RETARD-INSULINS FORMED BY COMPLEXING WITH POLYAMINO ACIDS

Günter Losse[a], Wolfgang Naumann[a], Heike Raddatz[a] and Joachim Koszowicz[b]

[a] *Technical University of Dresden, Chemical Section, Mommsenstrasse 13, Dresden, 8027, G.D.R. and*
[b] *VEB Berlin-Chemie, Glienicker Weg 129 PF 9, Berlin, 1199, G.D.R.*

Received December 10th, 1987
Accepted February 5th, 1988

Dedicated to the memory of Dr Karel Bláha.

Polyglycine, polyalanine, polyleucine, poly-α-glutamic acid, poly-γ-glutamic acid and poly-α--lysine were complexed with insulin under non-denaturating conditions. The liberation behaviour of the hormone was investigated in vivo and in vitro in dependence on the insulin content, molecular weight, ionic interactions and hydrophobicity of the polyamino acid. The in vitro results were confirmed by the in vivo experiments with animals. They clearly pointed out the influence of physicochemical parameters on the bioavailability of insulin. Complexes of poly-α--lysine and polyglycine were shown to be the most suitable retard forms, producing significant blood glucose lowering effects over 12 hours.

Complexing with proteins represents one of the possibilities of chemical modification of insulin[1,2] suitable for obtaining depot forms. Although recently the importance of the retard insulins has increased considerably[3], polymers enabling studies of the structural influences on the bioavailability of the hormone have not been applied methodically so far. In this work relevant studies are reported for the attempt to design polymer-based complexes of insulin with improved depot effects.

From the view of a broad variability in connection with good biological compatibility of the complexes to be synthesized, we have chosen poly-L-amino acids* (PAA) of various types of structure and different molecular weights as complexing agents. These compounds enable to study versatile influences with respect to the insulin binding capacity, retarding effect, biological compatibility and other physico--chemical and biological properties of the complexing agent.

* All the chiral amino acids mentioned in this work are of L-series. Nomenclature and abbreviations follow the recommendations of IUPAC-IUB-Commission on Biochemical Nomenclature: Eur. J. Biochem. *138*, 9 (1984). Additional abbreviations used are: DCCI dicyclohexylcarbodiimide, DCHA dicyclohexylamine, DCA dichloroacetic acid, DMF dimethylformamide, TFA trifluoroacetic acid.

We gave preference to the active ester[4] method for polycondensation of the monomers over the method using N-carboxy anhydrides, in order to limit the degrees of polymerization of the PAA and to obtain experimentally controllable solubility ratios. Pentachlorophenyl esters[5] which can be easily purified, have been utilized for the preparation.

As a starting compound for the preparation of poly(α-Glu) we used HCl.Glu-(OBzl)-OPcp prepared from glutamic acid γ-benzyl ester[6] according to the reaction sequence: Glu(OBzl) → Boc-Glu(OBzl) → Boc-Glu(OBzl)-OPcp → HCl.Glu(OBzl)--OPcp. Direct synthesis of poly-γ-glutamic acid via HCl.Glu(OPcp)-OBu^t or HCl.Glu-(OPcp)-OBzl was impossible as formation of the pyroglutamic acid prevailed in this case. Therefore the dipeptide HCl.Glu(Glu(OPcp)-OBu^t)-OBu^t accessible from Glp--Glu according to[7], has been chosen as the starting compound.

For the synthesis of poly(α-Lys), the diphenylphosphite method[8] proved to be more advantageous, allowing to start with the relatively easily accessible Lys(Boc) (ref.[9]) as the monomer. However, the polycondensation degrees obtained in this way do not reach quite the results obtained with active esters. Analytical data for the described polyamino acids are depicted in Table I.

Complexes with graduated insulin content were obtained from aqueous solutions of hydrophilic PAA or solutions of hydrophobic PAA in TFA or 85% acetic acid, and insulin. The complexes have been characterized in detail with respect to the insulin content and physical constants (Table II).

EXPERIMENTAL

The melting points and amino acid analyses *are corrected.* Polyamino acids (Table I) were characterized by IR spectroscopy by means of amide bands. In order to check the optical purity, values of rotation of the acidic polyamino acid hydrolysates (6M-HCl, 110°C, 24 to 72 h) were compared with those of analogously treated original amino acids; they did not exhibit any signicant deviations (relative error ± 3%).

Determination of the amino acid content possessed a value of 98·5% for all the preparations. Average molecular weight determination was performed by means of a modified method[13] for the quantitative detection of terminal amino groups utilizing dansylchloride and ultracentrifugation.

