We measured time-dependent concentration changes of human interferon-\(\alpha\) (IFN) and human tumor necrosis factor-\(\alpha\) (TNF) bound at the plasma membrane and internalized by human lung alveolar carcinoma A549 cells in the presence of excess free ligand. Concentration changes for these two ligands were substantially different. We modified our compartmental kinetic model encompassing receptor synthesis and receptor loss (Myers, A. C., Kovach, J. S., and Vuk-Pavlovic, S. (1987) J. Biol. Chem. 262, 6494–6499) to include receptor recycling. We solved analytically the equations of three variants of the model of receptor recycling. All parameters (rate constants) were identifiable when the data sets consisted of time-resolved concentrations of IFN and TNF at the cell surface and internalized by cells. By least squares fitting we derived the best fit values for the first order rate constants for internalization of the ligand-receptor complex, receptor recycling, turnover of free receptors, elimination of the ligand from cells, and the rate of insertion of free receptors into the membrane. The best fit to data for interactions of cells with IFN was obtained without inclusion of the term for recycling of receptors to the membrane. The simplest model including receptor recycling was necessary and sufficient for the fit to the respective data for TNF. These results demonstrate that the contribution of receptor recycling to the metabolism of the ligand and the receptor can be quantitated by compartmental modeling. Receptor recycling does not contribute to the kinetics of Type I IFN receptor in A549 cells. In contrast, recycling contributes significantly to endocytosis mediated by the TNF receptor.

Ligands such as growth factors, protein hormones, and trophic proteins interact with cellular receptors and trigger transduction of biochemical signals which elicit biological responses. These processes are usually accompanied by endocytosis of the ligand-receptor complex and by replenishment of free membrane receptors. In absence of ligand, the free receptors in the membrane maintain a steady-state concentration; this concentration is determined by the ratio of receptor insertion rate into the membrane and the rate of loss of free receptors from the membrane by internalization, shedding, or inactivation of receptors (1). The rate of internalization for any particular ligand will be determined by ligand concentration, receptor concentration, ligand-receptor affinity, rates of ligand synthesis and loss, receptor recycling (reutilization), the rate of endocytosis, and the transport capacity of the endocytic apparatus (1).

Understanding the regulatory factors critical for interactions of protein ligands with cells is particularly important for rational manipulation of extravascular protein trafficking, as in autocrine regulation (2, 3) and for biological response modifier therapy (4). For example, "sinking" of proteins into cells by receptor-mediated endocytosis might restrict the efficiency of diffusional and convective transport of proteins in tissues (5).

In order to resolve kinetic determinants of protein-cell interactions, we are studying interactions of proteins with cells in monolayer culture (6). Compartmental models provide means to analyze time-dependent concentrations of both ligand interacting with the cell surface and of internalized ligand. Recently we reported derivation of rate constants by computer-assisted modeling for interactions of IFN\(^1\) with the nonrecycling Type I IFN receptor in human carcinoma cells in vitro (6). We now report derivation of a similar mathematical model which includes recycling of receptors. We have considered applicability of simple models that include first-order rate constants for elementary processes in receptor metabolism. We have investigated identifiability of parameters, reported analytical solutions of the proposed models, tested these solutions on data (interactions of IFN and TNF with human alveolar lung carcinoma A549 cells in vitro), and investigated whether statistical criteria alone are adequate discriminants of nonrecycling receptors and recycling receptors. We also introduce methods for reduction in the number of free parameters per experimental point for compartmental modeling of receptor dynamics. A comparative kinetic analysis of interactions of IFN and TNF with human epithelial tumor cells in vitro will be reported separately.

**MATERIALS AND METHODS**

*Cells—* Human lung adenocarcinoma A549 cells were obtained from American Type Culture Collection (Rockville, MD). Cells were main-

\(^{1}\) The abbreviations used are: IFN, recombinant human interferon-\(\alpha\); TNF, recombinant human tumor necrosis factor-\(\alpha\).
tained in Dulbecco's modified Eagle's medium (GIBCO) containing 10% heat-inactivated fetal calf serum (GIBCO) at 37 °C in water-saturated 5% carbon dioxide, 95% air mixture.

**Radioiodination of Tumor Necrosis Factor—Recombinant human TNF (Lot 3056-55; specific activity: 5.02 x 10^6 Bq/ml) was iodinated according to a published procedure** (7). 

