

## Soluble, fatty acid acylated insulins bind to albumin and show protracted action in pigs

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**Summary** We have synthesized insulins acylated by fatty acids in the  $\epsilon$ -amino group of Lys<sup>B29</sup>. Soluble preparations can be made in the usual concentration of 600 nmol/ml (100 IU/ml) at neutral pH. The time for 50 % disappearance after subcutaneous injection of the corresponding Tyr<sup>A14</sup>(<sup>125</sup>I)-labelled insulins in pigs correlated with the affinity for binding to albumin ( $r = 0.97$ ), suggesting that the mechanism of prolonged disappearance is binding to albumin in subcutis. Most protracted was Lys<sup>B29</sup>-tetradecanoyl des-(B30) insulin. The time for 50 % disappearance was  $14.3 \pm 2.2$  h, significantly longer than that of Neutral Protamine Hagedorn (NPH) insulin,  $10.5 \pm 4.3$  h ( $p < 0.001$ ), and with less inter-pig variation ( $p < 0.001$ ). Intravenous bolus injections of Lys<sup>B29</sup>-tetradecanoyl des-(B30) human insulin showed a protracted blood glucose lowering effect compared to that of human insulin. The relative affinity of Lys<sup>B29</sup>-tetradecanoyl des-(B30) insulin to the insulin receptor is 46 %. In a 24-h glucose clamp study in pigs the

total glucose consumptions for Lys<sup>B29</sup>-tetradecanoyl des-(B30) insulin and NPH were not significantly different ( $p = 0.88$ ), whereas the times when 50 % of the total glucose had been infused were significantly different,  $7.9 \pm 1.0$  h and  $6.2 \pm 1.3$  h, respectively ( $p < 0.04$ ). The glucose disposal curve caused by Lys<sup>B29</sup>-tetradecanoyl des-(B30) insulin was more steady than that caused by NPH, without the pronounced peak at 3 h. Unlike the crystalline insulins, the soluble Lys<sup>B29</sup>-tetradecanoyl des-(B30) insulin does not elicit invasion of macrophages at the site of injection. Thus, Lys<sup>B29</sup>-tetradecanoyl des-(B30) insulin might be suitable for providing basal insulin in the treatment of diabetes mellitus. [Diabetologia (1996) 39: 281–288]

**Keywords** Insulin analogues, albumin binding, prolonged action, basal insulin, fatty acids, tetradecanoic acid, myristic acid, lysine<sup>B29</sup>, acylation, receptor affinity.

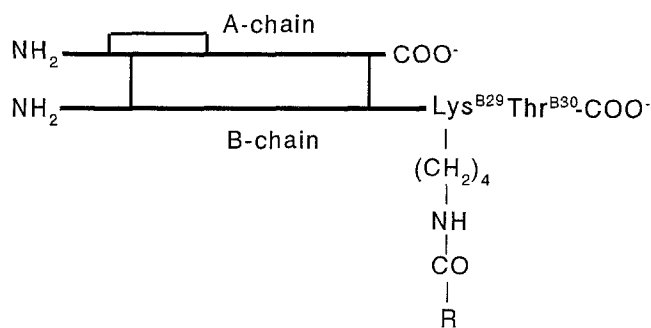
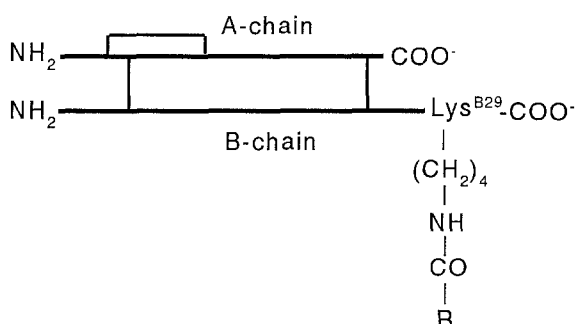
The Diabetes Control and Complications Trial (DCCT) study [1] has shown that intensive treatment

