fig. 3. Cryo-transmission electron microscopy of hyaluronan-insulin complex, preparation I, where the lower row shows magnifications of the objects in the upper row. (Clusters marked with an arrow and "I" are ice crystals.)

range of 1.3–1.5, it becomes positively charged and its structure is elongated from 65 to 105 nm. Adding Na_2SO_4 to the electrolyte solution helps to dissolve the hyaluronan. The DLVO theory predicts that both repulsive and attractive molecular interactions are weakened and become negligible when ionic strength exceeds ~0.1 M, which is probably why insulin and hyaluronan are soluble at low pH and the hyaluronan structure is more flexible. Insulin is dissolved at pH < 1.5, far below its isoelectric point at pH 5.4 (22). Thus, the negative charge of the acidic amino acids will be lost at low pH and the hydrophobicity of the molecules will be increased (16,23). Hyaluronan and insulin then form a homogeneous mixture/gel structure.

We suggest that the initiation of dialysis of insulin and hyaluronan against solutions of successively decreasing ionic strength and increasing pH triggers the formation of a complex between the molecules and the subsequent formation of a stable structure of the hyaluronan-insulin complex (Fig. 1). At pH 2 and during the start of the dialysis process, hyaluronan is either slightly positively charged or uncharged (Table I). During the course of the dialysis, the carboxyl groups of insulin dissociate with increasing pH, so that the molecule approaches its minimal net charge at the isoelectric point at pH 5.4. Hyaluronan becomes negative with increasing pH above 3. Thus, in the pH range 3–5.4, hyaluronan is negative and insulin is positive. Due to the lowering of ionic strength during the dialysis both molecules become surrounded by diffuse double layers of simple ions. The loss of ions decreases the double layer according to the DLVO theory (24,25), and increases the hydrophobic interactions between the molecular surfaces. Thus, when pH increases above 3 and the concentration of electrolytes is lowered further, the repulsive (positive) energy potential between the insulin and hyaluronan surfaces caused by the diffuse double layer decreases and the attractive energy potential begins to dominate. The total energy potential barrier at medium intersurface distances thus disappears or becomes small compared with the thermal energy of the insulin and hyaluronan molecules, leading to close contact and the squeezing out of water between the surfaces, and forming a stable complex between insulin and hyaluronan. Compact complexes between polycations and polyanions can actually be formed when the ionic strength is low; if mixed in the correct proportions, the complex will be neutral (7).

AFM and Cryo-TEM studies demonstrated the small size of the insulin-hyaluronan particulate complex. The particles of a similar complex, made earlier by complexing human growth hormone with hyaluronan, were also small (16). The particles cannot be seen by the naked eye, but form a totally transparent solution. The amino-acid analyses indicate that the particles might consist of a number of insulin molecules surrounded by a hyaluronan molecule.

The loss of zinc from the interior of the native hexamer insulin during dialysis and formation of hyaluronan-insulin complexes is an indication of conformational changes to the insulin structure. However, as shown in the next section, the insulin complex is still biologically active after oral administration to streptozotocin-diabetic rats.

Biological Effect of Hyaluronan-Insulin Complex

Table III summarizes the results of the *in vivo* glucose-lowering activity of the batches of the insulin complex and the

