Study of Nasal Enzyme Activity towards Insulin. In Vitro
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The possibility of insulin being enzymatically degraded in contact with the nasal mucosa has been studied in vitro. The insulin concentration was followed during 3h incubation at 37°C with freshly collected human nasal wash, isolated enzymes from pig and rabbit nasal mucosal tissue, leucine aminopeptidase and microsomal aminopeptidase, respectively. The rate of degradation with human nasal wash was found to be ≤0.02 µg/min, which indicates that less than 0.5% of an intranasally applied insulin dose may be destroyed by local enzymes during the time of absorption. The observed degradation was not found to be limiting for an intranasal application of insulin.

Keywords aminopeptidase; enzyme; insulin; intranasal; mucosa; nasal

Introduction
Among different non-parenteral routes, the intranasal route seems to be the most promising alternative for peptides and proteins.2) Insulin is an example of a peptide that has been extensively studied in recent years. The bioavailability of insulin is very low, when it is administered intranasally as a simple solution.3) Many investigations, however, have shown that insulin, in combination with an enhancer system4) or as a powder dosage form,5) can be delivered across the nasal mucosa in clinically relevant amounts. The reason for the observed low bioavailability could be: (1) the high molecular weight6) of insulin, in aqueous formulations, human insulin exists as dimers and tetramers (molecular weight: about 12 and 24 kDals, kDa)); (2) short contact time in the nasal cavity,7) the mucociliary clearance mechanism is extremely effective, inhaled particles (or droplets) are removed from the nasal cavity with a clearance half-life of 10—15 min8,9) or (3) local enzymatic degradation, the possibility exists that insulin may be degraded locally by peptidases prior to or during absorption.3,10,11)

Previous investigators have shown that insulin is degraded by enzymes isolated from rat9) and rabbit11) nasal mucosal tissue homogenate. The clinical relevance of this observation may have to be studied further, where some enzymes isolated from whole tissue may not be involved, in vivo, and the insulin doses studied may be too low.

The objective of this study is to investigate the enzymatic degradation of insulin at higher doses using the described animal tissue enzymes, as well as free enzymes isolated from the nasal cavity from humans after intranasal wash. The intention is to obtain a rough indication of whether enzymatic degradation is clinically important with respect to the low bioavailability of insulin after intranasal application.

Materials and Methods
Materials Zinc-free human insulin powder was obtained as a gift from Novo Nordisk A/S and prepared as a 0.08% w/v solution in 0.1 M Tris-buffer (Tris(hydroxymethyl)-aminomethane, pH = 7.4).
Leucine aminopeptidase (LAP, EC 3.4.11.2; 200000 U/mg protein), porcine kidney microsomal aminopeptidase (MAP, EC 3.4.11.2; 280000 U/mg protein) and t-leucine-4-methoxynaphthylamide were obtained from Sigma Chemicals (Sigma Chemie GmbH, Germany). The enzymes were dissolved by adding 0.04 M Tris-buffer (pH = 7.4), containing 0.008 M manganese sulphate (manganese(II) sulphate monohydrate) to either LAP or MAP to obtain a final concentration of 0.05% w/v.11) All other chemicals were of reagent grade.

Collection of Pig and Rabbit Mucosal Tissue Mucosal tissues from slain female DLR/Yorkshire bred pig (9 months, 90 kg) were kindly supplied and donated by Novo Nordisk A/S and stored at −70°C.

Rabbit mucosal tissue was obtained from male New Zealand white rabbits (about 2.3 kg), fasted overnight and killed by an injection of Nembutal. The mucosa was removed by making an incision along the length of the lateral wall on each side of the nasal septum followed by cutting and lifting the nasal bone frontally to expose the nasal cavity fully. Mucosal tissue was then carefully removed from underlying cartilage and bone and stored at −70°C.

Isolation of Nasal Enzymes The enzymes were isolated from the mucosal tissues as described by Stratford and Lee,12) but with slight modifications. Immediately before each experiment, about 400 mg of pig or about 100 mg rabbit mucosal tissue were thawed at room temperature for about 10 min and homogenized for 5 min in 1 ml of 0.1 M Tris-buffer at 4°C using an Ultra-turrax (TP 18/10 no: 71713) homogenizer. 3 ml Tris-buffer were added and this mixture was homogenized for a few more minutes using a manual Teflon-glass homogenizer. The homogenates were centrifuged at 7000 rpm (3640 x g) in a Beckman refrigerated centrifuge (model J-21) at 4°C for 10 min to remove cellular and nuclear debris. The resulting supernatants, were isolated and used directly to study the degradation of human insulin.