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*Abbreviations:* HI, Human insulin; HSA, human serum albumin; NN-304, Lys<sup>B29</sup>-tetradecanoyl des-(B30) human insulin; NPH, Neutral Protamine Hagedorn, a crystalline insulin-protamine preparation; T<sub>50%</sub>, time for 50 % disappearance; sIR, soluble insulin receptor (extracellular parts); TBS, Tris buffered saline, pH 7.6; Lys, lysine; Tyr, tyrosine; Gly, glycine;

aiming at normalization of blood glucose can prevent or delay diabetic complications. Basal insulins are crystalline preparations, the prevailing products being Neutral Protamine Hagedorn (NPH)- and Lente-type, using protamine [2] or Zn<sup>2+</sup> ions [3], respectively, to form crystals which dissolve slowly in the subcutaneous tissue fluid. The absorption rates of the prolonged-acting insulin products of NPH- and Lente-type fluctuate from day to day, impairing the efforts to achieve normoglycaemia. Crystalline suspensions have additional drawbacks; sedimentation requires shaking of the vial or cartridge before injection; the rate of dissolution at the injection site depends on the local blood flow in the capillaries, which is influenced

*Full B-chain**des-(B30)*

**Fig. 1.** Schematic representation of insulin and des-(B30) insulin acylated in the  $\epsilon$ -amino group of the side-chain of Lys<sup>B29</sup>. The A- and B-chains have 21 and 30(29) amino acids, respectively, each having an N-terminal  $\alpha$ -amino group. R designates the aliphatic carbon-chain of the fatty acid used for acylation of Lys<sup>B29</sup>, resulting in an amide bond connecting the fatty acid and insulin moieties. By removal of Thr<sup>B30</sup> the negative charge of the C-terminal gets closer to the aliphatic moiety R, whereby the modified site may mimic a non-esterified fatty acid better than in the presence of residue B30

Previously, we tried to develop preparations of insulin analogues, soluble at pH below 4, which would crystallize spontaneously upon neutralization in the tissue [6–9]. This was achieved by shifting the isoelectric point of the analogues from that of human insulin, which is about pH 5.4, to pH 7.2–7.3 by adding two positive charges. However, the blood glucose lowering effect of the selected insulin, OPID 174, proved unsatisfactory in the clinic situation [10].

Consequently, we had to find another mechanism that would slow down the absorption rate of a soluble insulin from the subcutaneous tissue, aiming at a longer and more reproducible action than that of NPH. In particular, intra-patient variability in bio-availability and absorption rate should be reduced as compared to conventional crystalline products. One such mechanism might be binding of a soluble insulin to a large protein that is a constituent of the subcutaneous tissue fluid. Albumin, 67 000 M, is present in

0.3 mmol/l. Due to its size and negative charge we expected its half-life in subcutis to be sufficiently long to be useful for insulin retardation. Albumin has multiple binding sites for non-esterified fatty acids, the binding constants ranging from  $10^8$  l/mol for the first to  $10^6$  l/mol for the fifth fatty acid [11]. In human plasma the normal loading of albumin is about 1 mole of fatty acid per mole of albumin [12], leaving a huge surplus of sites for interaction. Considering the slow disappearance rate of albumin, the abundance of albumin in subcutis and the surplus of fatty acid binding sites on albumin, we chose to modify insulin by fatty acid acylation, hoping that the analogues would mimic fatty acids and bind to albumin, be biologically active, stay in solution at neutral pH in concentrations of 600 nmol/ml and, consequently, show a retarded absorption and action.