**Measurement of Dissociable and Nondissociable TNF**—Measurement of dissociable and nondissociable TNF was performed in confluent cell layers in 35-mm plastic Petri dishes (Falcon, Oxnard, CA). Briefly, experiments were initiated by collection of the conditioned media overlaying the confluent cells. The medium was centrifuged at 9000 x g for 10 min at room temperature when the cell supernatant was removed. The medium was exchanged with medium containing 10% heat-inactivated fetal calf serum (GIBCO) at 37 °C in water-saturated 5% carbon dioxide, 95% air mixture. After incubation of the tube on ice for 10 min, 100 μl of the solution was transferred to another tube containing 10 μg (20 μl) of TNF. 10% heat-inactivated fetal calf serum (GIBCO) at 37 °C in water-saturated 5% carbon dioxide, 95% air mixture. Specific radioactivity of these preparations was -5 x 10^6 Bq/mol. On the average, 93% radioactivity was precipitable by 8.5% trichloroacetic acid in presence of 10% fetal calf serum as carrier protein. The biological activity of radiolabeled TNF was indistinguishable from native TNF based on lysis of murine L929 cells pretreated with actinomycin D (1 μg/ml) with 2-fold dilution series of native TNF and labeled TNF (7). 1^25I-TNF was stored at 4 °C and showed no change in binding and internalization over 4 weeks.

**Kinetic Models and Ligand-Receptor Interactions**—The indented rectangles represent specific membrane receptors and circles stand for the ligand molecules. The symbol R0 denotes the unoccupied surface receptors, (LR)s, the ligand-receptor complex at the cell surface, (LR)esc the ligand-receptor complex committed to internalization at the cell surface and (L)1 (square in Model III) represents internalized receptor free ligands. The meaning of symbols for the rate constants is described in the text.

![Fig. 1. Schematic representations of kinetic models of ligand-receptor interactions.](image-url)

**Equations for the Models**—The interactive computer graphics methods and the curve fitting program were described by Myers et al. (6). The best fit parameters were obtained as values of first order rates or rate constants for all parameters: k[L], k0, k2, k3, Vc, as well as k and k, when applicable.

Equations were fitted to the means of triplicate measurements ± S.E. These errors reflected variations in number of cells among dishes and the Poissonian nature of radioactive decay. When experimentally determined standard errors were smaller than predicted by the Poissonian distribution, the (radioactive) ligand and/or its degradation products accumulate within a compartment of intracellular receptor-free radioactivity (L). This compartment is included into Model III (Fig. 1) described by Equations 1a, 2b, and 4.

When ligand binding triggers delayed irreversible changes of the physical state of membrane receptors (e.g. aggregation; cf. Ref. 12), a distinct pool of membrane receptors committed to internalization (LR)esc is created. Conversion of (LR)s to (LR)esc is characterized by the first order rate constant k, (Model IV; Fig. 1, Equations 1a, 2b, and 5).

If the free ligand concentration is constant throughout experiments (or if the change is negligibly small), differential equations defining Models I–IV are first order and can be explicitly solved. The explicit solutions for the models are given in Appendix I.2 The interactive computer graphics methods and the curve fitting program were described by Myers et al. (6).
son distribution, we used the square root of the mean value as the standard error. The uncertainty in best fit parameter values was determined by standard methods (13). The goodness of fit is reported by values of $\chi^2$ ($x^2$ divided by the number of degrees of freedom). The significance of differences between $\chi^2$ values was evaluated by the $F$-test (15). The best fit curves were tested for serial correlations of error terms according to Durbin and Watson (14, 15).

RESULTS

In this paper IFN and TNF interactions with A549 cells have been studied as a paradigm for testing Models I-IV. A549 cells were selected because they provided easily measurable interactions both with IFN (6) and TNF.

Interactions of A549 Cells with IFN.—When IFN interacted with cells under pseudo-first order conditions, both [LR] and [LR] depended on time (Fig. 2 in Ref. 6). [LR] values reached a maximum approximately 30 min after exposure of cells to IFN and then declined to a steady-state concentration. [LR] peak values were observed around 100 min and declined to a steady-state level. For a discussion of mechanisms of these concentration changes cf. Refs. 1 and 6.

We fitted equations of Models I-IV to our previously published data (Fig. 2 in Ref. 6). The best fit parameters for Model I (nonrecycling receptors) are given in Table I. Coefficients of variation of these parameters were equal to or less than 15% of the mean value. The value of $[R]_0$ at $t = 0$ calculated from the ratio $V_0/k_0$ was $910 \pm 85$ receptors/cell; at steady state ($t = \infty$) it was $75 \pm 15$ receptors/cell. The value of $[R]_{\text{steady}}$ is a function of [L].