Collection of Human Nasal Wash Human nasal wash samples were collected by using a modification of the nasal washing method introduced by Rossen et al.13) While the volunteer was in a Moffs position14) with the head inclined forward, 2 ml of isotonic sodium chloride solution was instilled into each nasal cavity. After one min, specimens consisting of saline and a variable amount of secretion were collected into small glass vials while the volunteer’s head was still inclined forward. The volunteers did not blow their noses during the collection procedure. The specimens obtained were used immediately after sampling.

Design of Degradation Study The enzymatic degradation of insulin was determined by using the optimal conditions for the enzymes. A modified assay method for LAP was used.13) 500 µl of the above mentioned enzyme preparations were mixed with 1.0 ml of 0.1 M Tris-buffer, at 37°C. The reaction was initiated by adding 1.0 ml of 800, 80 or 10 µg/ml insulin solutions. The change in the insulin concentration was measured by withdrawal of 25 µl samples, at various time intervals up to 3h, for direct analysis on high performance liquid chromatography (HPLC).

Analysis of Insulin Insulin was analyzed by an HPLC method modified from the method described by Snel, Dangaard and Mollerup.15) The Hitachi HPLC-system was from Merck and consisted of a model 655A-11 pump, a model 655A variable wavelength ultraviolet (UV)-detector and a Rhodyne model 7125 injection valve. The column was a 4 x 250 mm LiChrosorb® RP-18, 5 µm, from Merck and the guard column was a 4 x 25 mm Purosorb® RP-8, 30—40 µm, from Merck.

The mobile phase was a mixture of two solutions A and B (4:3). Solution A consisted of 0.04 M phosphoric acid, 0.2 M sodium sulphate, 10% acetonitrile and H2O at 25°C with ethylalmine. Solution B was 50% acetonitrile solution. Detection at 214 nm; flow rate 1 ml/min; column temperature 25°C and injection volume 20 µl. The retention time (tR) was about 10 min. Sample concentration was calculated on the basis of peak height (hR) relative to external insulin standard (0.8 mg/ml in 0.1 N hydrochloric acid). The detection limit was found to be about 5 ng injected.
and the reproducibility was about 5%.

**Assay of Aminopeptidase Activity** Aminopeptidase activity was determined as described by Stratford and Lee, but with slight modifications. 100 μl of a tissue supernatant or nasal wash was preincubated in 2.8 ml 0.04 M Tris-buffer containing 0.008 M manganese sulfate (pH = 7.4) for 15 min in a water bath at 37°C. The reaction was initiated by adding the mixture, 100 μl of 10 mM L-leucine-methoxy-2-naphthylamide. The fluorescence intensity was monitored at an excitation wavelength of 342 nm and an emission wavelength of 426 nm (Hitachi, Fluorescence spectrophotometer F-2000). The initial fluorescence intensity was found to be linear for all samples studied, and was expressed in terms of mIU per mg protein after correcting for chemical hydrolysis.

The protein concentration for tissue supernatant or nasal wash was kindly determined by standard methods at the Department of Clinical Chemistry, Bispebjerg Hospital (Copenhagen, Denmark).

**Results**

The enzymatic activity in human nasal wash and in isolated enzymes from rabbit and pig nasal tissue homogenate are shown in Table I. The table shows that all preparations contain active enzymes under the experimental conditions, characterized as mIU/mg protein.

Table II shows the degradation rate of insulin after incubation with human nasal wash, enzymes isolated from pig and rabbit nasal mucosa, and pure enzymes, respectively. The rate of degradation, if any, in human nasal wash, isolated pig and rabbit enzymes, were measured to be ≤ 0.02, 0.09 and 0.03 μg/min, respectively. The table shows that the recovery of insulin, after 3 h incubation at 37°C, was above 96%, except for enzymes isolated from rabbit nasal mucosa. Experiments performed with rabbit nasal mucosa were carried out using amounts of insulin which are much lower than clinically relevant amounts.

**Discussion**

This study shows that clinically relevant amounts of insulin, incubated in freshly collected human nasal wash or in isolated enzymes from rabbit and pig nasal mucosa, do not show any pronounced degradation. The incubation of insulin with human nasal wash imitates the exposure of insulin to the enzymes present in the nasal cavity. The incubation with enzymes isolated from animal mucosal tissue also imitates the exposure taking place during the passage of insulin through the membrane into the circulation.

As shown in Table II the rate of degradation (if any) in human nasal wash, was found to be ≤ 0.02 μg/min. This rate is very slow in relation to one clinical intranasal dose of about 2000 μg insulin. Based on the above results, this would indicate that a max. 0.5% of the applied insulin may be destroyed by local enzymes during the time of absorption (about 30 min). Incubation with lower doses of insulin was not of clinical relevance.

