Binding of Insulin to Bovine Liver Plasma Membrane

USE OF INSULIN ANALOGUES MODIFIED AT THE A1 RESIDUE

Peter RÖSEN, Martina SIMON and Hans REINAUER

*Department of Biochemistry, Diabetesforschungsinstitut an der Universität Düsseldorf,
Auf dem Hennekamp 65, D-4000 Düsseldorf, Federal Republic of Germany*

and Heinz J. FRIESEN, Cornelia DIACONESCU and Dietrich BRANDENBURG

Deutsches Wollforschungsinstitut, Veltmannplatz 8, D-5100 Aachen, Federal Republic of Germany

(Received 14 September 1979)

Bovine liver plasma membranes [Rösen, Ehrich, Junger, Bubenzer & Kühn (1979) *Biochim. Biophys. Acta* **587**, 593–605] show similar insulin-binding characteristics, as evaluated by Scatchard analysis, to those of membrane systems from other species. However, the dissociation rate of bound insulin cannot be accelerated by the addition of insulin, in contrast with membranes isolated from rat liver. The dissociation rate is strongly dependent on the pH. Although dependent on temperature, the total capacity of binding sites is minimally changed, but the number of high-affinity sites is increased 2–3-fold, by lowering the incubation temperature. These data might be interpreted by assuming a single population of receptors whose distribution between different affinity states depends on temperature. In competition studies, most of the modified insulins examined show a close correlation between binding, determined in plasma membranes from bovine liver, and biological activity, measured in adipocytes. The hypothesis that a positive charge on the A1 residue may be favourable for binding is supported by experiments with an isosteric pair of insulins modified at this residue ([carbamoyl-Gly^{A1}]- and [amidino-Gly^{A1}]insulin) and with modified insulins carrying one or more positive charges on the A1 residue ([Arg-Gly^{A1}]-, [Arg-Arg-Gly^{A1}]-, [Arg-Arg-Arg-Gly^{A1}]- and [Lys-Arg-Gly^{A1}]insulin). The latter insulin derivatives show a higher binding activity for plasma membranes from bovine, porcine and rat liver than expected from their biological activities in adipocytes.

We have reported the large-scale preparation of plasma membranes from bovine liver, and presented results on the specific binding of insulin to these membranes (Rösen *et al.*, 1979). These purified plasma membranes are used as a source for the insulin receptor protein. Because of the metabolic differences between the livers of ruminants (Armstrong, 1965; Long, 1979) and other animals, it seemed necessary to characterize the interaction of insulin with its receptor integrated in plasma membranes from bovine liver in more detail.

As demonstrated in previous studies, chemically modified insulins have almost invariably shown a close correlation between binding activity and biological activity (Freychet *et al.*, 1971; De Meyts, 1976; Pullen *et al.*, 1976). Therefore we used a number of chemically modified insulins to study the binding characteristics of the plasma membranes of bovine liver. Derivatives modified at glycine-A1 were

of special interest to us because this region is thought to be strongly involved in receptor binding (Pullen *et al.*, 1976).

The present studies on the dissociation of bound insulin and on the dependence of binding on temperature provide more information about the interaction of the insulin receptor in bovine liver plasma membrane with insulin.

Materials

Insulin A- and B-chains were purchased from Serva (Heidelberg, W. Germany). Sheep insulin was from Novo (Copenhagen, Denmark). Bovine insulin was a gift from Hoechst A.G. (Frankfurt, W. Germany). Porcine [¹²⁵I]monoiodinated insulin (specific radioactivity 90–110 Ci/g; trichloroacetic acid-precipitability > 98%) was from New England Nuclear, Boston, MA, U.S.A. LAB Trol, a protein

mixture (69 mg/ml), was from DADE, Miami, FL, U.S.A. All other reagents were commercial products of analytical grade. Sources of insulin derivatives and analogues are given in Table 1. They were prepared as described in the references given or obtained from the sources indicated.

Methods

Preparation

Plasma membranes from bovine liver were prepared and analytical procedures were carried out as described in detail elsewhere (Rösen *et al.*, 1979). Plasma membranes from rat liver were prepared as described by Neville (1960) and those from porcine liver as described by Bubenzer *et al.* (1978).