Equations of Model II (recycling receptors) were fitted to data with two different sets of initial parameters. With best fit parameters from Model I and $k_0 = 0$ as initial estimates, the best fit parameters for Model II did not differ from initial estimates, indicating a local minimum. This fit was robust and the best fit parameters did not change if single initially entered parameters were varied within 1 order of magnitude. If several initial parameters were perturbed by several orders of magnitude, another best fit was reached for Model II and the $\chi^2$ value was 6.8 compared to 11.7 for Model I. However, the value of the recycling rate constant $k_r$ was negative indicating that this set of best fit parameters represented a physically unfeasible solution. This conclusion is corroborated by the negative sign of the calculated values of $[R]_0 = f(t)$. Similarly unrealistic sets of best fit parameters were obtained by fitting equations of Models III and IV to same data (not shown). Thus, Model I yielded the optimal fit to data and physically adequate parameter values, although smaller $\chi^2$ values were obtained in a fit which resulted in physically unrealistic best fit parameters.

Reduction in Number of Free Parameters.—The fit of equations of Model I to data (Fig. 2 in Ref. 6) was obtained with all parameters free and without limits imposed on values of acceptable solutions. However, when one or more parameters of the model are known a priori, they can be used as fixed parameters in the fitting procedure to reduce the number of free parameters. This manipulation results in fewer free parameters per experimental point and has been shown to result in better fits (11, 16).

In order to reduce the number of free parameters, we used the equilibrium dissociation constant $K_D$. This constant, usually derived by the Scatchard analysis of equilibrium binding data is known for many cell-protein interactions. We modified the format of parameters to include the value of $K_D/[L]$ as the fixed parameter. However, the extent to which the best fit parameters are sensitive to errors in the fixed parameter might limit the usefulness of the independently derived fixed parameter. Therefore, we investigated how the best fit parameters and $\chi^2$ value from Model I for data (Fig. 2 in Ref. 6) varied as the function of $K_D$.

Parameters and $\chi^2$ values from best fits of Model I to data (Fig. 2 in Ref. 6) are shown in Fig. 2 as a function of $K_D/[L]$. The plots of best fit parameters as a function of $K_D/[L]$ in Fig. 2 show that the best fit parameters depended on $K_D/[L]$. The value of $V_r$ diminished almost linearly with increasing $K_D/[L]$; other parameters exhibited a maximum ($k_r$ and $k_0$) or

![Fig. 2. Best fit parameters and $\chi^2$-value as a function of $K_D/[L]$ for fits of equations of Model I to time-dependent concentrations of IFN at the cell surface and internalized by cells (Fig. 2 in Ref. 6). Shaded areas denote the range of $\pm$S.D. For details see text.](image-url)
Kinetic Models of Ligand-Receptor Interactions

The best fit values were obtained by fitting equations of Model I and Model II, respectively, to data in Fig. 2 from Ref. 6. The value in italics denotes the fixed parameter. The coefficients of variability for each best fit value are given in parentheses. The errors in $k_2$ and $V_v$ are influenced also by the error in [L] (for $k_2$ and $V_v$) and number of cells per plate (for $V_v$ only). The error in [L] was not determined and variability of the number of cells/plate was ±10%.

| Model | [L] | $k_c$ | $k_r$ | $k_k$ | $k_d$ | $k_d$ | $V_v$ | $x^2$ | receptor-cell$^{-1}$ |
|-------|-----|-------|-------|-------|-------|-------|-------|-------|-------------------|
|       |     | $s^{-1}$ | $M^{-1}$ | $s^{-1}$ | $s^{-1}$ | $s^{-1}$ | $s^{-1}$ |       |                   |
| I     | $9.4 \times 10^{-11}$ | $3.6 \times 10^{6}$ | $(7)$ | $1.2 \times 10^{-3}$ | $(8)$ | $7.8 \times 10^{-4}$ | $(3)$ | 0.000 | $1.2 \times 10^{-6}$ | $(16)$ | $3.7 \times 10^{-4}$ | $(3)$ | 0.011 | $(13)$ | 11.7 |
| II    | $9.4 \times 10^{-11}$ | $5.9 \times 10^{2}$ | $(2)$ | 0.09 | $(9)$ | $7.5 \times 10^{-3}$ | $(3)$ | $-2.8 \times 10^{-4}$ | $(4)$ | $2.9 \times 10^{-4}$ | $(7)$ | $3.2 \times 10^{-4}$ | $(4)$ | 0.19 | $(4)$ | 6.8 |

**Fig. 3.** Time-dependent acid-dissociable (○) and internalized (□) radioactivity in the interaction of 125I-TNF with human lung carcinoma A549 cells. The circles represent the mean of three determinations ± S.D. The curves are computed from the best fits of equations of Model I (a) and Model II (b) to data, respectively. The extra line in b represents the computed concentration change in $R_a$. Inset in b is a blow-up of data and best fit curves during the first hour of the experiment.

The best fit values to six free parameters (Fig. 3c). The fit was characterized by the $x^2 = 18.8$. Coefficients of variability for $k_c$, $k_r$, $k_k$, and $V_v$ were unacceptably large. Moreover, the values of $k_d$ and $[R]_{LR = \infty}$ were negative, rendering the entire fit meaningless.

