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Practical considerations in analyzing radioreceptor assays by Scatchard analysis and capacity determination in irreversible hormone receptor interactions

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Abstract. The Scatchard plot in a radioreceptor assay depends upon the definition of specific binding and the quality of the iodinated hormone used. Iodination of protein hormones may alter it so that it no longer binds to the receptor and methods are available to measure the extent of this inactivation. When appropriate corrections are made for specific binding and the amount of inactive iodinated hormone in an assay, both qualitative and quantitative differences were observed in estimates of binding capacity and affinity in some well characterised hormone receptor systems.

Theoretical predictions derived from Scatchard analysis of irreversible unimolecular hormone-receptor interactions were applicable, both qualitatively and quantitatively to two irreversible hormone-receptor systems. A method described permits a more accurate estimate of capacity from radioreceptor assay data.

Keywords. Receptor assays; iodination of hormone; Scatchard analysis

Introduction

Previously it was reported that the reaction of human growth hormone (hGH) and ovine prolactin (oPRL) with solubilised or membrane bound receptor was irreversible (Murthy and Friesen, 1985; Van der Gugten *et al.,* 1980). The difficulties encountered in interpreting Scatchard plots when irreversible reactions occur was also considered and theoretical considerations have been developed which led to the development of a method for an accurate interpretation of the results. In the present study, we examined the influence of another component, namely the integrity of the iodinated hormone used as a tracer on the interpretation of receptor assays, and defined the necessary corrections which should be applied to obtain valid estimates of receptor capacity and affinity by using two hormone-receptor systems to experimentally verify, qualitatively and quantitatively the interpretations developed for Scatchard plots in irreversible systems (Murthy and Friesen, 1985).

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Abbreviations used; HR, Hormone receptor complex; PEG, polyethylene glycol 6000; BSA, bovine serum albumin; oPRL, ovine prolactin; hGH, human growth hormone; hPRL, human prolactin, bPL, bovine placental lactogen.

Materials and methods

All radioreceptor assays were carried out in Tris-HCl buffer, pH 7·6, 0·05 M $MgCl_2$ containing 0·1 % bovine serum albumin (BSA), as described by Shiu and Friesen (1974) and Posner *et al.* (1974). The time of incubation was 20 h at room temperature, unless otherwise indicated. The membrane protein content of the receptor preparations was generally 80–120 μg unless specified otherwise. When solubilised rabbit mammary gland receptor was used in the assay, separation of the bound and free hormone was carried out by using bovine γ-globulin (IgG) and polyethyleneglycol (PEG) (Shiu and Friesen, 1974). Scatchard analysis of the assay was performed using the necessary correction factors. With each assay the extent of damage of the iodinated hormone was determined by the saturation procedure as described below. Specific binding was defined as the radioactivity bound in excess of the radioactivity bound in the presence of 500 fold excess of hormone. All experimental determinations were set up in duplicate. For Scatchard plots only points upto 60–70 % displacement were considered and the line was drawn by linear regression analysis.

Radioimmunoassays were carried out by established procedures (Brige, 1967). Fixation of rat liver receptor and the absorption of oPRL by fixed and unfixed liver homogenate were carried out as already described (Salih *et al.,* 1979).

Determination of the extent of inactivation of iodinated hormone

Inactivation is defined as the inability of the hormone to bind to the receptor, due to the process of iodination itself, or due to radiation damage caused by [^{125}I] during storage.

Determination of maximum binding: The experimental procedure is similar to the one described earlier by others (Rao *et al.,* 1977; Diekman *et al.,* 1978). A known amount of tracer (generally corresponding to 100,000 cpm) was incubated with different amounts of receptor (concentrations ranging from 1–20 times those normally Used in receptor assays) for the determination of total binding. To determine nonspecific binding parallel incubations with 500 fold excess of unlabelled hormones were carried out. Incubations were continued overnight at room temperature and the binding of tracer to the receptor in the pellet was determined by procedures described (Posner *et al.,* 1974).

