The Effects of Digestive Enzymes on Characteristics of Placental Insulin Receptor

COMPARISON OF PARTICULATE AND SOLUBLE RECEPTOR PREPARATIONS

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The role of the surrounding membrane structure on the binding characteristics of the insulin receptor was studied by using several digestive enzymes. The effects observed with particulate membrane preparations are compared with those from soluble receptor preparations. β -Galactosidase and neuraminidase had no effect on insulin binding to either particulate or soluble receptors from human placentae. Exposure to 2 units of phospholipase C/ml increased insulin binding to particulate membranes, but was without effect on the soluble receptor preparation. The increase in binding to particulate membranes was shown to be due to an increase in apparent receptor number. After 5 min exposure to 500 μ g of trypsin/ml there was an increase in insulin binding to the particulate membrane fraction, owing to an increase in receptor affinity. After 15 min exposure to this amount of trypsin, binding decreased, owing to a progressive decrease in receptor availability. In contrast, this concentration of trypsin had no effect on the solubilized receptor preparation. Because of the differential effects of phospholipase C and trypsin on the particulate compared with the solubilized receptor preparations, it is concluded that the effects of these enzymes were due to an effect on the surrounding membrane structure. Changes in receptor configuration due to alterations within the adjoining membrane provide a potential mechanism for mediating short-term alterations in receptor function.

The first step in the action of polypeptide hormones is the binding of the hormone to a specific receptor located on the cell surface [for reviews see Roth (1973) and Kahn (1975)]. In many physiological and disease states alterations in biological sensitivity to a hormone appear to be associated with alterations in hormone-receptor number and affinity (Kahn *et al.*, 1972; Olefsky & Reaven, 1974; Bar *et al.*, 1976; Harrison *et al.*, 1976; Merimee *et al.*, 1976). The mechanisms whereby these changes are induced are at present unknown.

Peptide-hormone-binding sites form an integral part of the cell-membrane structure and hence interact with the adjoining protein, glycoprotein and phospholipid components of the membrane. Apart from direct alterations to the receptor site itself, it is possible that alterations in the configuration of the adjoining membrane areas may affect the nearby binding sites (Cuatrecasas, 1971*b*). Adjoining membrane components may mask binding sites, making them unavailable to the hormone, or the interaction with other membrane components may facilitate binding of hormone to receptor, thus apparently increasing the affinity of the binding reaction.

The present study describes the effects of enzymes that are active against several cell-membrane components on the insulin-binding characteristics of particulate placental membrane preparations and compares the effects with those observed in solubilized receptor preparations. Solubilization of hormone receptors allows at least partial dissociation of the receptor from the adjoining cell membrane (Ginsberg *et al.*, 1976; Harrison *et al.*, 1978). Differential effects on the particulate receptor preparation compared with the solubilized receptor preparation may therefore reflect changes in receptor function attributable to modifications of the surrounding membrane structure.

Materials and Methods

Neuraminidase (EC 3.2.1.18) (activity 0.6 unit/mg; from *Clostridium perfringens*) and β -galactosidase (EC 3.2.1.23) (activity 30 units/mg; from *Escherichia coli*) were purchased from Boehringer Mannheim Australia Pty Ltd., Mt. Waverley, Vic., Australia. Phospholipase C (EC 3.1.4.3) (activity 120 units/mg; from *Bacillus cereus*) was purchased from Sigma

Chemical Co., St. Louis, MO, U.S.A., and trypsin (EC 3.4.21.4) (activity 200 BzArgEt units/mg; from ox pancreas) was purchased from British Drug Houses, Poole, Dorset, U.K. Soya-bean trypsin inhibitor (activity 1mg inhibited 7000 BzArgEt units of trypsin activity) was purchased from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Na¹²⁵I was purchased from The Radiochemical Centre, Amersham, Bucks., U.K., and pig monocomponent insulin was a gift from the Novo Research Institute, Bagsvaerd, Denmark. Triton X-100 was obtained from Rohm and Hass Co., Philadelphia, PA, U.S.A., and poly(ethylene glycol) was obtained from the Merck Institute, Darmstadt, West Germany. Human serum albumin (Cohn fraction V) was supplied by the Commonwealth Serum Laboratories, Melbourne, Vic., Australia.