**Model II**—In attempting to fit equations of Model II (recycling receptors) to data in Fig. 3, it was useful to reduce the number of free parameters by introduction of experimentally determined value of $K_D$ as a fixed parameter. This decreased the number of free parameters in Model II from seven to six. Since fitting yielded the value of $k_{c5}$, we selected $K_D/[L]$ (=$k_c/k_r/[L]$) as the parameter most convenient for manipulations. We fixed the ratio $K_D/[L]$ at 1.000 because the value of $K_D$ for the interaction of TNF with human cells was determined by equilibrium binding experiments as $1.5 \times 10^{-10} M$ (17–21) and the concentration of free TNF in experiment shown in Fig. 3 was set at $[L] = K_D$. The $x^2$ value for the fit of the six-parameter Model II ($x^2 = 7.0$; Fig. 3b and Table II) was significantly lower than $x^2$ for the fit of the six-parameter Model I ($F = 44.88, p < 0.001$). All best fit parameters for Model II were positive and the coefficients of variability were smaller than 15%, except for $k_d$ (Table II).

**Model III**—The absence of an apparent steady state in $[LR]_1$ (Fig. 3) and the small value of $k_d$ obtained from Model II (Table II) indicated the possible presence of an internalized ligand pool. Therefore, we modified Model II to include such a pool created by the $k_c$ process and reduced by the $k_d$ process (Model III). The $x^2$ of 16.8 was more than twice as large as the corresponding value for Model II. The unrealistically high value of $k_d$ and the $x^2$ value as large as for Model I indicate that Model III was significantly less adequate for data in Fig. 3 than Model II.

**Model IV**—Association of protein ligands with specific receptors in plasma membrane is generally accompanied and/or followed by preendocytotic aggregation of the ligand-receptor complexes in the plane of the plasma membrane (cf. Refs. 11 and 12). Therefore, we investigated how introduction of the preinternalization step (as in the seven-parameter Model IV) influenced fits to data in Fig. 3.

Except for $k_d$, the best fit parameter values for Model IV agree well with the respective values obtained from Model II (Table II). Model IV yields $x^2 = 6$ (compared to $x^2 = 7$ in Model II; $F = 5.2, 0.05 > p > 0.025$) and a 6-fold larger value of $k_d$; however, the large coefficient of variability for $k_d$ (Model II) makes evaluation of the difference between $k_d$ values from Model II and Model IV difficult. The value of $k_d$, the rate constant for conversion of $[LR]_1$ to $[LR]_{LR = \infty}$, was of the order of $10^3 s^{-1}$ (with a >100% coefficient of variability) indicating that the $k_d$ process is not rate limiting for internalization under conditions of experiment in Fig. 3.

On the basis of data in Table II, we conclude that Model I (nonrecycling receptors) is not applicable to interactions of...
The best fit parameters for interactions of human tumor necrosis factor-α with A549 cells

TNF with A549 cells. Model II, the least complex among models which include receptor recycling, yields fits equivalent to or better than more complex Models III and IV.

Dependence of Best Fit Parameters on $K_D/[L]$—Similarly to the analysis of dependence of best fit parameters for Model I (Fig. 2), we investigated how the best fit parameters and $\chi^2$ value for Model II depended on $K_D/[L]$. The values of parameters and $\chi^2$ values from the best fits of Model II to data in Fig. 3 are shown in Fig. 4 as a function of $K_D/[L]$. The true values for $K_D$ and $[L]$ in the experiment in Fig. 4 were $1.5 \times 10^{-10}$ M. From plots in Fig. 4 it appears that the values of $k_3$ and $k_6$ were almost independent of $K_D/[L]$, whereas the values of $k_2$ and $k_5$ were reduced to $77 \pm 11\%$ and to $82 \pm 8\%$ of the respective value at $K_D/[L] = 0$; for $K_D/[L] > 1$, the values of $k_6$ change only slightly. The values of $k_2$ depended strongly on $K_D/[L]$ assuming negative values at $K_D/[L] > 2$; thus, the negative value of $k_6$ set an upper limit to the ratio $K_D/[L]$. Within the interval $0 < K_D/[L] \leq 2$, the values of $V_c$ and $\chi^2$ increased monotonously by $51 \pm 16\%$ and $33\%$, respectively. It is noteworthy that both $k_2$ and $k_5$ were resilient to variation in $K_D/[L]$. Moreover, for $K_D/[L] < 1$ variations in $k_2$, $k_3$, $V_c$, and $\chi^2$ were less than $30\%$. The strong dependence of $k_2$ on $K_D/[L]$ shows that additional constraints could be set upon the free parameter space by entering the experimentally determined value of $k_2$ as a fixed parameter. Thus, internalization experiments performed at $K_D/[L] < 1$ by use of large $[L]$, will limit deviations of the best fit parameter values from the values of true rate constants. If precision in determination only of $k_2$ and $k_5$ is required, the accuracy of $K_D$ is even less critical.