There are three free amino groups in insulin available for acylation, the two N-terminal  $\alpha$ -amino groups of the A- and B-chains, Gly<sup>A1</sup> and Phe<sup>B1</sup>, and the  $\epsilon$ -amino group of Lys<sup>B29</sup> (Fig. 1). We excluded acylations of Gly<sup>A1</sup> because the potency of insulin drops markedly when groups are introduced in the A-chain N-terminus [13]. However, B-chain acylation by small groups such as acetyl and succinyl has only a minor effect on the potency, both when introduced in the N-terminal Phe<sup>B1</sup> and in the side-chain of Lys<sup>B29</sup> [13]. The properties of Phe<sup>B1</sup>-octadecanoyl and Phe<sup>B1</sup>-hexadecanoyl insulins have been reported earlier [14, 15]. When tested in rabbits and rats, respectively, the potencies were reported to be 0 and 22%. Neither of these investigations attempted to measure the rate of absorption or the albumin binding of the fatty acid acylated insulins. Furthermore, we knew that modifications of Phe<sup>B1</sup> interfere with the formation of the insulin hexamer unit, which is the most desirable state due to its inherent stability [16]. Consequently, acylation of the  $\epsilon$ -amino group in the side-chain of Lys<sup>B29</sup> became the preferred target for modification with fatty acids. In a series of analogues we deleted Thr<sup>B30</sup>, which places the acylated Lys<sup>B29</sup> residue in the C-terminal position of the B-chain (Fig. 1).

## Materials and methods

**Insulins.** Insulins and des-(B30) insulins acylated in N <sup>$\alpha$</sup> -Phe<sup>B1</sup> and N <sup>$\epsilon$</sup> -Lys<sup>B29</sup> were prepared from porcine insulin or single-chain, biosynthetic precursors using conventional peptide chemistry and recombinant technology [17]. The corresponding <sup>125</sup>I-tracers, labelled in Tyr<sup>A14</sup>, were prepared as described earlier [18]. Solutions containing 600 nmol of acylated insulin per ml, 2 Zn<sup>2+</sup>/hexamer, 1.5% glycerol and 0.3% phenol were used in the testing of pharmacokinetic and pharmacodynamic properties. Tyr<sup>A14</sup>(<sup>125</sup>I)-human insulin was used for labelling of human NPH preparations.

**Binding studies.** Binding constants were determined using im-

**Table 1.** Binding of fatty acid acylated insulins to HSA and sIR (soluble, extracellular domain of the insulin receptor) and disappearance after subcutaneous injections in pigs

Insulin	Acyl-chain length	Binding to HSA	Binding to sIR	Disappearance in pigs		
	Number of C-atoms	Relative affinity <sup>a</sup>	Relative affinity	T <sub>50%</sub> (h)	c. v. <sup>b</sup> (%)	n
Soluble human insulin	0	0	1.00	2		
N <sup>ε</sup> B <sup>1</sup> -tetradecanoyl des-(B 30) HI	14	0.26	0.48	n. d.		
N <sup>ε</sup> B <sup>29</sup> -decanoyl HI	10	0.063	0.76	5.1	10	5
N <sup>ε</sup> B <sup>29</sup> -tetradecanoyl HI	14	0.58	0.41	11.9	7	4
N <sup>ε</sup> B <sup>29</sup> -decanoyl des-(B 30) HI	10	0.12	0.76	5.6	21	6
N <sup>ε</sup> B <sup>29</sup> -undecanoyl des-(B 30) HI	11	0.27	n. d.	6.9	23	6
N <sup>ε</sup> B <sup>29</sup> -dodecanoyl des-(B 30) HI	12	0.42	0.54	10.5	21	12
N <sup>ε</sup> B <sup>29</sup> -tridecanoyl des-(B 30) HI	13	0.71	n. d.	12.9	12	6
N <sup>ε</sup> B <sup>29</sup> -tetradecanoyl des-(B 30) HI	14	1.00	0.46	14.3	15	32
N <sup>ε</sup> B <sup>29</sup> -hexadecanoyl des-(B 30) HI	16	0.69	0.19	12.4	23	5
NPH, human insulin	0			10.5	41	44

The relative binding affinities for HSA and sIR varied less than 10 % in the two assays.