Determining the irreversibility of binding of oPRL to receptor

For determining irreversibility of the hormone receptor complex (HR) formed during incubation, the pellet in a radioreceptor assay was obtained by centrifugation, resuspended and incubated overnight with 500 μl of buffer containing 1 μg of oPRL (Van der Gugten *et al.,* 1980). The mixture was centrifuged and the supernatant discarded. The extent of irreversibility was defined as the extent of the radioactivity that still remained bound after incubation with oPRL.

Accurate determination of the binding capacity by modified Scatchard analysis

The apparent binding capacity was obtained by Scatchard plots drawn from the binding data as described. $(B/F)_{max}$ was also obtained by Scatchard plot. The extent of the error was then estimated as outlined previously (Murthy and Friesen, 1985). Using these data the exact capacity of the receptor was calculated.

Results

Figure 1 shows the binding of tracer to receptors ([^{125}I]-oPRL and rat liver receptor). As can be seen the specific binding (curve "c") increases linearly with protein (receptor) concentration and reaches a maximum with higher concentrations of receptor. Of the [^{125}I]-oPRL added (97000 cpm), a maximum of 34,000 cpm was bound.

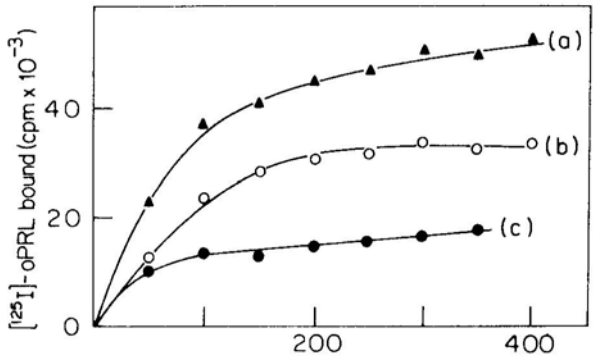

Figure 1. Binding of [^{125}I]-hormone to receptor as a function of receptor concentration. Binding of [^{125}I]-oPRL to rat liver receptor; (▲), total binding; (o), nonspecific binding; (●), specific binding. Receptor concentration: 800 μg/ml membrane protein.

Figure 2 shows a radioreceptor assay using oPRL and rat liver receptor. The data also indicates the binding in the presence of 0·4 μg unlabelled hormone and the radioactivity bound in the absence of receptor. Scatchard plots for this data are drawn in the inset. Curve "a" presents the Scatchard plot obtained assuming that all the [^{125}I]-oPRL used is capable of specifically binding to the receptor (an assumption that is generally made in literature). Curve "b" shows the plot drawn, assuming that 50 % of the [^{125}I]-oPRL is inactive, and also that all the radioactivity bound to the pellet in excess of what is bound to the glass tube is specific binding. Curve "c" is the plot obtained assuming that 50 % of the [^{125}I]-oPRL used is capable of binding to receptor and that only the excess tracer which is bound above that seen in the presence of 0·4 μg/rnl oPRL represents specific binding. In the above Scatchard analysis specific activity of the [^{125}I]-oPRL did not alter the plots. The plots drawn were nearly identical when the specific activity was assumed to be 50 μCi/μg, 100 μCi/μg or 150 μCi/μg of hormone.

Figure 3 presents radioreceptor assay curves for [^{125}I]-oPRL-oPRL (curve a) and [^{125}I]-hPRL-oPRL (curve b) with rat liver receptor. The data is analysed by conventional* (a' and b') and corrected** Scatchard Analysis (a and b). The common

* The assumptions used are that all the iodinated hormone used is capable of binding to the receptor and the radioactivity bound in excess of 2 μg blank is specific binding.

** The assumptions used are that only certain fraction of iodinated hormone is capable of binding to the receptor and the radioactive bound in excess of 2 μg blank is specific binding.