Insulin binding

This was studied as described in more detail previously (Rösen *et al.*, 1979). Briefly, the standard assay mixture contained ^{125}I -labelled insulin at the concentrations given in Figure and Table legends, 0.1 M-sodium phosphate buffer, pH 7.8, and 0.5% (w/v) bovine serum albumin. The binding reaction was started by addition of the membrane sample (final volume 0.3 ml). After incubation at the given temperature, the reaction was terminated by rapidly cooling the samples and quick filtration under reduced pressure (EAWP 025 membranes, 1 μm ; Millipore Corp., Bedford, MA, U.S.A.), which was followed by two wash steps with cold incubation buffer, containing 1% bovine serum albumin ($2 \times 3 \text{ ml}$). The filter-bound radioactivity was counted in a gamma counter (60% efficiency). Equilibrium data were analysed as described by Kahn *et al.* (1974), correcting binding data for non-specific binding, which was defined as radioactivity bound in the presence of unlabelled insulin (1 μM). Equilibrium constants were determined by Scatchard (1949) analysis. Non-specific binding accounted for less than 1% of the total radioactivity in the incubation mixture. Intra-assay variation was usually less than 5%.

For studies with insulin analogues, plasma membranes were incubated in duplicate with a fixed amount of ^{125}I -labelled insulin (0.25 nM) and variable concentrations of the insulin derivative examined (final volume 0.25 ml). The competition curves were graphically analysed from a representation of the binding data as the ratio bound/free (B/F) or the percentage of ^{125}I -labelled insulin bound $[(B/B_0) \times 100]$: i.e. ^{125}I -labelled insulin bound in the presence (B) and absence (B_0) of unlabelled insulin, times 100] relative to the total concentration of the insulin derivative. The radioactivity bound was corrected by subtracting the radioactivity bound to the filters.

The percentage binding was calculated from the

ratio between the concentration of bovine insulin giving half-maximal inhibition of ^{125}I -labelled insulin receptor binding (K_i) and the concentration of insulin analogues giving half-maximal inhibition, times 100:

$$\text{Binding (\%)} = \frac{K_i \text{ for bovine insulin}}{K_i \text{ for insulin analogue}} \times 100$$

Dissociation of bound insulin

Dissociation of bound insulin from the membranes was studied as described by De Meyts *et al.* (1973, 1976).

Preparation of insulin and its derivatives

Insulin and its derivatives were dissolved in 10 mM-HCl, neutralized with an equivalent volume of 10 mM-NaOH at a concentration of about 1 mg/ml. The exact concentration was determined spectrophotometrically (Beckman Acta M VI) by using the appropriate absorbance coefficients (Friesen, 1978).

The biological activity of the derivatives was determined as described by Moody *et al.* (1974) by measuring the incorporation of ^3H from D-[2- ^3H]glucose into the toluene-soluble lipids. Complete dose-response curves were determined in triplicate. Activities are expressed as percentages, taking the activity of insulin as 100 (27 i.u. = 1 mg).

Protein concentration was determined by the method of Lowry *et al.* (1951), with LAB Trol as standard.

Results

Table 1 shows the sources and biological potencies *in vitro* of the insulin derivatives studied.

Competition of insulin and its derivatives with ^{125}I -labelled insulin for binding to plasma membranes of bovine liver

For various preparations of plasma membrane the competition of ^{125}I -labelled insulin with native bovine insulin was studied. Table 2 shows the variation in the concentration of bovine insulin required for half-maximal competition (K_i). Because the K_i is different for each plasma-membrane preparation, the specific K_i for each preparation used was determined for calculation of the binding activity (%) of the insulin derivatives.

The competition curves for five of the insulin derivatives studied with decreasing biological activity in the isolated adipocyte are shown in Fig. 1. The K_i of the insulin derivatives increases with decreasing biological activity in adipocytes (Table 1). A close relationship between biological activity in adipocytes and relative binding activity in plasma

Table 1. *Insulin derivatives studied and their biological activities in adipocytes*

Abbreviations: Boc, t-butoxycarbonyl; thiazolidine, 2-dimethyl-3-formyl-L-thiazolidine-4-carbonyl; (B), bovine; (P), porcine.

