Changes in Immunospecificity and Biologic Activity of Bovine Insulin Due to Subsequent Removal of the Amino Acids B_1, B_2 and B_3

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SUMMARY

The influence of splitting off the N-terminal amino acids B1, B2 and B3 of bovine insulin on the binding of these derivatives to antibodies raised with bovine insulin in guinea pigs and on biologic insulin activities is investigated.

In comparison with bovine insulin the standard free energies $-\triangle F^{\circ}_1$ for complex formation between high affinity antibodies Ak_1 and des-Phe^{B1}-insulin or des-(Phe-Val)$^{B1-2}$-insulin are reduced to 77 per cent and 76.3 per cent respectively, with a further decrease to 68 per cent for des-(Phe-Val-Asn)$^{B1-3}$-[Pyr^{B4}] insulin.

The standard free energy $-\triangle F^{\circ}_2$ for complex formation between low affinity antibodies Ak_2 and des-Phe^{B1}-insulin drops to 48.4 per cent. Further splitting off of the N-terminal amino acids B2 and B3 completely abolishes the antigen binding to Ak_2.

The ability of both des-Phe^{B1}-insulin and des-(Phe-Val)$^{B1-2}$-insulin to lower the in vivo blood sugar level does not differ significantly from bovine insulin, whereas the in vivo activity of des-(Phe-Val-Asn)$^{B1-3}$-[Pyr^{B4}] insulin decreases to 70±7 per cent.

The in vitro biologic activity, tested on isolated fat cells, is slightly reduced to 89±9 per cent for des-Phe^{B1}-insulin, to 86±3 per cent for des-(Phe-Val)$^{B1-2}$-insulin and to 68±6 per cent for des-(Phe-Val-Asn) $^{B1-3}$-[Pyr^{B4}] insulin. DIABETES *23*:651-56, August, 1974.

Lindsay and Shall[16] studied the immunologic and biologic properties of bovine insulin modified by acetylation. Substitution of the N-terminal amino acid of the B-chain phenylalanine (Phe^{B1}) by acetyl-, acetoacetyl- and thiazolidine groups led to a large decrease in the immunoreactivity of bovine insulin for insulin antibodies raised in guinea pigs, whereas these substitutions did not affect biologic activities. Brandenburg[4] split off Phe^{B1} in bovine des-Phe^{B1}-insulin and found this derivative to be biologically active and immunologically identical with insulin.

In the present paper the effects of the loss of the N-terminal amino acids in des-Phe^{B1}, des-(Phe-Val)$^{B1-2}$- and des-(Phe-Val-Asn)$^{B1-3}$-bovine insulin on the binding with antibodies raised in guinea pigs with bovine insulin were examined.

To determine the changes in immunospecificity due to the chemical modifications, the standard free energies of both the complex formation of bovine insulin and the modified insulins with bovine insulin antibodies were measured.

MATERIAL AND METHODS

Crystalline bovine insulin (27 U./mg.) from Farbwerke Hoechst AG was used as standard as well as starting material for preparing the chemically modified insulins and the ^{131}I-labeled insulin (9.0-11.2 mCi/mg.).

Preparation of the insulins with shortened B-chain*: $N^{\alpha A1}$, $N^{\epsilon B29}$-Bis-Boc-insulin was reacted with phenylisothiocyanate to give $N^{\alpha B1}$-Ptc-$N^{\alpha A1}$, $N^{\epsilon B29}$-Bis-Boc-insulin which in the Edman degradation furnished des-Phe^{B1}-insulin.[4,8,10] The repetition of this cycle once or twice resulted in des-(Phe-Val)$^{B1-2}$-insulin and des-(Phe-Val-Asn)-$^{B1-3}$-insulin, the N-terminal glutamine of which, however, is unstable and is cyclized to pyroglutamic acid.

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**Abbreviations.* Bis-Boc-insulin: substituted bovine insulin with two tertiary butyloxycarbonyl radicals in position $N^{\alpha A1}$ and $N^{\epsilon B29}$; Ptc: *p*henyl*t*hio*c*arbamoyl residue; Pyr^{B4}: B4-pyroglutamyl residue.