Cofitting Multiple Data Sets for Reduction in Number of Free Parameters—Together with introduction of fixed parameters, fits can be improved by reduction in the number of free parameters per experimental point by fitting two or more related data sets together in the manner of global analysis (22). The condition necessary for this manipulation is that experimental systems be identical in one or more parameters. A simple design for such experiments includes measurements of $[L]_s$ and $[L]_I$, as a function of $t$ and $[L]$, i.e. data points are collected at two or more pseudo-first order concentrations of free ligand. Equations of appropriate mathematical models are then fitted to these data sets with the equivalent parameters linked in a ratio compatible with experimental values of $[L]$.

We fitted equations of Model II to data sets for A549 cells interacting with $[L] = 1.5 \times 10^{-10}$ M (same data as in Fig. 3) and $[L] = 3.0 \times 10^{-10}$ M TNF (Fig. 5). We entered the value of $K_D/[L] = 1.000$ as the fixed parameter with $K_D/[L] = (K_D/[L])/2$ and $k_6/[L] = 2k_6/[L]$. Other parameters for both data sets were free.

The best fit curves and parameters are shown in Fig. 5 and Table III, respectively. The $\chi^2$ value for the fit was $9.2$ compared to $7.0$ obtained by fitting Model II to the low concentration data only (Fig. 3b). The best fit parameters for low concentration data in Table III did not differ from parameters for Model II in Table II. The values of $k_2$, $k_3$, $k_6$, and $V_c$ were somewhat larger for the high concentration data. The

![Table II](image)

**Table II**

Best fit parameters for interactions of human tumor necrosis factor-α with A549 cells. The best fit values were obtained by fitting equations of Models I-IV to data in Fig. 3. Values in italics denote fixed parameters. Other conditions as in Table I.

| Model | $[L]$ | $k_0$ | $K_D/[L]$ | $k_2$ | $k_3$ | $k_5$ | $k_6$ | $k_7$ | $V_c$ | $\chi^2$ |
|-------|-------|-------|-----------|-------|-------|-------|-------|-------|-------|--------|
| I     | $1.5 \times 10^{-10}$ | 0.89 | $1.0 \times 10^{-3}$ | 0.000 | $9.7 \times 10^{-4}$ | $1.1 \times 10^{-4}$ | ND* | 0.35 | ($>100$) | 18.8 |
| II    | $1.5 \times 10^{-10}$ | 1.000 | $1.3 \times 10^{-3}$ | $1.7 \times 10^{-4}$ | $3.4 \times 10^{-4}$ | $5.7 \times 10^{-4}$ | ND | 2.24 | (7) | 7.0 |
| III   | $1.5 \times 10^{-10}$ | 1.000 | $1.8 \times 10^{-3}$ | $2.6 \times 10^{-4}$ | $2.3 \times 10^{-4}$ | $9.3 \times 10^{-4}$ | ND | 1.23 | (1) | 16.8 |
| IV    | $1.5 \times 10^{-10}$ | 1.000 | $1.3 \times 10^{-3}$ | $1.3 \times 10^{-4}$ | $4.2 \times 10^{-4}$ | $3.6 \times 10^{-4}$ | $1.2 \times 10^{-1}$ | 1.74 | (1) | 6.0 |

*Not determined.

*Coefficients of variance not determined because of ill-conditioned curvature matrix due to large $k_6.$
internal consistency of these best fit parameters is corroborated also by the fact that the calculated values of the ligand-independent \((R[L]_{i=0})_1 = V_i/k_i\) were indistinguishable between the two data sets \((R[L]_{i=0})_1 = 6800 \pm 800 \text{ receptors/cell}\); \((R[L]_{i=0})_2 = 7800 \pm 700 \text{ receptors/cell}\); \((R[L]_{i=0})_1/(R[L]_{i=0})_2 = 1.14 \pm 0.17\).

**Testing for Serial Correlation of Residuals in Least Squares Analysis**—The least squares analysis is valid only when successive residuals are distributed independently of one another (14). When residuals are not independent of each other, the variance of the best fit parameters does not attain the least value and the standard procedures for variance evaluation underestimate the true variance (14). Thus, analysis of serial correlation of residuals by the Durbin-Watson method (14, 15) can help to assess whether a particular function or set of functions provide an adequate model for a given set of data. The method has been successfully applied for analysis of multiexponential functions describing time-resolved data (23, 24).