<sup>a</sup> N<sup>ε</sup>B<sup>29</sup>-tetradecanoyl des-(B30) HI represents the relative affinity of 1.00. The K<sub>a</sub> for binding of this analogue to HSA is 2.4 ± 0.7 × 10<sup>5</sup> l/mol at 23 °C (n = 5) and 1.0 ± 0.3 × 10<sup>5</sup> mol/l at 37 °C (n = 4)

<sup>b</sup> Coefficients of variation (c. v.) refer to variations between pigs

human albumin was immobilized on MiniLeak (Kem-En Tec, Copenhagen, Denmark), which is divinylsulphone activated Sepharose 6B, to a concentration of 0.2 mmol/l in the gel. The immobilized HSA was suspended into buffer at seven different concentrations ranging from 0 to 10 μmol/l. The buffer contained 0.1 mol/l Tris, adjusted to pH 7.4, and 0.025 % Triton X-100 for prevention of non-specific adhesion. After incubation with tracer amounts of Tyr<sup>A14</sup>(<sup>125</sup>I)-labelled insulin analogues for 2 h at 23 °C, free and albumin-bound insulin was separated by centrifugation. Plots of bound/free insulin vs albumin concentration were linear. The apparent association binding constant, K<sub>a</sub>, is found from the slope of the plots. The relative binding affinities of the insulins in Table 1 are the average of two assays, each of which included all the analogues.

Receptor binding affinities relative to human insulin, using a high affinity variety of the soluble insulin receptor sIR [20, 21], were determined in a similar assay. The sIR was immobilized on MiniLeak to a concentration of 1 nmol/ml in the gel. The immobilized sIR was diluted into the buffer to concentrations ranging from 0 to 20 pmol/ml. This buffer contained 0.1 mol/l Hepes, adjusted to pH 7.8, plus 0.1 mol/l NaCl, 0.01 mol/l MgCl<sub>2</sub>, and 0.025 % Triton X-100. Incubation with tracer amounts of labelled insulins and calculation of binding constants were performed as in the albumin binding assay, and the binding constants expressed relative to that of human insulin. The receptor binding affinities of the insulins in Table 1 are the average of two assays, each of which included all the analogues.

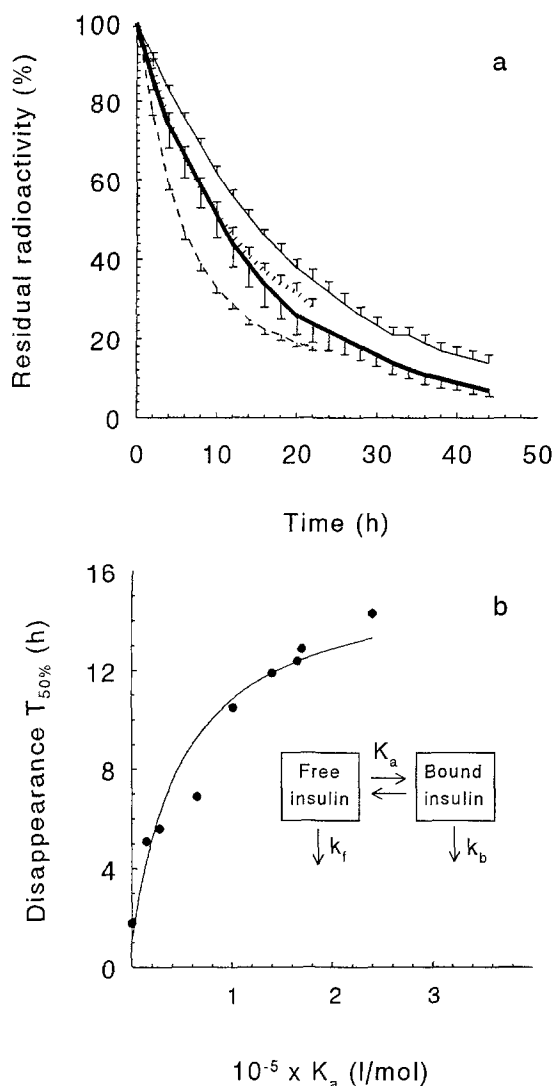
**Studies in pigs.** The principles of laboratory animal care were followed. Specific pathogen-free LYYD, non-diabetic female pigs, cross-breed of Danish Landrace, Yorkshire and Duroc, were used throughout (Holmenlund, Haarloev, Denmark). Pharmacokinetic and pharmacodynamic studies were carried out in conscious pigs 4–5 months of age and weighing 70–95 kg after being fasted overnight for 18 h. For tissue reaction studies pigs weighing 50–60 kg were used.