Preparation of the Insulin–Polyamino Acid Complexes

Insulin complexes with poly(Gly), poly(Ala), poly(Leu): For the preparation of the 38% insulin--PAA-complexes, 200 mg poly(Gly) (100 μmol, $\langle M \rangle$ = 2 000 and 16·7 μmol, $\langle M \rangle$ = 12 000, resp.) or poly(Ala) (100 μmol, $\langle M \rangle$ = 2 000) were dissolved in 5 ml 85% formic acid; 200 mg of poly(Leu) (10 μmol, $\langle M \rangle$ = 20 000) was dissolved in 5 ml of TFA.

For the preparation of the 20% insulin–PAA complex, 480 mg poly(Gly) (240 μmol, $\langle M \rangle$ = = 2 000) was dissolved in 12·0 ml, and to prepare the 17% insulin–poly(Gly) complex, 580 mg poly(Gly) (290 μmol, $\langle M \rangle$ = 2 000) was dissolved in 14·5 ml 85% formic acid. Insulin (120 mg,

i.e. 20 μmol), dissolved in 1·0 ml of the corresponding solvent was added to the PAA solution under stirring.

Subsequently, the 30% complexes were quantitatively precipitated with 20 ml, the 20% complexes with 30 ml, and the 17% insulin–poly(Gly) complex with 35 ml of absolute ether under continuous stirring. After the centrifugation of the pertinent insulin–PAA complex, it was

TABLE I

Analytical data of the synthesized poly-amino acids

PAA	$\langle M \rangle$ ref.[13]	$[\alpha]_D^{25}$ (c = 1·0)	IR Spectra cm^{-1}
Poly(Gly)	2 000		amide I 1 680 amide II 1 520 NH V 700 NH A 3 290
Poly(Ala)	2 000	−49·5 (DCA)	amide I 1 630 amide II 1 520 NH V 680
Poly(Leu)	20 000	−33·4 (TFA)	amide I 1 650 amide II 1 530 NH V 605 NH A 3 290
Poly(α-Glu(OBzl))	34 000	15·1 ($CHCl_3$) 15·0 ref.[2] −19·7 (DCA) −19·8 ref.[3]	amide I 1 620 amide II 1 520 NH V 690 C=O 1 720
Poly(α-Glu)	20 000	−38·1 (1M-NH_4OH) −15·0 (H_2O/pH 6·5)	amide I 1 620 amide II 1 540 NH V 630
Poly(γ-Glu-OBu^t)	44 000		amide I 1 660 amide II 1 530 C=O 1 730
Poly(γ-Glu)	30 000	−23·4 (H_2O/pH 7·5) (c = 0·5) −23·3 ref.[7]	amide I 1 670 amide II 1 520
Poly(α-Lys(Boc))		16·9 ($CHCl_3$)	amide I 1 620 amide II 1 527 Boc- 1 170
Poly(α-Lys)	4 000[a]	−30·2 (H_2O/pH 8·5)	amide I 1 640 amide II 1 540

[a] Determination of molecular weight by ultracentrifugation.

washed several times with absolute ether and dried at 1·3 Pa and 20°C over P_2O_5. Yield: 93·0 to 98·4%.

Insulin complexes with poly(α- and poly(γ-Glu): 200 mg poly(α-Glu) (10 μmol, $\langle M \rangle$ = 20 000) or 200 mg poly(γ-Glu) (7 μmol, $\langle M \rangle$ = 30 000) were dissolved in 10·0 ml distilled water, adding 1 to 2 drops of concentrated NH_4OH. Subsequently, 120 mg (20 μmol) of insulin was added to the poly(Glu) solution. After 30 min stirring at 20°C (until insulin became completely dissolved), the complex was lyophilized. Yield: 93·8 to 94·1%.

Insulin complex with poly(α-Lys): 200 mg poly(α-Lys) (50 μmol, $\langle M \rangle$ = 4 000) was dissolved in 10·0 ml distilled water and 120 mg insulin (20 μmol) was added to the stirred solution. Stirring was carried out for another 30 min at 20°C, after which the insulin–poly(α-Lys) complex begun to precipitate. Yield: 96·9% after lyophilisation.

Characterization of the Complexes

The complexes (Table II) were characterized by means of the specific optical rotation and the amide-band shifts due to intermolecular interactions of both components[14-16]. After the 24 h hydrolysis in 6M-HCl at 110°C, the insulin content in the complexes was determined by amino acid analyses, using glutamic acid or glycine as standards.