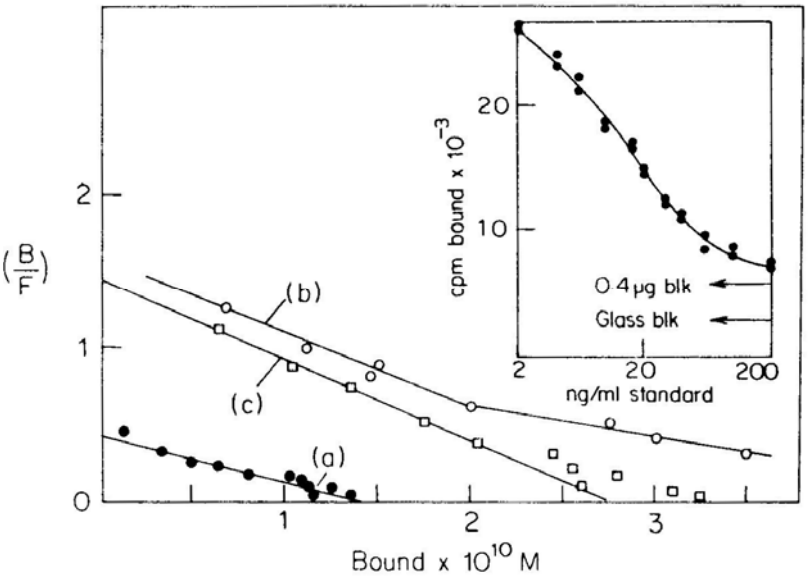

Figure 2. Scatchard plots of the interaction of oPRL with a rat liver receptor preparation. Inset presents the actural binding data from which Scatchard analysis was conducted. In the figure 0·4 μg blank represents the radioactivity bound in the presence of 0·4 μg/ml of oPRL. he glass blank represents the radioactivity bound to the glass tube (in the absence of receptor). Curve a; Scatchard plot drawn assuming the tracer to be 100 % bindable and binding at 0·4 μg blank represent nonspecific binding. Curve b; Correction applied for bindable trace (50% bindable) and binding at 0·4 μg blank represents nonspecific binding. Curce c; Correction applied for bindable trace (50 %) and glass binding represents nonspecific binding.

feature of the 4 plots is that all are reasonably linear. But the apparent capacity and binding constant are both very different. By conventional analysis the capacity of the receptor is nearly 10^{-10} M for both hormones. However, by the corrected Scatchard plot the capacity is increased nearly 2 fold. A casual glance of the radioreceptor assay data would also indicate that the radioactivity bound using [^{125}I]-hPRL (curve b) is greater than for [^{125}I]-oPRL (curve a) and hence would indicate higher K_a for hPRL, By conventional Scatchard analysis the binding constant for hPRL and oPRL is identical. But it becomes obvious from the corrected Scatchard plots that oPRL has infact higher affinity for the receptor than does hPRL.

Figure 4 shows the radioreceptor assay data for [^{125}I]-hGH -hGH (curve a) and [^{125}I]-bPL-hGH system (curve b) for rabbit liver receptor. Conventional and corrected Scatchard analysis are drawn for the two systems in the inset. It is seen that the plots for [^{125}I]-bPL-hGH is linear while the [^{125}I]-hGH-hGH is nonlinear by both types of Scatchards. The apparent capacity for bPL is increased nearly 3 fold by the correction, while the apparent binding constant remains nearly the same. However, in the case of hGH the plot is nonlinear, in both corrected and conventional plots. This nonlinearity is probably due to the presence of two kinds of receptors in the rabbit liver (Waters and Friesen, 1979; Maes *et al.,* 1983).