**Disappearance studies.** The disappearance from the subcutaneous injection site of a series of <sup>125</sup>I-labelled fatty acid acylated insulins, NPH and porcine albumin was measured using a modification [22] of the traditional external gamma-counting method [23, 24]. With this modified method it was possible to

a subcutaneous depot for several days using portable detectors. The measurements were performed at 1-min intervals, and the counting values were corrected for background activity. An insulin dose of 60 nmol (equal to 10 units of human insulin) was used and each pig received both one of the insulin derivatives and human NPH simultaneously in separate depots. The disappearance T<sub>50%</sub> values from 44 experiments using NPH did not follow the normal distribution, whereas the values from 32 experiments with NN-304 were normally distributed. Hence, the means and the variation in T<sub>50%</sub> for NPH and NN-304 were compared by the *t*-test and the two-sided variance ratio *F*-test of the log transformed values, respectively. The absorption of albumin was studied by injection of 5 nmol <sup>125</sup>I-labelled albumin in two separate subcutaneous depots.

**Glucose lowering effect after i. v. injection.** Lys<sup>B29</sup>-tetradecanoyl des-(B30) insulin (NN-304) or human insulin was injected intravenously through a catheter. The dose was 0.24 nmol/kg, and five pigs received both preparations in random order with a 1-week interval. Blood samples of 1 ml were drawn into heparinized glass tubes 15 and 10 min before administration and between 3 and 150 min after injection. The plasma glucose concentration for each time point was determined by a hexokinase method using a Technicon II analyser (Garges-les-Gonnesse, France). Since no assay was available capable of measuring NN-304 specifically in the presence of porcine insulin, no attempts were made to assess plasma insulin concentrations.

**Euglycaemic glucose clamp.** Five pigs received subcutaneous injections of NN-304 or human NPH in random order with an interval of 10 days. The dose was 216 nmol (equal to 36 units of human insulin), it was injected in three equal depots of 72 nmol, in order to administer a clinically relevant dose at each site and in order to counteract the large variation of the absorption of NPH. The pigs were kept euglycaemic at their individual fasting glucose levels (4.4 mmol/l) for 24 h by a variable rate intravenous infusion of a glucose solution, 1.5 mmol/l [25]. The infusion was given through a catheter inserted in the jugular vein and a Braun Infusomat Secura pump (Melsungen, Germany) was used. Depending on changes in plasma glucose concentrations observed during frequent plasma glucose mon-



**Fig. 2.** (a) Disappearance of radioactivity (mean  $\pm$  SEM,  $n = 6$ ) following subcutaneous injection of NPH insulin (thick line), Lys<sup>B29</sup>-decanoyl des-(B30) insulin (---), Lys<sup>B29</sup>-dodecanoyl des-(B30) insulin (····) and NN-304 (-·-·). (b) Correlation between the  $T_{50\%}$  for disappearance and the association constant,  $K_a$ , for binding of the acylated insulin to HSA. The curve is fitted assuming that the fraction of free insulin,  $\alpha_f$ , disappears with a first order rate constant  $k_f = 0.69 \text{ h}^{-1}$ , corresponding to  $T_{50\%} = 1 \text{ h}$  for the insulin monomer, and the fraction of albumin-bound insulin,  $(1-\alpha_f)$ , disappears with a rate constant  $k_b$ . The overall rate constant,  $k$ , for disappearance of the insulin can be expressed as:  $k = \alpha_f \times k_f + (1-\alpha_f) \times k_b$ ,  $\alpha_f$  depends on  $K_a$  and on the concentration of albumin in subcutis,  $[A] = 0.3 \text{ mmol/l}$ , and can be expressed as  $1/\alpha_f = K_a \times [A] + 1$ . The curve fits the experimental data points with a coefficient of correlation of 0.97 using the fitted  $k_b$  of  $0.043 \text{ h}^{-1}$ , which corresponds to a  $T_{50\%}$  of the albumin-bound fraction of about 16 h