Derivative	Activity in adipocytes (%)	References
1 Insulin (B)	100	Freychet <i>et al.</i> (1971)
2 Insulin (sheep)	100	Gliemann & Gammeltoft (1974)
3 Des-(B26-B30)-insulin (P)	17-27	Gattner (1975), preparation by Gattner
4 Des-(B23-B30)-insulin (B)	1.5	Freychet <i>et al.</i> (1971), Carpenter (1966)
5 Proinsulin (B)	4.5	Freychet <i>et al.</i> (1971), preparation by J. Helbig
6 [acetyl-Gly ^{A1}]Insulin (B)	37.5 ± 2.5	Brandenburg <i>et al.</i> (1972), Gliemann & Gammeltoft (1974); Lindsay & Shall (1971)
	32-50	
	18 ± 1	Preparation by J. Nowak
7 [Boc-Gly ^{A1}]Insulin (P)	17 ± 0.5	Pullen <i>et al.</i> (1976), Levy (1973), preparation by G. Krail
8 [thiazolidine-Gly ^{A1}]Insulin (B)	12.8 ± 1.6	Pullen <i>et al.</i> (1976), Lindsay & Shall (1970)
9 [amidino-Gly ^{A1}]Insulin (B)	88 ± 5	Friesen <i>et al.</i> (1977)
10 [carbamoyl-Gly ^{A1}]Insulin (B)	31 ± 2	Friesen <i>et al.</i> (1977)
	43 ± 5*	
11 [Lys-Arg-Gly ^{A1}]Insulin (B)	26 ± 2	Friesen <i>et al.</i> (1977)
	36 ± 7*	
12 [Arg-Gly ^{A1}]Insulin (B)	40-45	Friesen <i>et al.</i> (1977)
	39 ± 2*	
13 [Arg-Arg-Gly ^{A1}]Insulin (B)	42 ± 3	Friesen <i>et al.</i> (1977)
	35 ± 5*	
14 [Arg-Arg-Arg-Gly ^{A1}]Insulin (B)	40 ± 7	Friesen <i>et al.</i> (1977)
15 [amidino-Gly ^{A1} , amidino-Lys ^{B29}]Insulin	75-100	Friesen <i>et al.</i> (1979)
16 [biotinyl-Phe ^{B1}]Insulin	94 ± 9.6	Hofmann <i>et al.</i> (1977)
17 A-chain (oxidized) (B)	1	Freychet <i>et al.</i> (1971)
18 B-chain (oxidized) (B)	1	Freychet <i>et al.</i> (1971)

* Biological activity was determined in the course of the reported experiments.

Table 2. *Inhibition of ¹²⁵I-labelled insulin binding by bovine insulin in different preparations of plasma membranes from bovine liver*

Incubation mixtures contained membranes (0.4 mg of protein/ml), ¹²⁵I-labelled insulin (0.235 nM) and the given concentration of bovine insulin in 0.1 M-sodium phosphate buffer (pH 7.8)/0.5% bovine serum albumin. After incubation for 60 min (25°C), 0.25 ml portions were assayed for binding of ¹²⁵I-labelled insulin as described in the Methods section. Each value given is the mean of two different experiments done in duplicate.

Different preparations of plasma membranes from bovine liver	K _i (nM)
B5	1.99
B6	1.66
B7	3.31
B8	3.63
B9	2.29
Mean ± S.E.M.	2.58 ± 0.38

membranes from bovine liver was obtained with 14 of the insulin derivatives (Fig. 2).
The relative binding potencies of these materials agree well with those derived from studies with isolated adipocytes (Gliemann & Gammeltoft, 1974;

Fig. 1. *Competition between ¹²⁵I-labelled insulin and different insulin derivatives*
Incubation mixtures contained membranes (0.4 mg of protein/ml), ¹²⁵I-labelled insulin (0.235 nM) and the insulin derivative in 0.1 M-sodium phosphate buffer/0.5% bovine serum albumin (pH 7.8). After incubation for 60 min (25°C), 0.25 ml portions were assayed for binding of ¹²⁵I-labelled insulin as described in the Methods section. Each point represents the mean ± S.E.M. for three to seven different experiments done in duplicate. ●, des-(B23-B30)-insulin; △, [thiazolidine-Gly^{A1}]insulin; □, [t-butoxycarbonyl-Gly^{A1}]insulin; ○, [acetyl-Gly^{A1}]insulin; ▲, des-(B26-B30)-insulin; ■, bovine insulin.

Fig. 2. Relationship between biological and binding activity of 14 different insulin derivatives
Binding activity was calculated from:

$$\text{Binding (\%)} = \frac{K_i (\text{bovine insulin})}{K_i (\text{insulin analogue})} \times 100$$

For binding activity and biological activity the mean $\pm$ s.e.m. is given ($n = 3-7$). The line of identity is given. The numbers refer to insulin derivatives as described in Table 1.