Figure 5 presents the displacement curve in a receptor assay using oPRL and rat liver receptor. Curve "b" corresponds to specific binding of [^{125}I]-oPRL bound to the

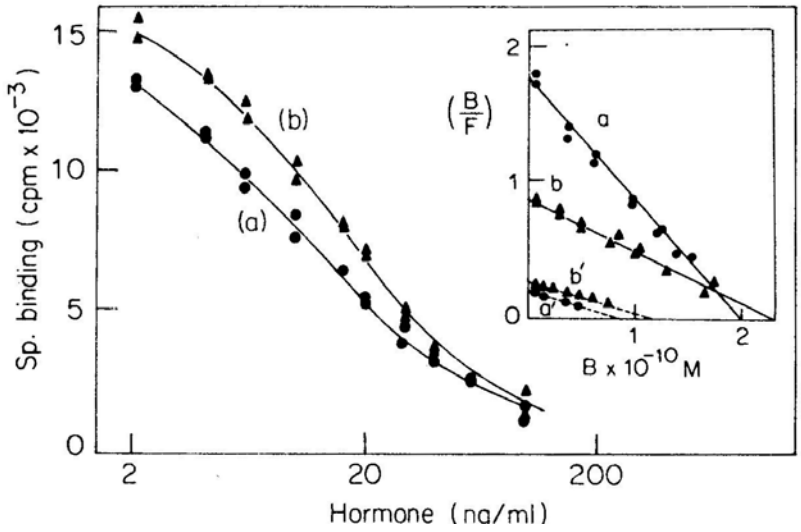

Figure 3. Binding data of [^{125}I]-oPRL displaced by oPRL (curve a) and [^{125}I]-hPRL displaced by oPRL (curve b) using rat liver repectors. The total radioactivity added was 100,000 amd 90,000 cpm for [^{125}I]-oPRL and [^{125}I]-hPRL respectively. The receptor active hormone was measured by repeated consecutive exposure to receptors as shown in table 1. The inset shows Scatchard plots for the same binding data, a and a' correspond to corrected and uncorrected Scatchard plots for [^{125}I]-oPRL-oPRL system; b and b' correspond to corrected and uncorrected plots for [^{125}I]-hPRL-oPRL system.

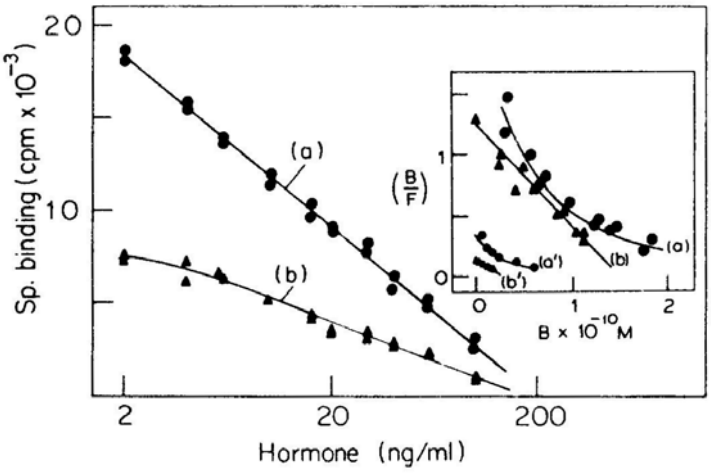

Figure 4. Binding data [^{125}I]-hGH displaced by hGH (curve a) and [^{125}I]-bPL displaced by hGH (curve b) using receptors obtained from pregnant rabbit liver membranes. Total radioactivity by [^{125}I]-hGH and [^{125}I]-bPL added were 95,000 and 75,000 cpm respectively. The maximal binding of the tracer was determined by the method outlined in table 1. Inset shows the Scatchard plots for the above binding data. a and a' correspond to corrected and uncorrected plots [^{125}I]-hGH-hGH system, b and b' correspond to corrected and uncorrected plots for [^{125}I]-bPH-hGH system.