glass tubes every 15 min, plasma was separated and glucose was determined within 1.5 min of blood sampling with a YSI (Yellow Springs Instrument, Yellow Springs, Ohio, USA) glucose analyser (glucose oxidase method). During the experiment the pigs were free to move in their pens. The paired  $t$ -test was used in the statistical comparison of NN-304 with NPH, assuming that the parameters were normally distributed

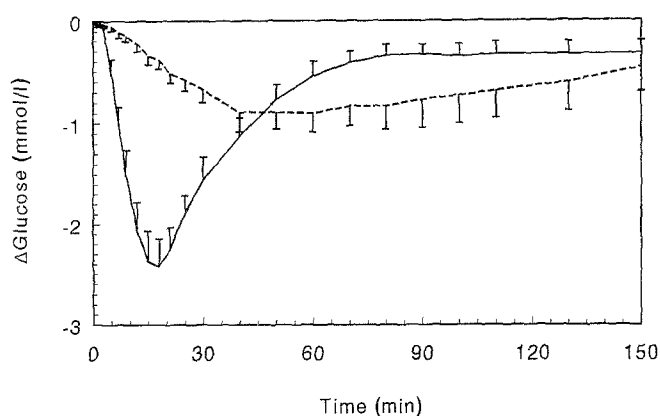
**Tissue reactions.** Four pigs were given subcutaneous injections of 200  $\mu\text{l}$  NPH (20 IU), NN-304 (120 nmol) and medium (1.5% glycerol, 0.3% phenol, 200 nmol  $\text{Zn}^{2+}/\text{ml}$ ) subcutaneously at distinct sites on the back 9, 5, 2, 1 day(s) and 12 h prior to killing by an overdose of pentobarbital. Human NPH and 0.9% NaCl were injected at adjacent sites as reference and control, respectively. All injections were given in a depth of 3 mm using a G28-cannula equipped with a stopper. Samples of skin ( $20 \times 20 \text{ mm}$ ) including underlying subcutis were fixed in Bouin's fixative.