The final product of this reaction step is therefore des-(Phe-Val-Asn)$^{B1-3}$- [B4-pyroglutamyl] insulin.[8,10] The compounds des-Phe^{B1}-insulin, des-(Phe-Val)$^{B1-2}$-insulin and des-(Phe-Val-Asn)$^{B1-3}$-[Pyr^{B4}] insulin were purified by gel filtration on Sephadex G-50 in acetic acid (column dimensions 2.5 x 200 cm.). In addition the compound des-(Phe-Val-Asn) $^{B1-3}$ -[Pyr^{B4}] insulin was subjected to partition chromatography on Sephadex LH 20 in a solvent system of n-butanol:acetic acid:water (8:4:40) (column dimensions 3.5 x 100 cm.). The compounds des-Phe^{B1}-insulin and des-(Phe-Val)$^{B1-2}$-insulin were crystallized in citrate buffer at pH 5.2. Compound des-(Phe-Val-Asn) $^{B1-3}$ -[Pyr^{B4}] insulin, which does not crystallize with the same ease, was finally crystallized after additional purification from phenolate buffer at pH 6.5.[10]

The splitting off of the N-terminal amino acids was confirmed in an amino acid analyzer (Unichrom, Beckman Instruments). The absence of Ptc residues was established by determining the quotient $E_{250}/E_{275} \cong 0.5$.[4]

Biologic tests

The in vitro insulin activity was measured using isolated fat cells.[17] The concentrations of the standard bovine insulin and of the modified insulins in the incubation medium were increased stepwise as follows: 0.037 ng., 0.111 ng., 0.333 ng., 1.0 ng., 3.0 ng., 30.0 ng. per milliliter. Formation of $^{14}CO_2$ from 1-^{14}C-glucose by the isolated fat cells, plotted semilogarithmically against the concentrations of the insulins, was used as a parameter of the biologic activity.

A concentration of 0.37 ng. (10 μU.) bovine insulin per milliliter was chosen for multiple estimations of the biologic activities of the modified insulins in relation to the standard bovine insulin (figure 1).

The in vivo biologic activities of the modified insulins were determined by the lowering of the blood sugar in rabbits.[19]

Immunologic technics

Twenty colony-bred guinea pigs were immunized with bovine insulin, each animal receiving four injections of 2 U. bovine insulin per 100 gm. body weight. The first application of antigen was carried out with complete Freund's adjuvant, the three following injections with incomplete Freund's adjuvant. Antisera, which were obtained eight days after the last injection of antigen, were pooled and stored at -20° C. without prior thawing.

The antibody binding of bovine ^{131}I-insulin was determined by preparative ultracentrifugation[13,15] at

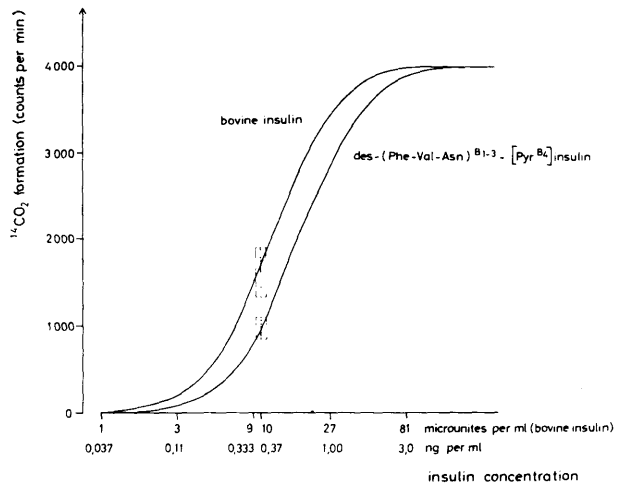

FIG. 1. Measurement of biologic activity of des-(Phe-Val-Asn)$^{B1-3}$-[Pyr^{B4}] insulin in comparison with bovine insulin using isolated fat cells.[17] The activities are calculated from multiple estimations of the rate of $^{14}CO_2$ formation within the steepest range of the bovine insulin calibration curve at insulin concentrations of 0.37 ng./ml.

constant antibody concentrations and increasing concentrations of bovine ^{131}I-insulin until approximate saturation of the antibody binding sites was reached. For this purpose the serum concentration (0.3 per cent serum in 0.04 Mols/L. phosphate buffer at pH 8.0 with addition of 0.25 per cent human serum albumin) was kept constant and the concentration of bovine ^{131}I-insulin increased in eleven steps from 1.28 to 1,290.0 x 10^{-9} Mols/L. The mixtures were allowed to come to an equilibrium between free and antibody-bound insulin during a thirty-six hour period at 4° C. Free and antibody-bound insulin were separated by subsequent ultracentrifugation (eight hours, 4° C., 120,000 x g.). During this procedure free insulin constitutes a concentration gradient due to its own gravity. This gradient was checked for each run in antibody-free ultracentrifuge tubes and found to be constant within the range of ^{131}I-insulin concentrations employed.