TABLE II

Complexes of poly-L-amino acids with insulin

Complex with	Mole ratio PAA/insulin	Insulin content %	$[\alpha]_D^{25}$ (c = 0·5)	IR Spectra cm^{-1}
Poly(Gly)	1 : 0·20	38	−22·8 (TFA)	amide I 1 620
	1 : 0·08	20	−14·2 (TFA)	amide II 1 520
	1 : 0·07	17	−10·9 (TFA)	NH V 700
	1 : 1·20	38	−22·9 (TFA)	NH A 3 280
Poly(Ala)	1 : 0·20	38	−21·3 (DCA)	amide I 1 620
				amide II 1 520
				NH V 685
				NH A 3 260
Poly(Leu)	1 : 2·00	38	3·4 (TFA)	amide I 1 640
				amide II 1 530
				NH V 605
Poly(α-Glu)	1 : 2·00	38	−34·6 (1M-NH_4OH)	amide I 1 660
				amide II 1 540
				NH A 3 430
Poly(γ-Glu)	1 : 2·99	38		
Poly(α-Lys)	1 : 0·40	38	−34·8 (1M-HCl)	amide I 1 650
				amide II 1 530

Insulin Liberation in vitro

The amount of each complex corresponding to 3·0 mg insulin was covered with 3·0 ml citrate/phosphate buffer (0·1M citric acid and 0·2M sodium hydrogenphosphate, 17·7 : 82·3 *v/v*, pH 7·0; 0·03% NaN_3). The insulin liberation was followed photometrically at 25°C on a Specord UV VIS at 278 nm in a closed system, in comparison with the corresponding amount of the unloaded PAA.

In vivo Blood-Glucose Lowering Activity in Rabbits

For each test series, the initial blood-glucose value of 10 rabbits of sound metabolism and of approximately equal weight (4 kg), age, race and sex was determined, after the check of their emptiness and sensitivity to insulin. This value was used as standard. Suspension of the insulin complex in HCl (0·032 mol l^{-1}, pH 5·0, 1% Nipagin) corresponding to 1·0 IU/kg was administered s.c. The blood-glucose values were determined in samples taken from the ear vena after 1·5, 3, 4, 5, 6, 7, 8, 10, 12 and 24 h using the Fermognost blood-glucose test.

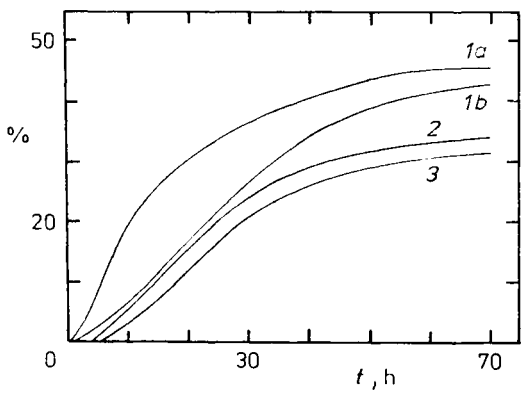

FIG. 1

Liberation of insulin (%) from poly(Gly) complexes in citrate/phosphate buffer (pH 7·0) in the dependence on insulin content and average molecular weight, determined at 278 nm. *1a* insulin content 38%, $\langle M \rangle$ = = 2 000; *1b* insulin content 38%, $\langle M \rangle$ = = 12 000; *2* insulin content 20%, $\langle M \rangle$ = = 2 000; *3* insulin content 17%, $\langle M \rangle$ = = 2 000

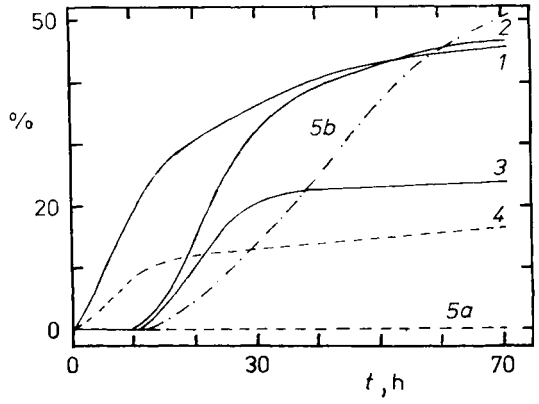

FIG. 2

Liberation of insulin (%) from complexes with different PAA (insulin content 38%) in citrate/phosphate buffer (pH 7·0), determined at 278 nm. Insulin complexes with: *1* poly(Gly), $\langle M \rangle$ = 2 000; *2* poly(Ala), $\langle M \rangle$ = 2 000; *3* poly(Leu), $\langle M \rangle$ = 20 000; *4* poly(α-Glu), $\langle M \rangle$ = 20 000; *5a* poly(α-Lys), $\langle M \rangle$ = 4 000; *5b* poly(α-Lys), $\langle M \rangle$ = 4 000 (in 0·1M HCl)

RESULTS AND DISCUSSION

The in vitro liberation of the hormone from the complexes was analyzed photometrically against citrate/phosphate buffer of pH 7·0. Special attention has been focused on the dependence of the hormone liberation on the insulin content and molecular weight of the PAA (Fig. 1), as well as on the type of the complex (Fig. 2).