Pullen *et al.*, 1976), hepatocytes (De Meyts, 1976) and plasma membranes isolated from rat liver (Freychet *et al.*, 1971, 1972; Kahn *et al.*, 1974). The binding curves obtained with all the derivatives studied appeared to be parallel with that for insulin.

Increasing the size of uncharged groups added to glycine-A1 of the insulin molecule is associated with decreased receptor binding (Table 3). The reason for

Fig. 3. Competition of ^{125}I -labelled insulin with [amidino-Gly^{A1}]insulin (●) and [carbamoyl-Gly^{A1}]insulin (■). Experimental details are given in Fig. 1. Each point represents the mean $\pm$ s.e.m. for three different experiments done in duplicate.

Table 3. Binding activities of modified insulin derivatives carrying uncharged groups of increasing size at glycine-A1
Experimental details are given in Fig. 1. The binding activity was calculated as described in the Methods section. Means $\pm$ s.e.m. for duplicate determinations of three separate experiments are given.

Derivative	Activity in adipocytes (%)	Binding activity (%)
[acetyl-Gly ^{A1}]Insulin	37.5 $\pm$ 2.5	23.2 $\pm$ 4.0
	18.0 $\pm$ 1	—
[Boc-Gly ^{A1}]Insulin	17.4 $\pm$ 0.5	15.2 $\pm$ 1.2
[thiazolidine-Gly ^{A1}]Insulin	12.8 $\pm$ 1.6	5.6 $\pm$ 0.5

Table 4. Binding activities of modified insulin derivatives carrying one or more basic amino acid residues on the A1 residue

Experimental details and abbreviations are given in the legend to Fig. 1. The binding activity was calculated as described in the Methods section. For plasma membranes from bovine liver means $\pm$ s.e.m. for duplicate determinations of three separate experiments are given.

Derivative	Activity in adipocytes (%)	Receptor binding activity to plasma membranes from bovine liver (%)
Insulin (B)	100	100
[Lys-Arg-Gly ^{A1}]Insulin	36 $\pm$ 7	79.5 $\pm$ 16.8
[Arg-Gly ^{A1}]Insulin	39 $\pm$ 2	107 $\pm$ 3.5
[Arg-Arg-Gly ^{A1}]Insulin	35 $\pm$ 5	90.5 $\pm$ 3.5
[Arg-Arg-Arg-Gly ^{A1}]Insulin	40 $\pm$ 7	125 $\pm$ 12.6

the low biological activity in one preparation of [acetyl-Gly^{A1}]insulin is not known.

The effect of a positive charge on the A1 residue was evaluated by studying an isosteric pair of insulin derivatives [amidino-Gly^{A1}]- and [carbamoyl-Gly^{A1}]insulin (Fig. 3). Both the biological potency in adipocytes ($88 \pm 5\%$ compared with $43 \pm 5\%$) and the binding determined by measuring competition with ¹²⁵I-labelled insulin ($84.2 \pm 3.4\%$ compared with $25.9 \pm 7.7\%$; Fig. 3) are greater for the analogue with the positive charge.

Analogues with basic amino acids added to the N-terminus of the A-chain had binding potencies that were higher than their potencies in the adipocyte assay with both bovine and porcine liver plasma membranes (Table 4). A similar dissociation between biopotency and binding activity was recently also observed for [Arg-Gly^{A1}]insulin with plasma membranes of rat liver (results not shown).

Dissociation of bound ¹²⁵I-labelled insulin from plasma membranes of bovine liver

Fig. 4 shows the dissociation curve for bound

Fig. 4. Dissociation of bound insulin from plasma membranes of bovine liver

Plasma membranes (2.1 mg of protein/ml) were incubated with ¹²⁵I-labelled insulin (50 pM) in 0.1 M-sodium phosphate buffer (pH 7.8)/0.5% bovine serum albumin for 60 min at 23°C. After separation of non-bound from bound ¹²⁵I-labelled insulin by centrifugation (Microfuge; 10000 rev./min; 5 min), the pellet obtained was rehomogenized and diluted with the same phosphate buffer or acetate buffer (0.1 M, pH 6.0) containing 0.5% bovine serum albumin in the presence (■, ●) or absence (□, ○) of unlabelled insulin (0.17 μM). At the times indicated three samples (1 ml) were taken and assayed for bound ¹²⁵I-labelled insulin as described in the Methods section. Each point represents the mean of triplicate determinations from two different experiments.