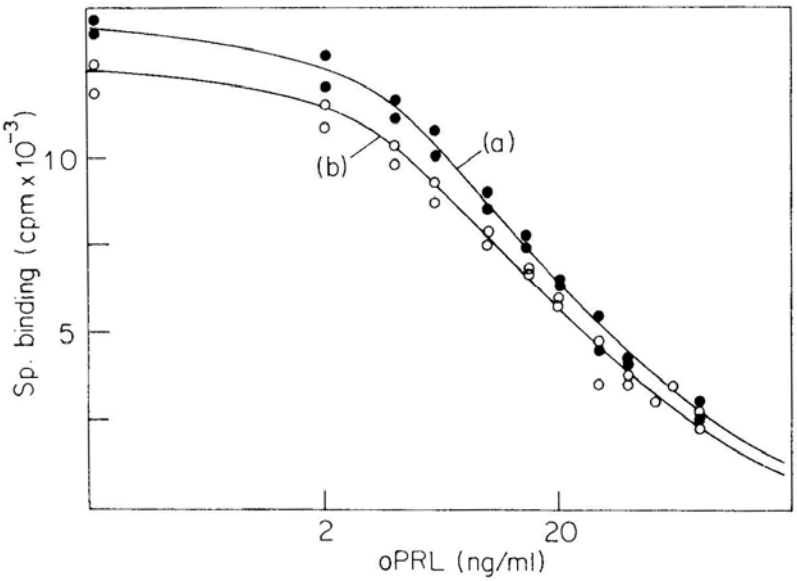

Figure 5. Binding of [^{125}I]-oPRL to rat liver membrane (72 μg protein) in radioreceptor assay (curve a). Irreversibility bound [^{125}I]-oPRL in the assay is shown in curve b. Irreversibility was determined as described in materials and methods.

receptor after incubation with 1 μg of oPRL overnight at room temperature. The data clearly indicate that 90 % of the oPRL bound to receptor cannot be displaced by added oPRL and that the reaction is largely irreversible. The other system which has already been shown to be completely irreversible (Murthy and Firesen, 1985) is the binding of [^{125}I]-hGH to rabbit mammary gland receptor. Hence these systems are appropriate ones to check the theoretical predictions for irreversible systems.

Figure 6 presents several experimental Scatchard plots. Figure 6A, B and C show the effect of time on the Scatchard plot in receptor assays for soluble rabbit mammary gland receptor and hGH. The time of the reactions were 3·5 h (figure 6A) 7·5 h (figure 6B) and 20 h (figure 6C) respectively. Likewise figure 6D, E and F show the effect of time on the Scatchard plot in receptor assay for rat liver receptor and oPRL. The time of the reactions were 5 h (figure 6D), 7·5 h (figure 6E) and 22 h (figure 6F) respectively. Figure 6G and H show the effect of receptor concentration on the Scatchard plot in receptor assays for rat liver receptor and oPRL. It is seen that $(B/F)_{max}$ increases as the time of the reaction (figure 6A–F) or concentration of receptor increase (figure 6G, H). In addition an increase of $(B/F)_{max}$ is associated with a decrease in apparent capacity in both rabbit mammary gland (figure 6A–C) and rat liver receptor (figure 6D–H) . Table 1 presents the capacity measured for the receptor preparation by conventional and modified methods.

Table 2 compares the capacity-measurement from receptor assays using conventional and modified Scatchard analysis. All the assays were carried out on different days. The receptor used for all the assays were from the same batch. With each assay, the integrity of the tracer and the extent of inactivation were determined by the receptor saturation procedure as described. The capacity measured (last column) by the