Fig. 1 demonstrates on the example of the poly(Gly) type complex a marked increase of the velocities and degrees of liberation proportionally to the insulin content and the decreasing molecular weight. A remarkable retarding effect of one order exhibited for poly(Gly) with an insulin content of 38%, average molecular weight of 2 000 and liberation period up to 40 hours, might be of practical interest. Furthermore, Fig. 2 clearly indicates that the polarity and hydrophobicity of the PAA are important factors affecting the liberation behaviour. Thus, poly(α-Glu) and poly(α-Lys) (curves *4* and *5a*) exhibit insulin liberations beginning very slowly and which are still incomplete at the end of the observed period. Poly(α-Lys) releases insulin even only when the incubation solution is changed to 0·1 M-HCl. This PAA has obviously the strongest binding capacity towards the 6 insulin carboxyles. On the other hand, in the case of the three non-polar PAA (curves *1* and *3*), the end of the liberation is reached substantially earlier. Here the amounts of liberated insulin are also considerably reduced in the order poly(Gly) > poly(Ala) > poly(Leu), i.e.

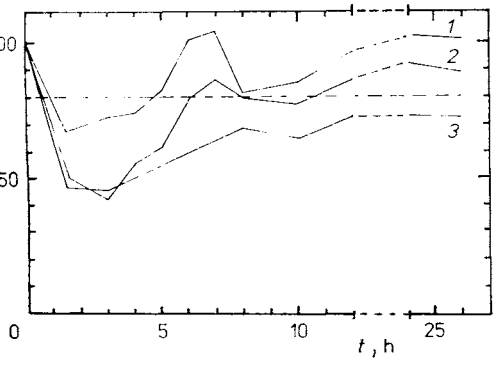

FIG. 3

Blood-glucose lowering effect (%) of insulin–poly(Gly) complexes in the dependence on insulin content and molecular weight in rabbits; subcutaneous application (1·0 IU/kg). *1* insulin content 17%, $\langle M \rangle = 2\,000$; *2* insulin content 38%, $\langle M \rangle = 12\,000$; *3* insulin content 38%, $\langle M \rangle = 2\,000$

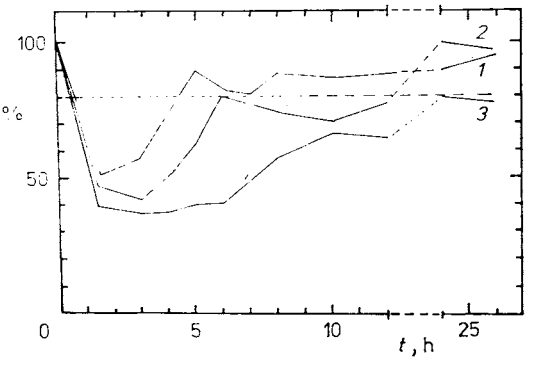

FIG. 4

Blood-glucose lowering effect (%) of the complexes with hydrophilic PAA in rabbits; subcutaneous application (1·0 IU/kg). *1* poly(γ-Glu); insulin content 38%, $\langle M \rangle = 30\,000$; *2* poly(α-Glu), insulin content 38%, $\langle M \rangle = 20\,000$; *3* poly(α-Lys), insulin content 38%, $\langle M \rangle = 4\,000$

with the increasing hydrophobicity and molecular weight. Additionally, inclusion processes should be considered.

As can be seen from Figs 3 and 5, the in vitro results were in principle confirmed by the animal experiments, concerning the dependence of the liberation on the insulin content and average molecular weight (as found with poly(Gly) as an example). In accordance, the representative with 38% insulin and $\langle M \rangle = 2\,000$ (Fig. 3) exhibits the highest liberation rate, and thus the strongest influence on the glucose level. Moreover, the in vivo results confirm the predominance of poly(α-Lys) as a retarding agent in the group of polar PAA (Fig. 4).

In contrast to this finding, poly(Ala) and poly(Leu) exhibit a considerably reduced liberation effect, due to their enhanced hydrophobicity and inclusion effects mentioned above (Fig. 5). Finally, the Fig. 6 shows the most effective complexes in comparison with a commercially available insulin preparation and insulin of the Lente type, demonstrating the superior efficiency of the complexes as promising depot forms.