The method of Durbin and Watson tests the null hypothesis of serial independence of residuals against the existence of positive or negative serial correlation. To test against positive correlation the value of \(d(=\frac{n}{\sum (r_i - r_{i-1})^2/\sum r_i^2} \text{ where } r_i \text{ denotes the residual})\) is compared to the upper and lower limits of critical values \((d_0 \text{ and } d_1, \text{ respectively})\). For \(d < d_0\), the value is significant; for \(d > d_1\), the value is not significant at the given significance level. For \(d_0 < d < d_1\), the test is inconclusive (14, 15).

**DISCUSSION**

Studies leading to definition of elementary steps in interactions of selected protein ligands with cells have been extensively reviewed (cf. Refs. 10, 11, 16, 25). Dynamic aspects of these interactions have been considered ideal for application of mathematical modeling in a biological system (cf. Refs. 1, 10, 16, 25–27). In this paper we used the pre-steady state models of interactions of cytokines with cells to evaluate the contribution of receptor recycling to the maintenance of membrane receptor concentration. We measured the time-resolved concentration of radiolabeled ligands bound to membrane receptors and of internalized ligand-receptor complex. We restricted ourselves to this type of data because we intended to apply these and similar models to protein transport in three-dimensional tissue models; we expect that in such more complex experimental systems, time-dependent concentrations of not more than two compartments would be measured.

We tested the applicability of models which took into account only experimentally verified compartments which could be individually measured by separation of the ligand bound to the membrane from internalized ligand. Thus, only the most essential phenomena in kinetics of interactions of cells with protein ligands were modeled; we neglected those which would have to be taken into account when convenient methods for more subtle time-resolved discrimination of cellular distribution of proteins become available. We believe that models presented in this paper are based on reasonable assumptions, are consistent with experimental evidence, are useful for derivation of relevant rate constants, and will serve as a basis for more refined and/or more realistic models. A quantitative comparison by kinetic modeling of interactions of biological response modifier proteins with human normal fibroblasts...
Kinetic Models of Ligand-Receptor Interactions

| Table IV |
|------------------------------------------------|
| **Durbin-Watson factors for best fit curves in Figs. 3 and 5** |
| Symbols $d$, $d_l$, and $d_v$ stand for the calculated Durbin-Watson factor and for the lower and upper limit of the critical value of $d$, respectively. Details in the text. |
| Data | Model | Curve | $d$ | $d_l$ | $d_v$ |
|------|-------|-------|-----|-------|-------|
| Fig. 3 | I | $S^*$ | 0.56 | | |
| | I | | 1.27 | | |
| | II | S | 1.36 | | |
| | III | S | 0.64 | 0.86 | 1.73 |
| | IV | S | 1.38 | | |
| | | I | 1.87 | | |
| Fig. 5 | II | $S_l$ | 1.29 | | |
| | $I_l$ | 1.79 | | |
| | $S_h$ | 1.38 | | |
| | $I_h$ | 2.01 | | |

* $S$, surface-associated radioactivity curve; $I$, internalized radioactivity curve; $L$ and $H$ refer to low concentration and high concentration data in Fig. 5, respectively.

and epithelial tumor cells in vitro will be published separately.

Models I to IV are based on the assumption that each of the constituent elementary processes is continuous and adequately described by a first order rate constant. This assumption was validated experimentally for most rate constants considered in interactions of proteins with specific receptors, particularly for human epidermal growth factor (1, 11, 16, 26, 28), transferrin and asialoorosomucoid (9, 10), and IFN (29).

Under our experimental conditions, concentrations of the free ligand changed up to 5%; thus, the assumption of pseudo-first order conditions with respect to concentration of the ligand is justifiable. Stable values of [L] resulted in rate constants of elementary processes described in Models I to IV to be de facto independent of concentration. However, this fact does not contradict the finding of different rate constant values for different (constant) values of [L] (Table III), in full agreement with Ref. 1.

Under conditions of constant [L] and of concentration-independent rate constants, Models I to IV could be described by systems of linked first order differential equations which were explicitly solved. Solution of equations of Model I was published previously (6); solutions of equations of Models II–IV can be found in Appendix I. As demonstrated by simulations in Figs. 2 and 4, the best fit parameter values of Models I and II were rather resilient to deviations from the pseudo-first order conditions of the magnitude encountered in our experiments. All parameters in Models I–IV were identifiable (Appendix II).

The $\chi^2$ values for fits in Figs. 2, 4, and 6 are rather large indicating that Models I and II are not perfect descriptions of the respective ligand-cell interactions. We could not find the $\chi^2$ values published for similar studies of ligand-receptor systems, so comparison of our models to those in the literature can be only qualitative. However, the $\chi^2$ values were sensitive discriminators among different models (Tables I–III). The $\chi^2$ value for TNF data (Model II) was reduced significantly if data points for surface-associated radioactivity collected within first 15 min were disregarded (not shown); thus, it appears that early membrane-associated events are probably oversimplified in our models. We could not resolve the nature of this oversimplification as the introduction of a rate-limiting step which could function as a delay for internalization (Model IV) did not change the best fit parameter values or reduce the $\chi^2$ value (Table II).