Table III. Characterization of the Hyaluronan-Insulin Complex

Batch[a]	Insulin/hyaluronan molar ratio		Loss of mass (%)	Biological characterization				
	Initial	Final		Dose (mg)	Time of treatment	$\Delta G_{or}(t_i)$ (mM)	$\Delta G_{c,a=0.05}(t_i)$ (mM)	Significance
A1[b]	12	12	46	1.0	07.00	1.8	–7.4	No
A2	12	15	45	1.5	07.00	–13.5	–7.4	Yes
B3[b]	15	38	69	1.1	12.00	–9.0	–14.2	No
B4	15	27	50	1.2	07.00	–7.0	–7.4	No
B4				1.2	07.00	–9.6	–7.4	Yes
B5	15	21	40	1.5	07.00	–4.7	–7.4	No
B5				2.3	10.00	–19.7[c]	–15.2[c]	Yes
B5				1.5	07.00	–16.6[c]	–10.9[c]	Yes
B5				1.5	07.00	–16.9[c]	–10.9[c]	Yes
B6	15	22	21	4.7	11.00	–7.9	–12.9	No
B6				4.7	07.00	–1.5	–7.4	No
B6				4.7	07.00	–23.6	–7.4	Yes
B7	15	20	76	1.2	07.00	–3.1	–7.4	No
B7				1.5	07.00	–8.8	–7.4	Yes
B7				1.5	07.00	–8.0	–7.4	Yes
C8	—	15	—	3.1	14.00	–0.4	–12.6	No
C8				3.1	14.00	–3.9	–12.6	No
C8				3.1	14.00	–1.9	–12.6	No
C8				3.1	14.00	–2.1	–12.6	No

[a] Two formulation compositions, A (batches 1 and 2) and B (batches 3–7), of hyaluronan-insulin complex were used. A third formulation, C (batch 8), of a mixture of native insulin and hyaluronan, was used as control.

[b] These batches did not comply with the required specifications and were consequently not studied further.

[c] Initial values, $\Delta G_{or}(t_0)$, were not available and were substituted as described in Ref. 11.

control batch of a mixture of insulin and hyaluronan. In preparing the insulin complex, the initial molar ratio of insulin: hyaluronan was either 12 (batch A) or 15 (batch B). The final ratio increased in the approved formulations up to a maximum of 27. Two batches were rejected because of poor yield and unacceptable insulin:hyaluronan ratio. None of the rats given insulin from the control batch showed any significant decreases.

The evaluation of the biological activity was experimentally complicated, essentially due to variations in the individual rat blood glucose concentrations during the course of the day. It was also apparent that a dose-activity relationship existed after oral administration, but this has not yet been fully characterized. As is evident from Table III, the estimated effect on blood glucose concentrations in different rats varied within the same batch of insulin; some analyses did not meet the requirement that $\Delta G_{or}(t_i)$ be greater than $\Delta G_{c,\alpha=0.05}(t_i)$. However, it is quite evident that orally administered, hyaluronan-insulin complex given in appropriate doses is biologically active. A subsequent paper discusses a comparative biological study of the insulin complex and native insulin given both subcutaneously and orally to rats; this study found improved biological activity for the insulin complex (11).

This new method appears to offer potential as a general method for the formulation of proteins intended for oral administration. However, before conclusions can be drawn on the possible role of these formulations in clinical practice, further studies in both animals and humans are required, particularly in the area of mechanism of protein uptake, transport through the gut mucosa and bioavailability.

In conclusion, the size of the insulin is reversibly decreased when insulin and hyaluronan are dissolved in an acid environment followed by a decrease in the ion concentration and increased pH during dialysis. The hyaluronan-insulin complex appears as a colloidal phase, an aqua sol, containing a solid phase as precipitate. The preparations appeared as a totally transparent solution, although clusters of particles less than a few nm in diameter were seen using AFM and Cryo-TEM. A significant decrease in blood glucose concentration was obtained in rats after oral administration of the hyaluronan-insulin complex, but not the native insulin.