Previously, Hirai et al. and Hayakawa et al. have studied the enzymatic degradation in isolated enzymes from rat and rabbit nasal mucosa, respectively. Hirai et al. incubated 1.67 μg insulin with the enzyme preparations finding that 0.025 μg/min was degraded. Hayakawa et al. incubated 0.3 and 0.03 μg insulin, and showed that the rates of degradation were 0.00035 and 0.00098 μg/min, respectively. Compared to Table II, the rates of degradation from various preparations were found to be about 0.017 to 0.092 μg/min. Having in mind that one clinically relevant dose is about 2000 μg, these rates may not be able to degrade significant amounts of insulin within the time of absorption.

As seen in Table II, incubation at higher concentrations makes rate determination difficult because the observed degradation of insulin after 3 h is only a few %, which is close to the analytical variation of about 5%. Although not all tissue enzymes may be relevant, the observed low degradation rate for insulin may indicate a low degradation potential. Besides, enzymes may leak out from the cells in vivo and some penetration enhancers may promote transcellular absorption of insulin. In order to estimate a significant degradation rate 10 μg/ml insulin was incubated with rabbit tissue enzymes. The total amount of incubated insulin was 10 μg, which is only about 0.5% of a possible clinical intranasal dose of 2000 μg insulin. Only about 0.025 μg/min was degraded. Results obtained with enzymes from rabbit mucosal homogenate were, as mentioned, performed with a very low concentration so as to estimate the rate of degradation more precisely and to compare our observations with previously published results. The experiments showed a linear decrease in the insulin concentration with time, indicating that the degradation of insulin followed zero order kinetics. Furthermore, our results did not differ from those previously published.

Hirai et al. reported that the pure enzymes, such as LAP, cleave the B-chain of insulin. No significant degradation of insulin was found together with pure LAP or MAP, when clinically relevant amounts of insulin were used.

In conclusion, only a relatively small degradation of

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**Table I. The Enzymatic Activity towards L-Leucine-4-methoxy-2-naphthylamide and Protein Concentration in Various Preparations**

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Enzymatic activity (mIU/mg protein)</th>
<th>Protein concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human nasal wash</td>
<td>37</td>
<td>0.35</td>
</tr>
<tr>
<td>Isolated pig enzymes</td>
<td>25</td>
<td>2.8</td>
</tr>
<tr>
<td>Isolated rabbit enzymes</td>
<td>36</td>
<td>1.0</td>
</tr>
<tr>
<td>No enzymes</td>
<td>0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

**Table II. Recovery of Insulin during 3h Incubation at 37°C in the Presence of Various Nasal Enzymes**

<table>
<thead>
<tr>
<th>Preparations</th>
<th>Insulin conc. (μg/ml)</th>
<th>Number of experiments</th>
<th>Degradation rate (ng/min)</th>
<th>% recovery</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human nasal wash</td>
<td>32</td>
<td>6</td>
<td>17</td>
<td>96</td>
<td>94—98</td>
</tr>
<tr>
<td>Isolated pig enzymes</td>
<td>320</td>
<td>3</td>
<td>92</td>
<td>98</td>
<td>95—102</td>
</tr>
<tr>
<td>Isolated rabbit enzymes</td>
<td>4</td>
<td>2</td>
<td>46</td>
<td>99</td>
<td>99—99</td>
</tr>
<tr>
<td>LAP (EC 3.4.11.1)</td>
<td>320</td>
<td>5</td>
<td>46</td>
<td>99</td>
<td>86—107</td>
</tr>
<tr>
<td>MAP (EC 3.4.11.2)</td>
<td>320</td>
<td>6</td>
<td>46</td>
<td>89</td>
<td>85—93</td>
</tr>
</tbody>
</table>
intranasal insulin was observed in this study. These observations may be studied further, e.g., in tissue and enzymes isolated from human nasal mucosa and at other insulin concentrations. The apparently low degradation of insulin in combination with the various isolated enzymes indicates that the suggested "enzymatic barrier" to the absorption of insulin via the nasal route may not be limiting. It is therefore likely that the low absorption rate in combination with a rapid ciliary clearance of insulin from the nose are responsible for the low bioavailability of nasally applied insulin in simple solutions. Our results seem to correlate with the results observed by Hirai et al. and Hayakawa et al., and due to the observed low degradation, even with lysosomal enzymes, it may be concluded that enzymatic degradation in the nasal cavity is not limiting for the intranasal application of insulin.

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References and Notes