**Light microscopy procedures.** All tissues were embedded in paraffin wax according to standard histological procedures. Sections 6–8  $\mu\text{m}$  thick were stained by haematoxylin eosin and iron haematoxylin Picro-Acid Fuchsin. Adjacent sections were stained immunohistochemically for insulin by subsequent incubations of the deparaffinized sections with 0.05 mol/l tris buffered saline (TBS) (5 min), 10% rabbit serum in 0.05 mol/l TBS (35 min), monoclonal mouse anti-human insulin (HUI.018; Novo Nordisk, Bagsvaerd, Denmark), diluted 1:50 in 10% rabbit serum in 0.05 mol/l TBS (40 min), 0.05 mol/l TBS containing 0.1% Triton X-100 ( $2 \times 5 \text{ min}$ ), 0.05 mol/l TBS (1 min), alkaline phosphatase conjugated to rabbit anti-mouse immunoglobulin (D314; Dako, Copenhagen, Denmark), diluted 1:50 in 10% rabbit serum in 0.05 mol/l TBS (30 min), and finally with 0.05 mol/l TBS ( $3 \times 5 \text{ min}$ ). As chromogen New Fuchsin was prepared from stock solutions of: (a) 1.52% New Fuchsin in 2 mol/l HCl; (b) 2.24% sodium nitrite; (c) 0.1 mol/l Tris/HCl, pH 8.2; (d) Tris 12.33 g, naphthol AS-MX-phosphatase 9.5 g in 440 ml 0.1 mol/l HCl, distilled water to a total volume of 1.0 litre, (BioGenex GmbH, Mainz, Germany) and (e) Levamisole 1 mol/l (Sigma, St. Louis, USA). Working solutions were prepared from 50  $\mu\text{l}$  (a), 50  $\mu\text{l}$  (b), 5000  $\mu\text{l}$  (c), 400  $\mu\text{l}$  (d) and 10  $\mu\text{l}$  of (e). Incubation for 11 min was followed by three washings for 5 min with 0.05 mol/l TBS, containing 0.1% Triton X-100, and after additional washing in running tap water for 10 min the sections were counterstained in Mayer's haematoxylin for 45 s. Finally, after 10 min of washing in running tap water the sections were dehydrated in ethanol, cleared in xylene and coverslips were mounted with Eukitt (O. Kindler & Co GmbH, Freiburg, Germany). All washings and incubations were carried out at room temperature. Three control sections without (a) primary antibody (b) secondary antibody or (c) chromogen, respectively, were treated similarly. All controls turned out negative, meaning that endogenous alkaline phosphatase had been sufficiently inhibited.

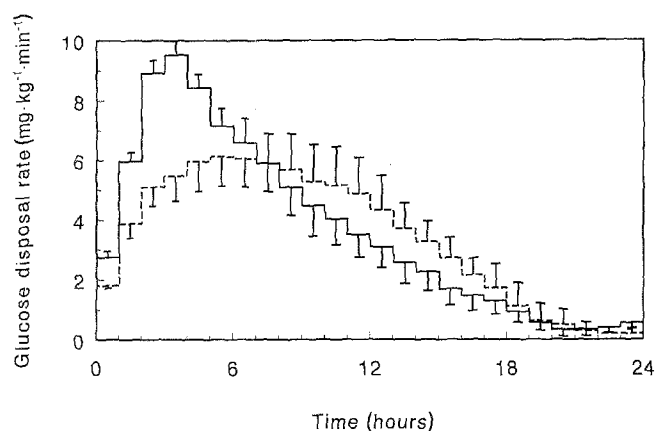
## Results

**Disappearance and binding studies.** The  $T_{50\%}$  for the des-(B30) insulins was found to increase with the number of C-atoms in the fatty acid up to 14 (Table 1). The fatty acid acylated insulins bound to HSA with binding constants in the order of  $10^4$  to  $10^5$  l/mol. The relative affinities for HSA varied less than 10% between the two assays. The maximal binding affinity was obtained for Lys<sup>B29</sup>-tetradecanoyl des-(B30) insulin, NN-304. The absolute values of the binding constants were different from those valid in vivo, because the  $K_a$  was determined in aqueous buffer at 23 °C. The  $T_{50\%}$  times were strongly correlated to the albumin binding constants, the coefficient of





**Fig. 3.** The plasma glucose lowering effect in five pigs after i.v. administration of 0.24 nmol/kg of human insulin (—) and NN-304 (- - -), respectively. Bars represent SEM. Initial glucose concentrations were  $4.6 \pm 0.1$  mmol/l for human insulin and  $4.5 \pm 0.4$  mmol/l for NN-304



**Fig. 4.** Euglycaemic glucose clamp in five pigs after subcutaneous injection of human NPH (—) and NN-304 (- - -), respectively. Bars represent SEM. The glucose target levels were set to the initial values, which were 4.4 mmol/l in both groups. The achieved controls were  $4.4 \pm 0.1$  mmol/l for NPH and  $4.4 \pm 0.2$  for NN-304 (mean  $\pm$  SD). The coefficients of variation of glucose concentrations within animal were  $13.6 \pm 3.7\%$  for NPH and  $11.2 \pm 2.9\%$  for NN-304 (mean  $\pm$  SD)

$T_{50\%}$  time ( $14.3 \pm 2.2$  h), significantly longer than that of NPH ( $10.5 \pm 4.3$  h) ( $p < 0.001$ ). The coefficients of variation of the  $T_{50\%}$  were 15% for NN-304 and 41% for NPH. Using the two-sided  $F$ -test on the log transformed data the  $F$ -value was calculated to 8.0, highly significantly different at  $p < 0.001$ .