This concentration gradient allows the calculation of free insulin (F) in the sediment from the measured ^{131}I-insulin concentration in the supernatant. The concentration of antibody-bound insulin (B) is given as the difference between the measured ^{131}I-insulin concentration in the sediment and (F).

Changes in the antibody binding of modified insulin antigens were determined in two further steps. Firstly, nonlabeled bovine insulin was added in equimolar concentrations at each concentration step of the bovine ^{131}I-insulin binding curve. Secondly, the

nonlabeled bovine insulin was replaced by equimolar concentrations of the modified bovine insulin under test. The antibody binding of ^{131}I-insulin with and without addition of unlabeled insulin, as well as with addition of modified insulin, was measured within the same ultracentrifugation run.

The experimental results were interpreted according to the conclusions of Berson and Yalow,[2] that is, that the two main components of the antibody combining sites react with a univalent insulin antigen, which does not necessarily imply that there is only a single antigenic site. The average association constants for the binding of bovine insulin

$$k_1 = \frac{[Ak_1 - insulin]}{[insulin_{free}] * [Ak_{1\,free}]}$$

$$k_2 = \frac{[Ak_2 - insulin]}{[insulin_{free}] * [Ak_{2\,free}]}$$

for each main component of antibodies Ak_1 and Ak_2 were determined by plotting B/F vs. B according to the Scatchard plot[18] using the modification of the plot and the method of curve fitting by Berson and Yalow.[2]

The readings of the intercepts of the asymptotic straight lines with ordinate and abscissa to determine k's and Ak's were done on curve drawings with amplified scales. Following Berson and Yalow,[2] the construction of the asymptotic straight lines was adjusted until the binding curves calculated from Ak's and k's gave a good fit to the experimental points.

The added nonlabeled insulin competes with the ^{131}I-insulin for antibody binding sites. This causes a shift of the extrapolation curve for the antibody binding of bovine insulin toward the ordinate if the amount of antibody-bound ^{131}I-insulin alone is taken into account. As shown in figure 2, the addition of nonlabeled bovine insulin in an equimolar ratio (1:1) to each concentration step of the binding curve brings about a reduction of the antibody-bound ^{131}I-insulin precisely by one half. Inhibition of antibody binding of ^{131}I-insulin by a modified insulin is determined from the change in the intercepts of the asymptotic straight lines on the abscissa. Concerning the antibody binding to Ak_1, the inhibitory action of nonlabeled insulin is given by intercept a, the inhibitory action of the modified insulin by intercept b in figure 2.