The observed binding effects are attributed to the fact that under the selected conditions of complex formation, the insulin conformation is stabilized by disulphide bridges and thus does not denaturate, whilst PAA are transformed into their random

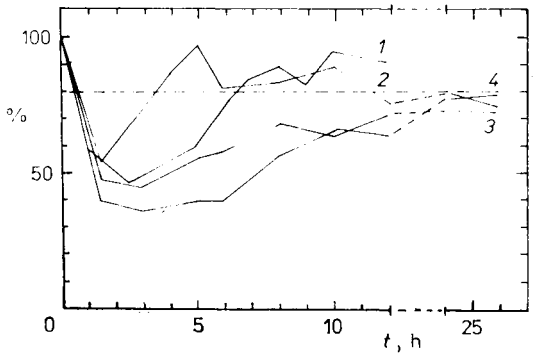

FIG. 5

Blood-glucose lowering effect (%) of the complexes with poly(Gly) and hydrophobic PAA in rabbits; subcutaneous application (1·0 IU/kg). *1* poly(Leu), insulin content 38%, $\langle M \rangle = 20\,000$; *2* poly(Ala), insulin content 38%, $\langle M \rangle = 20\,000$; *3* poly(Gly), insulin content 38%, $\langle M \rangle = 2\,000$

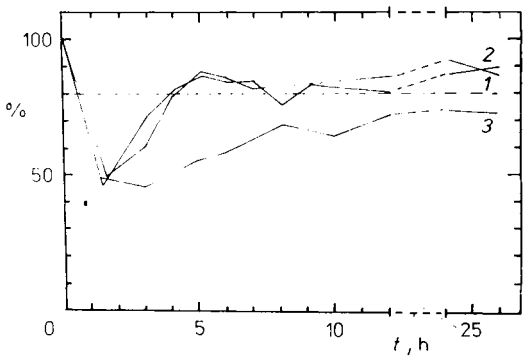

FIG. 6

Blood-glucose lowering effect (%) of selected insulin–PAA complexes in comparison with commercial insulin and a standard depot preparation (*L*-insulin S.N.C.) in rabbits; subcutaneous application (1·0 IU/kg). *1* *L*-insulin S.N.C.; *2* insu in; *3* insulin–poly(Gly) complex (38% insulin, $\langle M \rangle = 2\,000$); *4* insulin–poly(α-Lys) complex (38% insulin, $\langle M \rangle = 4\,000$)

coil form[10-12]. After the subsequent precipitation and lyophilisation, the compact insulin molecule is thus enclosed by the unfolded PAA into a stable complex.

In conclusion, these studies have pointed out that PAA complexes offer a variable spectrum of factors which influence the retard effects of insulin preparations, so that the insulin-liberating system may be adapted suitably to the requirements of practical application. Further investigations on these compounds are in progress.

REFERENCES

1. Losse G., Mätzler G.: Z. Chem. *21*, 394 (1981).
2. Naumann W., Losse G.: Z. Chem. *21*, 209 (1981).
3. Losse G., Müller F., Koszowicz J.: Pharmazie *42*, 521 (1987).
4. Prack A., Spach G.: Bull. Soc. Chim. Fr. *1971*, 4489.
5. Johnson B., Trask E.: J. Org. Chem. *33*, 4521 (1968).
6. Guttmann St., Boissonnas R. A.: Helv. Chim. Acta 41, 1852 (1958).
7. Kovacz J., Schmit G., Johnson B.: Can. J. Chem. *47*, 3690 (1969).
8. Yamazaki N., Higashi F., Kawabata J.: Makromol. Chem. *175*, 1825 (1974).
9. Moroder L., Hallett A., Wünsch E., Keller O., Wersin G.: Hoppe-Seyler's Z. Physiol. Chem. *357*, 1651 (1976).
10. Chao Y.-Y. H., Holtzer A., Mastin S. H.: J. Am. Chem. Soc. *99*, 8024 (1977).
11. Peretti J. A., Ninham B. B.: Macromolecules *3*, 30 (1970).
12. Noji S., Nomura T., Yamacka K.: Macromolecules *13*, 1114 (1980).
13. Naumann W., Fischer I., Kroschwitz H., Losse G.: Z. Chem. *26*, 108 (1986).
14. Fujie A., Komoto T., Oya M., Kawai T.: Makromol. Chem. *169*, 301 (1973).
15. Kikuchi Y., Kubota N.: Makromol. Chem., Rapid Commun. *6*, 387 (1985).
16. Miyazawa T., Blout E. R.: J. Am. Chem. Soc. *83*, 712 (1961).