Particulate membranes were prepared from fresh human placenta by a differential-centrifugation method described in detail elsewhere (Harrison *et al.*, 1978).

To solubilize the receptor, membranes were extracted in 1% Triton at a protein concentration of 8–12mg/ml for 1h at 24°C. The extract was centrifuged at 100000g in a Beckman L5-65 ultracentrifuge for 2h at 4°C and the supernatant retained as the soluble receptor preparation. This material contains insulin receptors whose properties are essentially the same as those in whole cells or intact membranes from a variety of species (Harrison *et al.*, 1978).

All protein measurements were by the method of Lowry *et al.* (1951), with human serum albumin standards. A modification of the method involving the addition of sodium dodecyl sulphate to prevent precipitation of the Triton was used for the solubilized receptor preparations (Wang & Smith, 1975).

Pig monocomponent insulin was iodinated to a specific radioactivity of 100–150 μ Ci/ μ g by stepwise addition of chloramine-T (Roth, 1975). The insulin was purified on cellulose (Yalow & Berson, 1960) followed by Sephadex G-50 chromatography.

The membrane preparations were incubated with the enzymes at 37°C for times as specified in the legends to the Figures, after which a standard binding assay was performed. The assay buffer was 0.1M-sodium phosphate, pH7.4, containing 0.1% human serum albumin for particulate membranes and 0.05% Triton for soluble receptors. A sample of membrane (200 μ g of protein) was incubated with a tracer amount (approx. 10000 c.p.m./ml, equivalent to 60pg of insulin) of ¹²⁵I-labelled insulin in the presence or absence of an excess (10 μ g/ml) of unlabelled insulin in a total volume of 1ml for 16h at 4°C.

¹²⁵I-labelled insulin bound to receptors was separated from free ¹²⁵I-labelled insulin by centrifugation of particulate membranes at 3000g for 30min, or by precipitation of soluble receptors with

poly(ethylene glycol) (Desbuquois & Aurbach, 1971). In either case the pellet contained the receptor-bound ¹²⁵I-labelled insulin. The bound and free radioactivity were measured in an Autogamma spectrometer (Packard Instrument Co.). As is usually accepted (De Meyts, 1975), specifically bound ¹²⁵I-labelled insulin was taken to be the difference between the total radioactivity bound and the radioactivity bound in the presence of a large excess (10 μ g/ml) of unlabelled insulin (non-specific binding). The non-specific binding was never more than 15% of the total binding in the absence of added unlabelled insulin. Results obtained from binding assays with increasing concentrations of unlabelled insulin were treated by the method of Scatchard (1949).

In the experiments where the effects of trypsin were studied, the control experiments consisted of receptor preparations exposed to soya-bean trypsin inhibitor before the addition of trypsin.

In the experiments testing the effects of the other enzymes, it was impossible to wash solubilized receptors to remove these enzymes; consequently, after the initial enzyme incubation, all tubes were immediately placed in ice to stop the enzyme activity and kept at 4°C for the subsequent binding experiments. Control studies were performed by using 'washed' and 'unwashed' particulate placental membranes to show that the omission of the washing step did not significantly alter the results.

To eliminate the possibility that Triton itself played any part in the effects observed with soluble receptors, particulate membranes were incubated in 0.05% Triton (equivalent to the final concentration of Triton in the solubilized receptor preparation); at this concentration there was no significant solubilization of the particulate membranes.

Results

Neuraminidase and β -galactosidase

Table 1 shows that, whether used individually or together, these enzymes had no effect on the binding of insulin by either particulate or solubilized receptor preparations, at the concentrations tested.

Phospholipase C

As shown in Table 2, phospholipase C (2 units/ml) acting on particulate membranes increased insulin binding by 31% ($P < 0.001$) compared with control values. There was no significant effect of this enzyme on binding to solubilized receptors. When the enzymic action on the particulate membranes was inhibited by decreasing the temperature rather than by washing before the binding assay, similar results were obtained (results not shown). This made the

Table 1. Specific ¹²⁵I-labelled insulin binding to particulate placental membranes and solubilized placental receptors after exposure to neuraminidase or β-galactosidase

Membranes were exposed to enzymes for 30min at 37°C. The tubes were cooled to 4°C, and a binding assay was performed as described in the text. The enzymes were used separately or together as indicated. Each value is the mean of triplicate determinations from two separate experiments.