The best-fit curves to data in Figs. 3 and 5 were assessed by the Durbin-Watson analysis of serial correlation of residuals. It is noteworthy that curves of internalized radioactivity for Models II and IV displayed negative correlation of residuals showing that data were described by appropriate mathematical functions. Tests of the respective curves for surface-associated radioactivity yielded inconclusive results indicating more complex kinetics of the receptor-ligand interactions at the cell surface. However, refinement of kinetic equations must rely on advances in understanding of elementary steps of these interactions.

Our models include the influx of the ligand into the system and the efflux from it, but include also the influx and efflux of receptors (by the $V_p$ and $k_h$ processes, respectively). The influx and efflux of receptors were originally postulated by Wiley and Cunningham (1) in the model which was successfully applied to interactions of IFN with the nonrecycling Type I receptor in course of 6 h (6). The alternative assumption that the sum of receptors in all cellular compartments is constant was used in studies of recycling receptors for asialoglycoproteins and transferrin (9, 10), respectively. By use of experimentally derived rate constants, changes in concentration of total (occupied and free) surface receptors and free surface receptors in course of 11 min of receptor cycling in absence of protein synthesis were successfully simulated (9, 10). The same assumption of constant total number of receptors was used by Gevrey and DeLisi to formulate a more elaborate model of interactions of epidermal growth factor with fibroblasts (11, 16). Decision on which of the models to apply to interactions of a particular ligand-cell pair will depend on experimental evidence for distinct patterns of receptor metabolism as well as on statistical criteria.

In conclusion, on the basis of time-resolved measurements of concentrations of protein ligands associated with the cell surface and internalized ligands we formulated simple mathematical models of the kinetics of ligand metabolism in the cell. By fitting equations of these mathematical models, we quantitated the contribution of receptor recycling to the maintenance of receptor concentrations on the surface of the cell; this method made it possible to discriminate between receptors which did recycle between the plasma membrane and interior of the cell and receptors which did not recycle. We studied how the best fit parameters depended on perturbation of the fixed input parameter; the $k_h$ and $k_v$ values were the most resilient, making it possible for the method to be applied particularly to studies of kinetic aspects of endocytosis and receptor recycling.

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Kinetic Models of Ligand-Receptor Interactions

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Supplement to
Binding, Internalization, and Intracellular Processing of Proteins
Interacting with Recycling Receptors.
A Kinetic Analysis

Jelko Badger, Andrew C. Myer and Stanimir Vuk-Pavlović

Appendix I: Analytical Solution for Models I to IV

Models I to IV are represented by sets of linked first-order differential equations (Eqns. 1 to 5). For brevity, let us assume \( p_1 = (R1, 2, 3) \) \( p_2 = (R1, 3, 2) \) \( p_3 = (R1, 2, 3) \) and \( p_4 = (R1, 3, 2) \). Parameters are determined at \( t = 1 \) : \( a = A \), \( b = B \), \( c = C \), \( d = D \), \( e = E \), \( f = F \), \( g = G \), \( h = H \), \( i = I \), \( j = J \), \( k = K \), \( l = L \), \( m = M \), \( n = N \), \( o = O \), \( p = P \), \( q = Q \), \( r = R \), \( s = S \), \( t = T \), \( u = U \), \( v = V \), \( w = W \), \( x = X \), \( y = Y \), \( z = Z \).

Models I to IV are expressed as the following matrix equations (9):

(A1) \[ \begin{bmatrix} x(t) \end{bmatrix} = \begin{bmatrix} A \end{bmatrix} \begin{bmatrix} x(t) \end{bmatrix} \]

(A2) \[ \begin{bmatrix} y(t) \end{bmatrix} = \begin{bmatrix} B \end{bmatrix} \begin{bmatrix} y(t) \end{bmatrix} \]

(A3) \[ \begin{bmatrix} z(t) \end{bmatrix} = \begin{bmatrix} C \end{bmatrix} \begin{bmatrix} z(t) \end{bmatrix} \]

(A4) \[ \begin{bmatrix} w(t) \end{bmatrix} = \begin{bmatrix} D \end{bmatrix} \begin{bmatrix} w(t) \end{bmatrix} \]

(Roman numerals refer to Models I, II, III, IV.) The symbol \( y(t) \) refers to the observed surface-averaged activity of the system. The parameters \( a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z \), and \( \omega \) are identical to Models II. Model I can be obtained from Model II by setting \( p = 0 \).