¹²⁵I-labelled insulin in the presence and absence of unlabelled insulin during the dissociation phase at two different pH values (pH 6.0 and 7.8). The dissociation curves cannot be explained by a single exponential equation. A fast primary phase (t_1 approx. 3–5 min) is followed by a very slow reaction (t_2 approx. 90 min). The dissociation is accelerated by a low pH, but not by the addition of unlabelled native insulin (Figs. 4 and 5). In contrast with these results, the rate of dissociation of insulin bound to plasma membranes of rat liver is enhanced by the addition of native insulin.

Dependence of binding on temperature

Variation in temperature causes large changes in binding data as shown in a Scatchard plot (Fig. 6). By calculating the affinity and number of receptor sites on the basis of a model that assumes heterogeneities, we obtained the results shown in Table 5. Whereas the total capacity of binding sites is not, or minimally, changed, the number of high-affinity sites is doubled.

Fig. 5. Acceleration of dissociation of insulin bound by liver plasma membranes by insulin at pH 7.8

Experimental details are given in the legend to Fig. 4. The concentration of insulin in the dissociation experiment was varied between 10^{-10} and 10^{-6} M; 30 min after the beginning of the dissociation experiment, samples were taken and assayed as described in the text. Calculations were done as described by De Meyts *et al.* (1976):

$$\text{Increase in dissociation (\%)} = \frac{B_D - B_1}{B_0 - B_D} \times 100$$

where B_0 is insulin bound at time 0 (immediately after dilution), B_D is insulin bound 30 min after dilution in the absence of insulin and B_1 is insulin bound 30 min after dilution in the presence of insulin. Each point represents the mean of triplicate determinations in two different experiments. ●, Rat liver plasma membranes; ■, bovine liver plasma membranes.

Fig. 6. Scatchard plots of insulin binding to plasma membranes at three different temperatures

Plasma membranes from bovine liver (0.4 mg of protein/ml) were incubated at pH 7.8 (0.5% bovine serum albumin present) with a fixed amount of ^{125}I -labelled insulin (50 pM) and increasing concentrations of unlabelled insulin: —, 3 h, 25°C; ···, 16 h, 15°C; ----, 16 h, 4°C. Separation of bound and free insulin and calculations are described in the Methods section. The curves were calculated from the mean of triplicate determinations from three different experiments. The bars represent the difference between the largest and smallest value.

Fig. 7. Reversibility of binding curves after preincubation at different temperatures

Plasma membranes of bovine liver (0.4 mg of protein/ml) were incubated with a fixed amount of ^{125}I -labelled insulin (50 pM) and an increasing concentration of unlabelled insulin in 0.1 M-sodium phosphate buffer/0.5% albumin. Δ , Preincubation for 3 h at 25°C, followed by incubation for 17 h at 4°C; $\blacktriangle$, incubation for 17 h at 4°C; $\square$, preincubation for 17 h at 4°C followed by incubation for 3 h at 25°C; $\blacksquare$, incubation for 3 h at 25°C. Each point represents the mean of triplicate determinations from two different experiments.

The K values for the low-affinity site ranged between 4.8×10^7 and $9.1 \times 10^7 \text{ M}^{-1}$. These observations are compatible with the hypothesis that

Table 5. Dependence of the total capacity and apparent affinity constants of insulin binding sites from bovine liver plasma membranes on temperature

Temperature (°C)	High-affinity site	
	K (nM^{-1})	Numbers of receptors (pmol/mg of protein)
4	2.8	0.46
15	1.0	0.29
25	1.5	0.20

cooling allows a transition of some receptors from a low- to a high-affinity state. The reversibility of such a temperature-induced transition is shown in Fig. 7.