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REFERENCES

1. O. Pillai and R. Panchagnula. Insulin therapies—past, present and future. *Drug Discov. Today* **6**:1056–1061 (2001).
2. M. A. Clark, M. A. Jepson, and B. H. Hirst. Exploiting M cells for drug and vaccine delivery. *Adv. Drug Deliv. Rev.* **50**:81–106 (2001).
3. B. Furugren. *Biomolecules Studied by Small-Angle X-ray Scattering*. Swedish University of Agricultural Sciences, Uppsala, 1987.
4. G. Jederstrom and G. Russell. Size exclusion chromatography of liposomes on different gel media. *J. Pharm. Sci.* **70**:874–878 (1981).
5. S. Friberg, L. Rydhag, and G. Jederstrom. Liquid crystalline phases in aerosol formulations. I. Phase equilibria in propellant compositions. *J. Pharm. Sci.* **60**:1883–1885 (1971).
6. G. Jederstrom, L. Rydhag, and S. Friberg. Liquid crystalline phases in aerosol formation. II. Influence of liquid crystalline phases on foam stability. *J. Pharm. Sci.* **62**:1979–1982 (1973).
7. K. Thalberg. *Polyelectrolyte–Surfactant Interactions.* Division of Physical Chemistry 1. Chemical Center, University of Lund, Lund, 1990, pp.180.
8. W. D. Comper and T. C. Laurent. Physiological function of connective tissue polysaccharides. *Physiol. Rev.* **58**:255–315 (1978).
9. T. C. Laurent. Biochemistry of hyaluronan. *Acta Oto-Laryngologica. Suppl.* **442**:7–24 (1987).
10. D. Miller and R. Stegman (eds.). *Healon (Sodium Hyaluronate): A Guide To its Use in Ophthalmic Surgery*. Wiley, New York, 1983.
11. G. Jederstrom, J. Gråsjö, A. Nordin, I. Sjöholm, and A. Andersson. Efficacy of oral hyaluronan-insulin complex in diabetes rats. *Diabetologia* (2004).
12. J. P. Roubroeks, D. I. Mastromauro, R. Andersson, B. E. Christensen, and P. Åman. Molecular weight, structure and shape of oat (1-3),(1-4)-β-d-glucan fractions obtained by enzymatic degradation with lichenase. *Biomacromolecules* **1**:584–591 (2000).
13. R. P. B. Berne. *Dynamic Light Scattering*. Wiley, New York, 1976.
14. J. Jakes. Testing of the constrained regularization method of inverting Laplace transform on simulated very wide quasielastic light-scattering auto-correlation function. *Czech. J. Phys.* **B38**: 1305–1316 (1988).
15. M. E. Aulton. *Pharmaceutics: The science of dosage form design.* 2nd ed. Churchill Livingstone, Edinburgh, London, New York, Philadelphia, St. Louis, Sidney, Toronto, 2002.
16. G. Jederstrom. *Hydrophobe Biomolecular Structure*, United States Patent. *US* **6448093**:1–28 (2002).
17. M. Bergkvist. *Orientation and Conformation of Single Macromolecules on Unmodified and Functionalized Surfaces*. Faculty of Science and Technology, Uppsala Universitet, Uppsala, 2002, pp. 62.
18. M. Almgren, K. Edwards, and G. Karlsson. Cryo transmission electron microscopy of liposome and related structures. *Colloids and Surfaces. A: Physicochemical Engineering Aspects* **174**:3–21 (2000).
19. E. D. G. Bentley, G. Dodson, D. Hodgkin, and D. Mercola. Structure of insulin in 4-zinc insulin. *Nature* **261**:166–168 (1976).
20. H. B. Olsen, S. Ludvigsen, and N. C. Kaarsholm. Solution structure of an engineered insulin monomer at neutral pH. *Biochemistry* **35**:8836–8845 (1996).
21. U. Derewenda, Z. Derewenda, E. J. Dodson, G. G. Dodson, C. D. Reynolds, G. D. Smith, C. Sparks, and D. Swenson. Phenol stabilizes more helix in a new symmetrical zinc insulin hexamer. *Nature* **338**:594–596 (1989).
22. J. Brange. *Galenics of Insulin*. Springer-Verlag, Berlin, Heidelberg, 1987.
23. J. B. Honing, K. Sharp, and A.-S. Yang. Macroscopic models of aqueous solutions and chemical applications. *J. Phys. Chem.* **97**: 1101–1109 (1993).
24. B. V. Derjaguin. I. L. Abricosova and E. M. Lifshitz. Direct measurement of molecular attraction between solids separated by a narrow gap. *Q. Rev. Chem. Soc.* **10**:295–329 (1956).
25. B. V. Derjaguin. The forces between molecules. *Sci. Am.* **203**:47–53 (1960).