Assuming that the PDE to be solved is in the steady state, the system of equations (A) satisfies the following differential equations (A3) \[ \begin{bmatrix} x(t) \end{bmatrix} \]

(A4) \[ \begin{bmatrix} y(t) \end{bmatrix} \]

(A5) \[ \begin{bmatrix} z(t) \end{bmatrix} \]

(A6) \[ \begin{bmatrix} w(t) \end{bmatrix} \]

where the model parameters \( a, b, c, d, e, f, g, h, i, j, k, l, m, n, o, p, q, r, s, t, u, v, w, x, y, z, \) and \( \omega \) are identical to Models II. Model I can be obtained from Model II by setting \( p = 0 \).

The system of differential equations (A) can be solved analytically by the Laplace transform method (Ref. 30). Thus Eq. (A1) becomes the system of linear equations:

(A7) \[ \begin{bmatrix} A \end{bmatrix} \begin{bmatrix} v(t) \end{bmatrix} = \begin{bmatrix} v(t) \end{bmatrix} \]

which can be solved to yield a solution of the form

(A8) \[ \begin{bmatrix} v(t) \end{bmatrix} = \begin{bmatrix} v(t) \end{bmatrix} \]

where \( \omega = 1 \) for Models I and II and \( \omega = 4 \) for Models III and IV. \( p(t) \) and \( q(t) \) are polynomials.

Appendix II: Identifiability of Parameters in Models I to IV

A model is uniquely identifiable when there is only one set of parameters yielding solution for \( \theta(t) \) and error in the estimation of the parameter \( \omega \). For models I to IV, the solution is identical to Models I to IV.
Kinetic Models of Ligand-Receptor Interactions

We describe the testing of identifiability on the example of Model II which following the similarity transformation becomes

\[ y(t) = Q^T \dot{x} + \dot{x}, \quad \tau_{\text{act}}(t) = C y(t), \quad \tau(t) = \tau^* \]

\[ Q = \left( \begin{array}{cccc} p_0 & 0 & 0 & 0 \\ 0 & p_1 & 0 & 0 \\ 0 & 0 & p_2 & 0 \\ 0 & 0 & 0 & p_3 \end{array} \right) \]

When there is a nonsingular matrix \( Q \) such that \( Q^T = Q^{-1} \) and

\[ \tau_{\text{act}}(t) = \tau^T(t) \]

for any \( t \geq 0 \), then the model is identifiable. Taking the Laplace transform of Eq. (A10) and by use of Eq. (A6), the condition in Eq. (A12) leads to

\[ C(s-I)^{-1} \dot{x}(s) + u = Q^T(s-I)^{-1} \dot{x}^* + Q^T \dot{x}^* \]

for any \( u \). This condition can be rewritten with A14 for the \( Q^T \) are simultaneously satisfied.

\[ A = \left( \begin{array}{cccc} 0 & 1 & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 \end{array} \right) \]

Moreover, the structure of the new model matrix \( Q^T A \) should be identical to that of the original model matrix \( A \) in order to describe the same experimental model. Then, in case of Model II

\[ Q^T A = \left( \begin{array}{cccc} p_0 & p_1 & p_2 & 0 \\ p_0 & p_1 & p_2 & 0 \\ 0 & p_1 & p_2 & 0 \\ 0 & 0 & p_2 & 0 \end{array} \right) \]

\[ p_1 > 0, \quad p_2 > 0, \quad p_3 > 0 \]

(cf. Eq. A2.8). By use of the algebraic programming system REDUCE (Rand Corp., Santa Monica, Calif.) we found that Eqs. (A14) and (A15) can be satisfied for \( Q = I \) only if \( p_3 > p_2 \). The complete results can be summarized as follows:

- \( p_3 = p_2 \)
- Model II is identifiable in all parameters;
- \( p_3 = p_2, p_3 > 1 \)
- Model II is identifiable in all parameters;
- \( p_3 = p_2, p_3 > 1 \)
- Model II is identifiable.

When Model II is not identifiable, it is still identifiable for the parameter \( p_3 = p_2, i.e. p_3 = p_2 \). Identifiability of Model III can be demonstrated by an analogous procedure. However, it is more convenient to replace the equation for \( y_3 \) by the equation for \( y_2 + y_3 \), which is the observable quantity. Then Model III can be equivalently described by

\[ A = \left( \begin{array}{cccc} 0 & 1 & 0 & 0 \\ 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 1 \\ 0 & 0 & 0 & 0 \end{array} \right) \]

Model III is uniquely identifiable in all parameters even when \( p_3 = p_2 \).

For Model IV it is more convenient to replace the equation for \( y_3 \) by the equation for \( y_2 + y_3 \), which is the observable quantity. Model IV is identifiable in all parameters if \( p_3 = p_4 \). For \( p_3 > p_4 \), testing of identifiability of parameters was precluded by the complexity of algebraic equations.