The disappearance  $T_{50\%}$  of  $^{125}\text{I}$ -labelled porcine albumin after subcutaneous injection was found to be 44 h, substantially longer than that of any insulin.

The removal of the C-terminal residue of the B-chain (B30) resulted in an increase in the affinity for albumin by a factor of 1.7–1.8, as demonstrated for decanoylation and tetradecanoylation (Table 1). Tetradecanoylation of N<sup>ε</sup>-Lys<sup>B29</sup> in des-(B30) insulin resulted in a four times greater affinity for albumin

the des-(B30) series an increase in binding constant was correlated with fatty acid chain length from 10 to 14 carbon atoms, whereas a chain lengthening to 16 apparently failed to improve binding any further.

The affinity for the insulin receptor decreased somewhat in the des-(B30) series, from 76 to 19% relative to human insulin, as the fatty acid substituent of Lys<sup>B29</sup> increased from 10 to 16 C-atoms (Table 1). Both Phe<sup>B1</sup> and Lys<sup>B29</sup> tetradecanoylations resulted in the same slight decrease in affinity, indicating that neither type of substitution made close contact to the active site of insulin.

**Intravenous bolus.** The blood glucose lowering effect (Fig. 3) of NN-304 was clearly protracted as compared to the effect of human insulin, and the action appeared to continue towards the end of the study, 150 min after injection.

**Euglycaemic glucose clamp (Fig. 4).** The glucose disposal rate after a subcutaneous bolus of the soluble NN-304 was compared to that of human NPH in a 24-h euglycaemic glucose clamp. The areas under the curves, i.e. the total amounts of glucose infused,  $454 \pm 226$  g and  $469 \pm 114$  g, respectively, are not significantly different ( $p = 0.88$ ). The times when 50% of the glucose was infused,  $7.9 \pm 1.0$  h and  $6.2 \pm 1.3$  h were significantly different ( $p = 0.04$ ), as were the maximal infusion rates,  $6.6 \pm 2.2$  and  $9.9 \pm 0.8$   $\text{mg} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  ( $p = 0.01$ ) and the times to reach the maximal infusion rate,  $6.4 \pm 2.2$  h and  $3.4 \pm 0.2$  h ( $p = 0.03$ ), respectively for the two insulins, provided these last three parameters distributed normally.

**Histology (Fig. 5).** NPH gave a reaction in subcutis with numerous macrophages surrounding condensed heaps of NPH-crystals left in the interstitium and between fat cells in subcutis. The reaction was strong on day 1 (Fig. 5a) and turned to granulation tissue with mild formation of collagen without macrophages on day 5 and diminished on day 9. Insulin reaction was positive in NPH-crystals, macrophages with uptake and digestion of NPH-crystals, fibroblasts, reticular fibres, surface of collagen fibres, fat cell cytoplasm (but not in fat vacuoles) and vascular walls. Insulin reaction almost disappeared after 5 days. NN-304 showed a mild oedematous cellular infiltrate with granulocytes after 12 h, 1 day (Fig. 5b) and 2 days. The findings were uniform in all four pigs. After 5 and 9 days, only a mild formation of granulation tissue was seen. No macrophages were involved. Insulin reaction was positive in granulocytes and otherwise as above, bearing in mind the absence of crystals and macrophages. At all time points investigated, the medium itself showed a weak cellular infiltrate and oedema. In all pigs some injection sites, except those for the saline injections, had foci of foreign body reaction with formation of

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