Membrane type	Enzyme concn. (μg/ml) ...	Specific ¹²⁵ I-labelled insulin binding (pg/mg)							
		Neuraminidase			β-Galactosidase			Neuraminidase (a) followed by β-galactosidase (b)	
		0	5 μg/ml	10 μg/ml	0	25	125	(a) 0, (b) 0	(a) 10, (b) 25
Particulate placental membranes		82.2	79.2	80.7	78.0	84.0	83.1	73.2	75.3
Solubilized placental receptors		63.9	70.5	63.0	44.1	43.8	46.2	69.9	75.9

Table 2. Specific ¹²⁵I-labelled insulin binding to particulate placental membranes and solubilized placental receptors after exposure to phospholipase C

Membranes were exposed to 2 units of phospholipase C/ml for 30min at 37°C, after which the tubes were cooled to 4°C and a binding assay was performed as described in the text. Differences between the mean values were evaluated by Student's *t* test; *P* values are shown in the Table. Abbreviation: n.s., not significant (*P*>0.05). Each value is the mean of triplicate determinations from two separate experiments.

Membrane type	Specific ¹²⁵ I-labelled insulin binding (pg/mg)		<i>P</i>
	Control	Phospholipase C-treated	
Particulate placental membranes	75.6	99.0	<0.001
Solubilized placental receptors	53.4	50.1	n.s.

experimental procedure analogous to that used for the solubilized preparation.

To determine the mechanism for the increased receptor binding in the particulate preparation after exposure to phospholipase C, equilibrium data obtained by using increasing concentrations of unlabelled insulin were studied and a Scatchard plot was drawn (Fig. 1). The two curves obtained are similar in shape, but, if projected, the curve for the phospholipase C-treated membranes would intercept the horizontal axis at a higher concentration of bound insulin than did that of the control; this intercept represents the maximum number of binding sites available (De Meyts, 1975). The results obtained indicate that phospholipase C treatment increased the number of available receptor sites without altering receptor affinity.

Trypsin

Fig. 2 shows the effects of 15min exposure to increasing concentrations of trypsin on insulin binding. There was a significant decrease in binding

Fig. 1. Scatchard plot derived from equilibrium studies with control (●) or phospholipase C-treated (▲) particulate membranes

Particulate membranes were treated with 2 units of phospholipase C/ml at 37°C for 30min. The tubes were cooled to 4°C and a binding assay was performed with increasing concentrations of unlabelled insulin as described in the text. Each point is the mean of triplicate determinations from two separate experiments.

to the particulate membrane preparation after exposure to 250 μg of trypsin/ml; at 500 μg of trypsin/ml this had fallen to 30% of the binding to control membranes, with abolition of binding after exposure to 1000 μg of trypsin/ml. This is in marked contrast with the solubilized receptor preparation, where no significant decrease was observed after exposure to 250 or 500 μg of trypsin/ml. Although a significant decrease was observed at 1000 μg of trypsin/ml this was much less than with the particulate material.

In contrast with the decrease in binding observed after 15min exposure to trypsin, exposure to 500 μg of trypsin/ml for 5min led to a significant increase of insulin binding to the particulate preparations. No significant effect was observed on binding to the solubilized receptor preparation after 5 or 15min

Fig. 2. Dose-response curve for particulate and soluble membrane receptors treated with trypsin

Particulate (■) and soluble (●) membrane preparations were incubated with increasing concentrations of trypsin at 37°C for 15 min, at which time an excess of soya-bean trypsin inhibitor was added. A binding assay was performed as described in the text. Specific ^{125}I -labelled insulin binding is expressed as a percentage of that in the control (soya-bean trypsin inhibitor added before the addition of trypsin). Each point represents the mean $\pm$ S.E.M. of triplicate determinations from two separate experiments. Values that differ significantly from the controls are shown: * $P < 0.01$, ** $P < 0.001$.

exposure to this concentration of trypsin (Fig. 3). This time-dependence was entirely reproducible, and qualitatively identical results were found in each of two separate experiments. Table 3 shows the effect of trypsin on the particulate membranes over shorter time intervals.