Discussion

As we have previously reported (Rösen *et al.*, 1979), plasma membranes of bovine liver show similar binding characteristics, as evaluated by Scatchard analysis, to those of liver cells and membrane systems from other species (Cuatrecasas, 1972; Andersen *et al.*, 1977; Gliemann & Gammeltoft, 1974; Pullen *et al.*, 1976; Freychet *et al.*, 1971, 1972; De Meyts, 1976). As for other systems, the Scatchard plot of the results of equilibrium binding experiments is clearly non-linear. It has been suggested that this observation in other receptor preparations may be due to site-site interactions of the negative co-operative type (De Meyts *et al.*, 1973, 1976). Experimental support for this suggestion is afforded by the observation that the rate of dissociation of radioactively labelled insulin is increased in the presence of an excess of native insulin (De Meyts *et al.*, 1973, 1976). We have confirmed these observations in our preparation of rat liver plasma membranes, but, significantly, bovine plasma membranes do not show this effect. We cannot explain the non-linearity of the Scatchard analysis observed with this preparation on the basis of negative co-operativity.

The existence of more than one body of receptors with different intrinsic affinities could be another interpretation (Kahn *et al.*, 1974). We think that our results strongly suggest an alternative explanation. We assume a single class of receptors whose distribution between different affinity states depends on temperature. Changes in temperature would induce alterations in the affinity state and therefore determine the balance of receptors in high- and low-affinity states. It may be that changes in the fluidity of the membranes have a critical influence on the affinity state of the insulin receptors (Singer, 1972; Jacobs & Cuatrecasas, 1976; Luly & Shinitzky, 1979). Interestingly, negative co-operative interactions have been observed at low tem-

perature, but not at 37°C (Gammeltoft *et al.*, 1978).

The studies with chemically modified insulins show that the derivatives can be divided into two groups. The major group exhibits the familiar good correlation between adipocyte bioactivity and membrane-binding potency (Freychet *et al.*, 1971, 1972; Kahn *et al.*, 1974; Gliemann & Gammeltoft, 1974; De Meyts, 1976; Pullen *et al.*, 1976). This group includes derivatives with neutral groups of different sizes attached to glycine-A1. The materials have lost the natural positive charge of the *N*-terminal amino group. Deletion of between five and nine amino acids from the *C*-terminus of the B-chain also results in parallel losses of bioactivity and binding. These results are in line with previous observations (Freychet *et al.*, 1971, 1972; Kahn *et al.*, 1974; Gliemann & Gammeltoft, 1974; Pullen *et al.*, 1976). The decreased potency of the neutral [carbamoyl-Gly^{A1}]insulin as compared with the isosteric [amidino-Gly^{A1}]insulin which carries a positive charge supports the suggestion (Friesen *et al.*, 1977) that this charge at the *N*-terminus of the A-chain is important for activity related to binding. A positive charge at the A1 residue might enhance the fixation of the ligand at or near the receptor site, which is followed by conformational rearrangements of the partly bound ligand, leading to an appropriate binding of the insulin molecule to the receptor (Burghen *et al.*, 1975). Such a mechanism would allow the high specificity and speed that is necessary for the information transfer (Pörschke & Eigen, 1971).

The addition of further basic amino acids by peptide linkage to the *N*-terminus of the A-chain produces apparent dissociation of binding potency, studied in plasma membranes from bovine, porcine and rat liver, from biological activity as determined in adipocytes. In these cases, there are, in addition to the *N*-terminal amino group, between one and three further positive charges on the side chains. In this respect these analogues differ from the insulin described by Lesniak *et al.* (1979), which shows surprisingly high activity with no dissociation of binding from activity. The observed dissociation cannot be explained by different rates of degradation of the insulin derivatives studied, as insulin-degrading activity in bovine liver plasma membranes is very low, and degradation of insulin derivatives with different biological activities is only minimally changed (Rösen *et al.*, 1979).

It is tempting to speculate that a single positive charge on the A1 residue is important for binding, but that an increased density of such charges impairs the translation of binding into biological effect.

We thank Mrs. U. Bucker and Mr. M. Werner for the excellent technical assistance in the preparation of the membranes and some binding studies. Furthermore, we

thank Dr. R. J. Jones, St. Thomas's Hospital, London, for his help in preparing this manuscript and Professor T. Blundell and Dr. S. P. Wood, Birkbeck College, University of London, for helpful discussions and providing us with some insulin derivatives. This work was supported by the Deutsche Forschungsgemeinschaft (SFB 113), the Ministerium für Wissenschaft und Forschung des Landes NRW and the Bundesministerium für Familie, Jugend und Gesundheit.