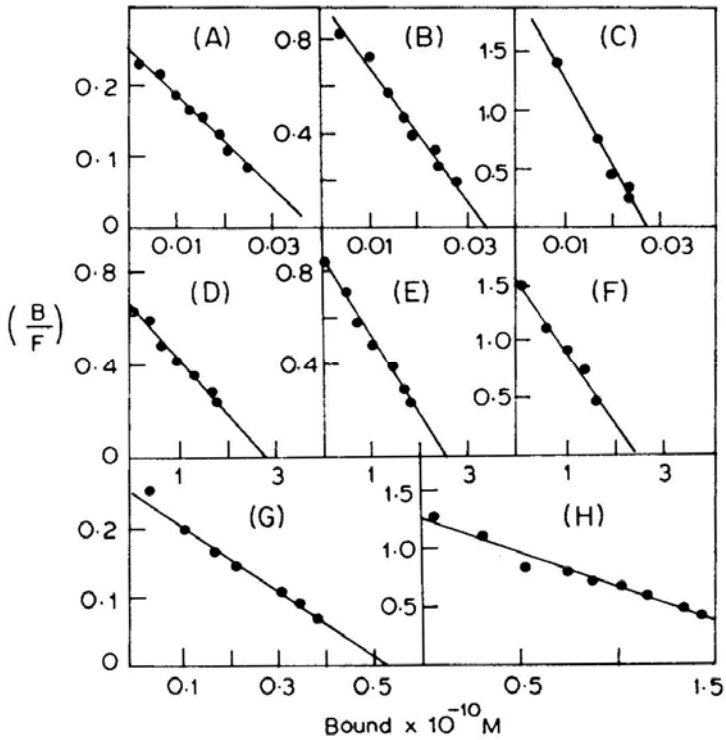

Figure 6. Scatchard plots drawn for competitive binding data of [^{125}I]-oPRL to membrane receptors from rat liver, and of [^{125}I]-hGH to solubilized receptors from rabbit mammary gland in receptor assays. The lines were drawn by least squares method. The corrections for inactive tracer and nonspecific binding were applied as described in the text. A, B and C are the Scatchard plots for the competitive binding data to soluble rabbit mammary gland receptor. 80 μg membrane protein was used in each assay. The time of the incubation was 5 h (A), 7·5 h (B) and 22 h (C) respectively. D, E and F show the effect of time of the reaction on the Scatchard plot for competitive binding data of [^{125}I]-oPRL to rat liver membrane. Membrane protein concentration in the assay was 100 μg. Time of the reaction was 5 h (D), 7·5 h (E) and 20 h (F) respectively. G and H show the effect of receptor concentration on the Scatchard plots in rat liver receptor membrane—[^{125}I]-oPRL receptor assay. Reactions were carried out overnight at room temperature. G and H show the plot obtained using 20 μg of membrane protein and 100 μg of membrane protein respectively.

modified Scatchard procedure was the same as should be expected for the same receptor preparation. However, the capacity obtained by the conventional Scatchard analysis was different in every assay. The capacity is underestimated in assay nos. 2, 3 and 4 by nearly 25–60% , and is overestimated by nearly 65 % in assay no. 1. Thus depending upon the integrity of the tracer used as well as the percent specific binding observed in an assay an overestimation or an underestimation is made by conventional Scatchard analysis.

Discussion

Analysis of hormone radioreceptor assay data to determine affinity constants by Scatchard's analysis is a widely employed technique. Estimates of affinity constant or

Table 1. Capacity estimations in irreversible hormone-receptors reactors by Scatchard plots.

Receptor system used		$(B/F)_{max}$	Uncorrected* capacity $\times 10^{-10}$ M	Over ± estimation (%)	Corrected* capacity $\times 10^{-10}$ M
Solubilised Rabbit mammary gland receptor-hGH	Time 3·5 h	0·262	0·38	52	0·25
	Time 7·5 h	0·487	0·34	42	0·24
	Time 22 h	2·070	0·27	18	0·23
Rat liver membranes-oPRL	Time 5 h	0·663	2·79	36	2·04
	Time 7·5 h	0·865	2·51	32	1·90
	Time 22 h	1·512	2·46	23	2·00
Rat liver membrane-oPRL	Receptor conc. 20 μg	0·262	2·62	62	1·72
	Receptor conc. 100 μg	1·257	2·09	26	1·66

* Expressed as concentration of the receptors. This is the value obtained after making corrections for Scatchard analysis as described previously (Van der Gugten *et al.*, 1980).
+ Values read from figure 8 of Murthy and Freisen (1985).

Table 2. Comparison of capacity measurements using conventional Scatchard analysis and modified Scatchard analysis*.