The effect on the particulate membrane of exposure to trypsin for 5 or 15 min was studied further by constructing a Scatchard plot from equilibrium data with increasing concentrations of unlabelled insulin. Fig. 4(a) shows that the stimulatory effect on binding after 5 min exposure to trypsin was associated with an increased mean slope of the Scatchard plot, indicating an increase in apparent affinity of the receptors (De Meyts, 1975). There was also some loss of receptor availability after 5 min, as indicated by the position of the intercept on the horizontal axis. After exposure to trypsin for 15 min (Fig. 4b), the apparent affinity was still increased, but the number of available receptors had decreased further and this had become the dominant effect.

Curvilinear Scatchard plots make it difficult to define affinity constants, so an operational index of

Fig. 3. Time-dependence of trypsin action on particulate and soluble membrane preparations

Particulate and soluble membrane preparations were exposed to 500 µg of trypsin/ml at 37°C. At the times shown samples containing 200 µg of protein were removed and added to tubes containing soya-bean trypsin inhibitor. Subsequently a binding assay was performed as described in the text (controls, open columns; trypsin-treated, stippled columns). Each bar represents the means $\pm$ S.E.M. of duplicate determinations from two separate experiments. Values that differ significantly from the controls are shown: ** $P < 0.001$.

Table 3. Time course of trypsin action on the binding of ^{125}I -labelled insulin to particulate membranes

Particulate membranes were exposed to 500 µg of trypsin/ml at 37°C. At the times shown samples containing 200 µg of protein were added to tubes containing soya-bean trypsin inhibitor. Subsequently a binding assay was performed as described in the text. The values shown are the means $\pm$ S.E.M. of duplicates from two separate experiments. Differences between the mean values were evaluated by Student's *t* test; *P* values are shown in the Table.

Time (min)	Specific insulin binding (pg/mg)		<i>P</i>
	Control	Trypsin-treated	
0.25	90.9 $\pm$ 1.8	102.3 $\pm$ 2.1	<0.01
0.5	95.1 $\pm$ 1.2	111.6 $\pm$ 1.2	<0.01
2	96.3 $\pm$ 0.9	125.1 $\pm$ 2.1	<0.001
5	95.7 $\pm$ 1.5	128.4 $\pm$ 1.8	<0.001
15	93.3 $\pm$ 1.2	71.1 $\pm$ 1.5	<0.001

affinity was used which at least allows comparison with other authors' work. This was obtained by determining the concentration of unlabelled insulin that displaced 50% of the specifically bound ^{125}I -labelled insulin (Harrison *et al.*, 1978; Ginsberg *et al.*, 1977). In control particulate membranes, such displacement was produced by an insulin concentration of 2.0 nM; this decreased to 0.38 nM after exposure to 500 µg of trypsin/ml for 5 min. After 15 min treatment with trypsin, 50% displacement was

Fig. 4. Scatchard plots derived from equilibrium studies with control (●) and trypsin-treated (■) particulate membranes

Particulate membranes were treated with 500 μ g of trypsin/ml at 37°C for (a) 5 min or (b) 15 min. After these times an excess of soya-bean trypsin inhibitor was added and a binding assay performed with increasing concentrations of unlabelled insulin as described in the text.

produced by 0.28 nM-insulin, compared with 2.5 nM-insulin in the concurrent control experiment.

Discussion

This study has revealed marked differences in the response to phospholipase C and trypsin treatment of two different insulin-receptor preparations. In the particulate preparation, where the receptor is still an integral part of the membrane, there was a much greater effect of these enzymes than in the soluble preparation, where the receptor is at least partially released from the surrounding membrane structure.

Before these differences can be ascribed to effects related to modifications of the insulin-binding sites by changes in the adjoining membrane structure, other possible explanations must be considered. One possibility is that the presence of detergent in the soluble preparation may have led to the formation of detergent micelles incorporating the receptor, and

perhaps protecting it from the enzymes. This seems unlikely to be the explanation, as control experiments (Table 4) with particulate membranes in 0.05% Triton gave results that did not differ significantly from those obtained with particulate membranes alone. A significant difference ($P < 0.01$) was observed between particulate membranes in 0.05% Triton and the soluble receptor preparation for both phospholipase C and trypsin treatment. Therefore Triton did not appear to protect the receptor from the action of these enzymes. Moreover it has been demonstrated that Triton does not inhibit the enzymic action of trypsin (Rinderknecht *et al.*, 1970).