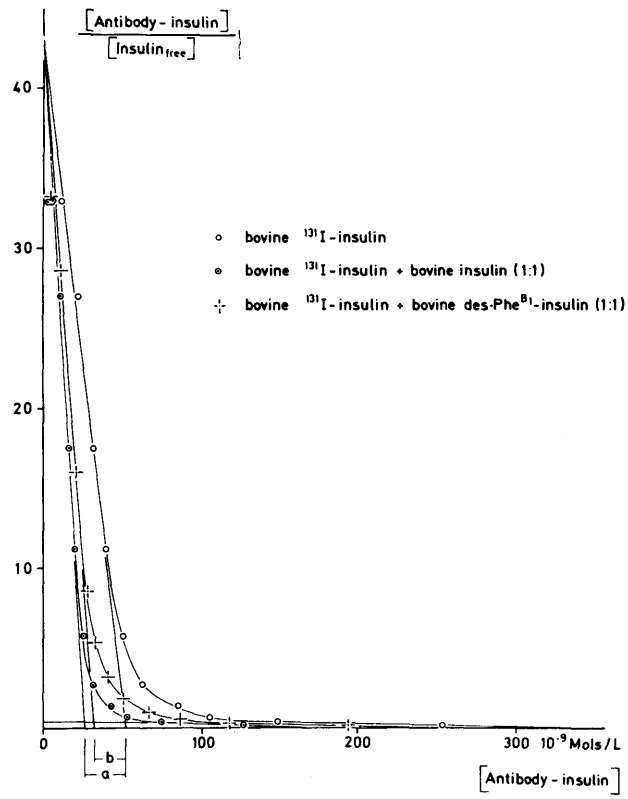

FIG. 2. Experimental results for the determination of the association constants k_1 and k_2 and of the free energies $-\Delta F^\circ_1$ and $-\Delta F^\circ_2$ for the binding of des-Phe^{B1} -insulin to beef insulin antibodies from guinea pigs. The $-\Delta F^\circ$ values are calculated from the inhibitory activity of the homologous bovine insulin compared with that of bovine des-Phe^{B1}-insulin upon the binding of bovine ^{131}I-insulin.

According to Kabat,[11] the inhibition of the antibody binding of the homologous antigen by a modified antigen is proportional to its standard free energy. Therefore the following equation holds:

$$\frac{\text{inhibitory action of insulin}}{\text{inhibitory action of modified insulin}} = \frac{-\Delta F^\circ_{(insulin)}}{-\Delta F^\circ_{(modified\ insulin)}} \quad (1)$$

From the measured association constants k_1 and k_2 for the binding of bovine ^{131}I-insulin, it is possible to calculate the values of $-\Delta F^\circ_1$ (insulin) and $-\Delta F^\circ_2$ (insulin) using equation (2)

$$-\Delta F^\circ = R \cdot T \cdot \ln k \quad (2)^*$$

*R = gas constant; T = absolute temperature.

By substituting these values in equation (1), the standard free energies $-\Delta F_1^\circ$ (modified insulin) and $-\Delta F_2^\circ$ (modified insulin) are obtained. Finally the association constants k_1 and k_2 for the antibody binding of the modified insulin are calculated using equation (2).

RESULTS

Biologic activities

The results obtained for the modified bovine insulins by the fat-cell test are compiled in table 1.

The fat-cell test shows a small but significant reduction of the in vitro biologic activities of des-Phe^{B1}-insulin and des-(Phe-Val)$^{B1-2}$-insulin. Splitting off of the three N-terminal amino acids of the B-chain in des-(Phe-Val-Asn)$^{B1-3}$-[Pyr^{B4}] insulin decreases the biologic activity to a greater extent.

The blood sugar lowering in vivo activity is reduced to 70 ± 7 per cent for compound des-(Phe-Val-Asn)-$^{B1-3}$-[Pyr^{B4}] insulin, whereas the in vivo activities of des-Phe^{B1}-insulin (97 ± 5 per cent) and des-(Phe-Val)$^{B1-2}$-insulin (95 ± 5 per cent) do not differ significantly from that of bovine insulin.

Immunologic activities

The antibody binding curve of bovine ^{131}I-insulin recorded in the presence of unlabeled des-Phe^{B1}-insulin in equimolar concentrations deviates noticeably from the binding curve recorded in the presence of equimolar concentrations of unlabeled bovine insulin, showing that des-Phe^{B1}-insulin competes to a lesser extent than bovine insulin for antibody binding sites (figure 2). In comparison with bovine insulin, k_1 for the complex formation with des-Phe^{B1}-insulin drops from 8.32 x 10^8 L./Mols to 7.39 x 10^6 L./Mols and k_2 from 1.34 x 10^6 L./Mols to 0.92 x 10^3 L./Mols. The further splitting off of valine in des-(Phe-Val)$^{B1-2}$-insulin reduces k_1 from 7.39 x 10^6 L./Mols to 6.40 x 10^6 L./Mols, whereas k_2 drops from 0.92 x 10^3 L./Mols to zero (table 2).

TABLE 1

Biologic activities of 0.37 ng./ml. of the modified insulins compared with 0.37 ng./ml. bovine insulin as measured by $^{14}CO_2$ formation from 1-^{14}C-glucose by isolated fat cells

bovine insulin (n = 17)	100% ± 8%*
des-Phe^{B1}-insulin (n = 11)	89% ± 8%*
des-(Phe-Val)$^{B1-2}$-insulin (n = 11)	86% ± 3%*
des-(Phe-Val-Asn)$^{B1-3}$-[Pyr^{B4}] insulin (n = 9)	68% ± 4%*

*Standard deviation s_x. Student t-test for each comparison with bovine insulin: $p < 0.005$.