References

- Andersen, O., Gliemann, J. & Gammeltoft, St. (1977) *Diabetologia* **13**, 589–593
- Armstrong, D. S. (1965) in *Physiology of Digestion in Ruminants* (Dougherty, R. W., ed.), pp. 272–288, Butterworth, New York
- Brandenburg, D., Gattner, H.-G. & Wollmer, H. (1972) *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 599–617
- Bubbenzer, H. J., Junger, E. & Kühn, L. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 1065
- Burghen, A. S. V., Roberts, G. C. K. & Feeney, J. (1975) *Nature (London)* **253**, 753–755
- Carpenter, F. H. (1966) *Am. J. Med.* **40**, 750–758
- Cuatrecasas, P. (1972) *J. Biol. Chem.* **247**, 1980–1991
- De Meyts, P. (1976) *Methods Mol. Biol.* **9**, 301–383
- De Meyts, P., Roth, J., Neville, D. M., Jr., Gavin, J. & Lesniak, M. A. (1973) *Biochem. Biophys. Res. Commun.* **55**, 154–161
- De Meyts, P., Bianco, A. R. & Roth, J. (1976) *J. Biol. Chem.* **251**, 1877–1888
- Freychet, P., Roth, J. & Neville, D. M., Jr. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 1833–1837
- Freychet, P., Kahn, C. R., Roth, J. & Neville, D. M., Jr. (1972) *J. Biol. Chem.* **247**, 3953–3961
- Friesen, H.-J. (1978) Doctoral Thesis, Rheinisch-Westfälische Technische Hochschule, Aachen
- Friesen, H.-J., Brandenburg, D., Diaconescu, C., Gattner, H.-G., Naithani, V. K., Nowak, J., Zahn, H., Dockerill, S., Wood, S. P. & Blundell, T. L. (1977) *Proc. Am. Peptide Symp.* **5th**, 136–140
- Friesen, H.-J., Naithani, V. K. & Gattner, H.-G. (1978) *Hoppe-Seyler's Z. Physiol. Chem.* **359**, 103–111
- Friesen, H.-J., Saunders, D. J., Naithani, V. K., Rösen, P. & Diaconescu, C. (1979) *Proc. Am. Peptide Symp.* **6th**, in the press
- Gammeltoft, S., Kristensen, L. O. & Sestoft, L. (1978) *J. Biol. Chem.* **253**, 8406–8413
- Gattner, H.-G. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 1397–1404
- Gliemann, J. & Gammeltoft, S. (1974) *Diabetologia* **10**, 105–113
- Hofmann, K., Finn Francis, M., Friesen, H.-J., Diaconescu, C. & Zahn, H. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 2697–2700
- Jacobs, St. & Cuatrecasas, P. (1976) *Biochem. Biophys. Res. Commun.* **433**, 482–495
- Kahn, C. R., Freychet, P., Roth, J. & Neville, D. M., Jr. (1974) *J. Biol. Chem.* **249**, 2249–2257
- Lesniak, M. A., Gliemann, J., Roth, J., Easter Barrat, R. D., Sutton, A. D. & Drewes, S. E. (1979) *Hoppe-Seyler's Z. Physiol. Chem.* **360**, 467–471
- Levy, D. (1973) *Biochim. Biophys. Acta* **328**, 107–113

- Lindsay, D. G. & Shall, S. (1970) *Eur. J. Biochem.* **15**, 547–554
- Lindsay, D. G. & Shall, S. (1971) *Biochem. J.* **121**, 737–745
- Long, R. A. (1979) *Adv. Vet. Sci. Comp. Med.* **24**, 209–260
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Luly, P. & Shinitzky, M. (1979) *Biochemistry* **18**, 445–450
- Moody, A. J., Stan, M. A., Stan, M. & Gliemann, J. (1974) *Horm. Metab. Res.* **6**, 12–16
- Neville, D. M., Jr. (1960) *J. Biophys. Biochem. Cytol.* **8**, 413–422
- Pörschke, D. & Eigen, M. (1971) *J. Mol. Biol.* **62**, 383–401
- Pullen, R. A., Lindsay, D. G., Wood, S. P., Tickle, I. J., Blundell, T. L., Wollmer, A., Krail, G., Brandenburg, D., Zahn, H., Gliemann, J. & Gammeltoft, St. (1976) *Nature (London)* **259**, 369–373
- Rösen, P., Ehrich, B., Junger, E., Bubenzer, H. J. & Kühn, L. (1979) *Biochim. Biophys. Acta* **587**, 593–605
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- Singer, S. J. (1972) *Science* **175**, 720–731