Another possible explanation which cannot be excluded is that the surrounding membrane may hold the receptor site in a conformation making it particularly vulnerable to the effects of phospholipase C and trypsin. However, it is hard to understand how solubilization could make the receptor site less exposed to enzymes (except by the micelle mechanism discussed above) than when it is surrounded by, and embedded in, the membrane. It therefore seems more likely that the digestive enzymes act predominantly on the surrounding membranes in the particulate preparations.

The interpretation of the effects of the enzymes depends in part on the interpretation of a curvilinear Scatchard plot. Our data do not allow differentiation between the alternative explanations of negatively co-operative interactions between binding sites (De Meyts, 1975) and multiple classes of binding sites of differing affinity. In the phospholipase C experiments, if the curvilinear Scatchard plots are attributed to negative co-operativity, the simplest explanation for the effect of phospholipase C on the particulate preparation is that it increases the apparent number of receptor sites without altering the average affinity (as shown by the similar curves in Fig. 1). This effect could be interpreted as being due to exposure of previously masked binding sites by digestion of phospholipid membrane components (Cuatrecasas, 1971b). However, it is also possible to interpret the results as showing an increase in the affinity of high-affinity binding sites, and an increase in number of low-affinity sites. Again this interpretation indicates a marked influence of the surrounding membrane on the binding characteristics of the receptor site, as no similar changes were observed in the soluble preparation.

The effects of trypsin are more complex. The increased affinity after exposure to trypsin suggests that the adjoining membrane proteins might normally decrease the facility of insulin binding to its receptors by holding the receptor in a lower-affinity configuration. Alternatively, if the curvilinear Scatchard plot is taken to represent multiple classes of binding sites, then the increased affinity could be due to trypsin unmasking high-affinity sites. The decrease in

Table 4. *Percentage change in ^{125}I -labelled insulin binding to different membrane preparations after enzymic digestion*
 Particulate membranes, particulate membranes in 0.05% Triton or solubilized placental receptors were incubated with phospholipase C (2 units/ml) for 30 min or with trypsin (500 $\mu\text{g}/\text{ml}$) for 15 min at 37°C. After these times the enzymes were inactivated and a binding assay was performed as described in the text. Each value is derived from the mean of triplicate determinations from two separate experiments. Differences between membrane preparations were evaluated by Student's *t* test; *P* values are shown in parentheses in the Table. Abbreviation: n.s., not significant (*P* > 0.05).

Membrane type	Percentage change in ^{125}I -labelled insulin binding	
	Phospholipase C-treated	Trypsin-treated
Particulate placental membranes	+ 16%	- 46%
	(n.s.)	(n.s.)
Particulate membranes in 0.05% Triton	+ 17%	- 50%
	(<i>P</i> < 0.01)	(<i>P</i> < 0.01)
Solubilized placental receptors	- 6%	- 11%

receptor numbers, which becomes more prominent with time (Fig. 4), may reflect an effect of trypsin on the overall conformation of the membrane, with such disruption of its structure that previously available sites become progressively unavailable. After a short time of exposure to trypsin, the increased affinity of binding is the predominant effect, producing increased receptor binding, whereas with increased time of exposure the loss of a number of available receptor sites leads to an overall decrease in receptor binding. Higher concentrations of trypsin affect the solubilized as well as the particulate receptor, suggesting that at these concentrations of trypsin there is direct digestion of the receptor site itself (Cuatrecasas, 1971a; Kono & Barham, 1971b).

Cuatrecasas (1971c) found a decrease in insulin binding to fat-cells after exposure to neuraminidase (4 or 10 $\mu\text{g}/\text{ml}$) and β -galactosidase (20 $\mu\text{g}/\text{ml}$), either used together or when neuraminidase was used first followed by β -galactosidase. The lack of effect of these enzymes observed in the present investigation (Table 1) suggests a different responsiveness of placental membranes compared with fat-cells. However, as the activities of the enzymes used in the Cuatrecasas (1971c) study were not stated, differing enzyme concentrations may have accounted for the results.