Assay No.	Specific binding (%)	Capacity obtained by conventional Scatchard analysis $\times 10^{-10}$ M	Capacity obtained by modified Scatchard analysis $\times 10^{-10}$ M
1	44	3·779	Nonlinear (concave)
2	14·3	1·485	2·330
3	18·7	1·435	2·272
4	15	1·056	2·320

The percentage of the tracer capable of binding to the receptor in assays no. 1,2,3 and 4 was 48 %, 34 %, 38 % and 24 % respectively.
* Capacities are all expressed as concentration of receptor when 120 μg membrane protein is used in the receptor assay.

capacity for the same HR interaction vary considerably, often by more than an order of magnitude from laboratory to laboratory, and even within one laboratory significant variation is observed. Based on the present study likely sources of error in these estimates arise from the use of partially inactive iodinated hormone used as tracer in the radioreceptor assay, and the specificity conditions used in the assay.

Data from figure 2 clearly demonstrates that any meaningful assessment of radioreceptor assay data by Scatchard analysis should take into account the proportion of tracer which is able to bind to receptor. Likewise an appropriate estimate of specific binding is important. Thus before any receptor assay be quantitatively analysed it is important to determine the extent to which the tracer is capable of binding to receptor and also to choose appropriate criteria and estimates for specific binding.

The extent of damage even when mild iodination procedures are used varies

considerably for different hormones (Maes *et al.,* 1983). Storage of iodinated hormones also leads to inactivation. In order to conduct proper analysis of radioreceptor assays when determining receptor affinity or capacity it is necessary to calculate the ability of the tracer to bind to receptors using the methods as outlined.

The results presented clearly indicate the nonspecific binding in the presence of excess cold hormone as the appropriate choice (table 3) and the nonspecific binding can be defined as the binding of [^{125}I]-hormone in the presence of excess cold hormone.

The specific activity of the iodinated hormone does not have a major influence on the Scatchard plot. This result is a likely reflection of the insensitivity of the assay.

Table 3. Binding of [^{125}I]-oPRL to receptors in fresh or fixed rat liver homogenate.

Receptor preparation	Fresh liver membrane	Fixed liver membrane	Ratio of binding normal/fixed membrane
Protein concentration (μg)	155	122	—
Specific binding %	12·5	8·5	1·47
Total binding %	15	26	0·57
PRL adsorbed/100 μl* of receptor preparation (ng)	4·8	2·8	1·7

Specific binding is defined as the difference in the radioactivity bound to the receptor, in absence and presence of 2 μg/ml cold hormone expressed as a percentage of the radioactivity added.
Total binding is defined as the difference in radioactivity bound to the receptor and glass tube expressed as a percentage of the radioactivity added.
* Determined by radioimmunoassay

The systems in which the hormone and receptor binding is demonstrably irreversible needs further corrections in the estimates of capacity as detailed below. One of the consequences of irreversibility of the reaction between hormone and receptor was that the $(B/F)_{max}$ increased and B_{max} decreased as the parameters *t* (time) and a (receptor concentration) increased (Murthy and Firesen, 1985. Table 1 also shows that the corrected capacity calculated according to the method previously suggested is independent of the $(B/F)_{max,}$ *t* and *a.* The errors in true capacity estimates in most cases are in the order of ± 5 %.

A comparison of the capacity measurements made by conventional and modified Scatchard analysis (Murthy and Friesen, 1985) indicates that (table 2) the capacities estimated by the former method overestimated or underestimated the true capacity to varying degrees, depending upon the activity of the tracer and the specific binding observed in any particular assay. The data presented in table 3 indicate that the overestimation was very significant when the tracer prepared had high ability to bind and the specific binding was high in the assay. Thus in a physiological experiment one can envisage a situation where the capacity measurement in treated and untreated receptor preparations are underestimated in one case and overestimated in another case. Thus in a radio-receptor assay a correction for the label that can be bound should be incorporated in each assay, as described in this paper.

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