TABLE 2

Association constants and standard free energies for the binding of bovine insulin and its derivatives by antibodies raised with bovine insulin in guinea pigs

	k_1 L./Mols	$-\Delta F^\circ_1$ kcal/Mols (4° C.)	k_2 L./Mols	$-\Delta F^\circ_2$ kcal/Mols (4° C.)
bovine insulin n = 3	8.32 x 10^8 s_x: ±0.5	11.30 ±0.03	1.34 x 10^6 ±0.07	7.76 ±0.03
des-Phe^{B1}-insulin	7.39 x 10^6	8.70	0.92 x 10^3	3.76
des-(Phe-Val)$^{B1-2}$-insulin	6.40 x 10^6	8.62	0	0
des-(Phe-Val-Asn)$^{B1-3}$-[Pyr^{B4}] insulin	1.16 x 10^6	7.68	0	0

The loss of the three N-terminal amino acids of the B-chain in des-(Phe-Val-Asn)$^{B1-3}$-[Pyr^{B4}] insulin, compared with des-(Phe-Val)$^{B1-2}$-insulin, brings about a further reduction of k_1 by nearly one power of ten from 6.4 x 10^6 L./Mols to 1.16 x 10^6 L./Mols. Thus k_1 for this derivative is nearly three orders of magnitude lower than the association constant k_1 for the complex formation with bovine insulin. As in the case of des-(Phe-Val)$^{B1-2}$-insulin, k_2 for des-(Phe-Val-Asn)$^{B1-3}$-[Pyr^{B4}] insulin remains at zero.

The $-\Delta F^\circ$ values decrease to a lesser degree than the k's, because $-\Delta F^\circ$ is proportional to ln k [equation (2)].

DISCUSSION

1. *Biologic activities*

In agreement with the effects of various substitutions at the Phe^{B1} position,[5,16] splitting off Phe^{B1} as reported by Weber and Weitzel[20] and Brandenburg,[4] did not affect the biologic activity. The biologic activity of des-Phe^{B1}-bovine insulin, now produced by a different method, remains at about 100 per cent in blood sugar lowering effect and at 89 per cent in the isolated fat-cell test. Splitting off the two N-terminal amino acids in des-(Phe-Val)$^{B1-2}$-bovine insulin does not lead to a further reduction of the biologic activities, whereas des-(Phe-Val-Asn)$^{B1-3}$-[Pyr^{B4}] insulin retains only about 70 per cent of biologic activity in both assays. From these results it may be concluded that the region B1 and B2 of the B-chain is not of great importance for binding the molecule to the receptor. This is in agreement with studies on the molecular structure of insulin insofar as the N-terminal part of the B-chain is situated on the sur-

face of the molecule at some distance from the molecular regions constant for different species, which are held to be responsible for the insulin binding to the receptor.[3] On the basis of the structural model,[3] it may also be assumed that splitting off the two N-terminal amino acids of the B-chain would hardly introduce conformational changes in bovine insulin. Removal of the third amino acid, however, may introduce slight conformational changes within the molecule.[10]

2. *Immunologic activities*

The test system allows a quantitative determination of the loss in affinity calculated from changes in the standard free energy $-\Delta F^\circ$ and in the association constants k for binding between modified bovine insulin antigens and specific antibodies. These changes in affinity can be measured separately for each of the main components of insulin binding IgG-antibodies Ak_1 and Ak_2, occurring simultaneously in all species investigated, also being directed against different regions of the insulin molecule[14] and different in clinical effects.[12]

Comparison of the binding of bovine insulin and modified insulins by the antibody component Ak_1 with high affinity reveals that Phe^{B1} contributes 2.6 kcal/Mols i.e. 23 per cent of the total standard free energy $-\Delta F^\circ_1$ of the antigen-antibody linkage. Splitting off $(Phe\text{-}Val)^{B1\text{-}2}$ does not bring about a further diminution of $-\Delta F^\circ_1$ in comparison with des-Phe^{B1}-insulin. The additional loss of the amino acid Asn^{B3} in des-$(Phe\text{-}Val\text{-}Asn)^{B1\text{-}3}$-$[Pyr^{B4}]$ insulin results in a further decrease of standard free energy ΔF°_1. The share of $(Phe\text{-}Val\text{-}Asn)^{B1\text{-}3}$ amounting to 3.62 kcal/Mols accounts for about one third of the total standard free energy for the binding of the bovine insulin antigen to Ak_1.