Cuatrecasas (1971a) interpreted the effect of trypsin on fat-cells as being due to a decrease in

affinity of hormone binding; however, his results are drawn not from competition experiments between a trace amount of ^{125}I -labelled insulin and increasing concentrations of unlabelled insulin, but from direct binding studies of ^{125}I -labelled insulin over a narrow concentration range of 10–100 pM-insulin. His experiments were performed with trypsin of unstated activity over a 15 min time period, where we, too, showed an overall decrease in binding. Our experiments clearly indicate opposing effects of trypsin on affinity (which was increased) and receptor number or availability (which was decreased). Kono & Barham (1971b) similarly found a decrease in insulin binding to fat-cells after a very short (15 s) incubation with 1 mg of trypsin/ml (unstated activity). It is impossible to compare their Scatchard plots with the present data, as their experimental design was somewhat different. They used a whole-cell system which they allowed to 'recover' for 2 h after trypsin treatment and before the binding assay. They also obtained a straight-line Scatchard plot, which is not usually the case for fat-cells (Harrison *et al.*, 1976), and differs markedly from the curvilinear plots obtained in the present series of experiments.

No direct conclusions about the effect of the enzymes on the biological responsiveness to insulin can be drawn from these experiments. It has been shown that trypsin directly stimulates glucose

oxidation in fat-cells initially, followed by a decrease in glucose oxidation after 15min exposure to trypsin (Kono, 1969; Kono & Barham, 1971a). Our results show that trypsin may also affect glucose metabolism indirectly by an effect on insulin binding, which appears to follow a similar time course.

In conclusion, although alternative explanations are possible, our results support the concept of influences of surrounding membrane structure on receptor affinity and availability. Although no direct conclusions can be drawn about the physiological relevance of these observations, alterations of adjoining membrane structure might provide one short-term regulatory mechanism affecting hormone-receptor interactions.

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References

- Bar, R. S., Gorden, P., Roth, J., Kahn, C. R. & De Meyts, P. (1976) *J. Clin. Invest.* **58**, 1123-1135
- Cuatrecasas, P. (1971a) *J. Biol. Chem.* **246**, 6522-6531
- Cuatrecasas, P. (1971b) *J. Biol. Chem.* **246**, 6532-6542
- Cuatrecasas, P. (1971c) in *Insulin Action* (Fritz, I. B., ed.), pp. 137-171, Academic Press, New York
- De Meyts, P. (1975) *Methods Mol. Biol.* **9**, 301-373
- Desbuquois, B. & Aurbach, G. D. (1971) *J. Clin. Endocrinol. Metab.* **33**, 732-738
- Ginsberg, B. H., Cohen, R. M. & Kahn, C. R. (1976) *Diabetes* **25**, Suppl. 1, 322
- Ginsberg, B. H., Kahn, C. R. & Roth, J. (1977) *Endocrinology* **100**, 82-90
- Harrison, L. C., Martin, F. I. R. & Melick, R. A. (1976) *J. Clin. Invest.* **58**, 1435-1441
- Harrison, L. C., Billington, T., East, I. J., Nichols, R. J. & Clark, S. (1978) *Endocrinology* in the press
- Kahn, C. R. (1975) *Methods Membr. Biol.* **3**, 81-146
- Kahn, C. R., Neville, D. M., Jr., Gorden, P., Freychet, P. & Roth, J. (1972) *Biochem. Biophys. Res. Commun.* **48**, 135-142
- New York and London
- Kono, T. (1969) *J. Biol. Chem.* **244**, 5777-5784
- Kono, T. & Barham, F. W. (1971a) *J. Biol. Chem.* **246**, 6204-6209
- Kono, T. & Barham, F. W. (1971b) *J. Biol. Chem.* **246**, 6210-6216
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Merimee, T. J., Pulkkinen, A. J. & Lofton, S. (1976) *J. Clin. Endocrinol. Metab.* **43**, 1190-1192
- Olefsky, J. M. & Reaven, G. M. (1974) *J. Clin. Invest.* **54**, 1323-1328
- Rinderknecht, H., Silverman, P., Geokas, M. C. & Haverback, B. J. (1970) *Clin. Chim. Acta* **28**, 239-246
- Roth, J. (1973) *Metabolism* **22**, 1059-1073
- Roth, J. (1975) *Methods Enzymol.* **37**, 223-233
- Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660-672
- Wang, C.-S. & Smith, R. I. (1975) *Anal. Biochem.* **63**, 414-417
- Yalow, R. S. & Berson, S. A. (1960) *J. Clin. Invest.* **39**, 1157-1175