Concerning the antibody component Ak_2 with low affinity, the Phe^{B1}-share of the standard free energy for the antigen-antibody binding amounts to 4.0 kcal/Mols, i.e. more than half the total standard free energy. Splitting off $(Phe\text{-}Val)^{B1\text{-}2}$ or $(Phe\text{-}Val\text{-}Asn)^{B1\text{-}3}$ reduces the free energy of binding to nonmeasurable values.

In contrast to a nearly preserved in vivo activity and a slightly decreased in vitro activity of des-Phe^{B1}- and des-$(Phe\text{-}Val)^{B1\text{-}2}$-insulin, the antibody binding of these compounds is strongly reduced for k_1 and $-\Delta F^\circ_1$ and even more for k_2 and $-\Delta F^\circ_2$ (table 3).

From these findings it can be concluded that the removal of the two N-terminal amino acids Phe^{B1} and Val^{B2} from bovine insulin affects the biologic and immunologic properties to a different extent. The

TABLE 3

Comparison of biologic activity and immunospecificity due to subsequent splitting off the N-terminal amino acids B1, B2 and B3 of bovine insulin.

The biologic in vivo and in vitro activities of bovine insulin, the association constants k_1 and k_2 and the standard free energies $-\Delta F^\circ_1$ and $-\Delta F^\circ_2$ for the antibody binding of the homologous insulin antigen are made equal to 100 per cent.

	Biologic		Immunologic			
	in vivo	in vitro	k_1	$-\Delta F^\circ_1$	k_2	$-\Delta F^\circ_2$
bovine insulin	100±6%	100±8%	100%	100%	100%	100%
des-Phe^{B1}-insulin	97±5%	89±9%	0.89%	77%	0.69%	48.4%
des-$(Phe\text{-}Val)^{B1\text{-}2}$-insulin	95±5%	86±3.5%	0.77%	76.3%	0%	0%
des-$(Phe\text{-}Val\text{-}Asn)^{B1\text{-}3}$-$[Pyr^{B4}]$ insulin	70±7%	68±6%	0.14%	68%	0%	0%

biologic activities of bovine insulin molecules modified by splitting off the first two N-terminal amino acids and also the ease with which they can be crystallized, suggest that no conformational changes have taken place. Consequently it must be concluded from the considerable loss of standard free energy between the modified antigens and the two main components of insulin-binding antibodies that the N-terminal region of the B-chain of bovine insulin makes a major contribution to the points of insertion and to one of the determinant molecular regions. Studies on antigenic sites of peptide antigens have shown that a complete determinant group consists of five to six[1] or more[6,7] amino acids. Therefore it must be assumed that amino acid sequences such as A9 - A10 and A12 - A15, both in the vicinity of the N-terminal region of the B-chain and situated on the surface of the molecule, contribute to the formation of a complete determinant group in this area of the molecule.

For the derivative des-$(Phe\text{-}Val\text{-}Asn)^{B1\text{-}3}$-$[Pyr^{B4}]$ insulin, the decrease in the standard free energy $-\Delta F^\circ_1$ is almost identical with the reduction of the biologic in vivo and in vitro activities. $-\Delta F^\circ_2$, being reduced to nonmeasurable values, shows no correspondence to the degree of diminution of biologic activities. From the identical diminution of the biologic activities and of the standard free energy $-\Delta F^\circ_1$, one might conclude that the change in the association constants for the binding of des-$(Phe\text{-}Val\text{-}Asn)^{B1\text{-}3}$-$[Pyr^{B4}]$ insulin to the biologic receptor and to the antibody component Ak_1 would be identical. From this one might assume that the chemical alteration of insulin due to splitting off $(Phe\text{-}Val\text{-}Asn)^{B1\text{-}3}$ concerns a molecular region being involved in binding both to the biologic recep-

tor and also to antibody binding site Ak1 with high affinity. However, in relation to these considerations it must be taken into account that des-(Phe-Val-Asn)$^{B1-3}$[Pyr^{B4}] insulin does not crystallize with the same ease as des-Phe^{B1}- and des- (Phe-Val) $^{B1-2}$-insulin, [10] so that small conformational changes are possible. The decrease in biologic and immunologic activity of des-(Phe-Val-Asn) $^{B1-3}$-[Pyr^{B4}] insulin should therefore not necessarily be interpreted as a direct consequence of the local operation on the molecule.

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