stage appear to require interaction with the target in order to remain responsive to ACh. If a similar requirement exists in vivo, either during development or throughout the neuronal life cycle, target interaction may be seen as a mechanism particularly suited to the formation and maintenance of appropriate neuronal circuitry. Activity through a synaptic pathway has been implicated as an important influence on the maintenance and relative efficacy of neuron-to-neuron and neuromuscular transmission (*15*). Neurons failing to compete successfully for target interaction might lose transmitter responsiveness and also lose a sustaining influence gained from tonic activity. This would be particularly true if the lack of contact with a target caused a loss of responsiveness to all transmitters, resulting in transmission failure at all incoming synapses. Because interaction with membrane remnants is as effective as interaction with live myotubes, at least in the short-term maintenance of neuronal ACh sensitivity, it is hard to envision a major role for tonic activity in this instance.

In cell culture, ciliary ganglion neurons can be released from the normal dependency on the target for survival by the addition of target-derived proteins (*16*). In addition, several other "trophic" proteins or activities support aspects of neuronal differentiation (*17*). These observations, along with studies of nerve growth factor (*18*), suggest an important role for readily soluble proteins in the development of embryonic neurons. However, for the ciliary ganglion, protein factors added to the culture medium are not capable of supporting neuronal development equivalent to that seen in vivo (*3, 19*). The failure of the soluble trophic factors to foster normal development in cell culture may be the result of other deficiencies in the culture environment, insufficient neuronal access to the factors, or as yet obscure factors that act in concert to promote neuronal differentiation. Nonetheless, the present results indicate that contact with the target membrane has a strong influence on neuronal development.

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References and Notes

1. S. Carbonetto, D. Fambrough, K. Muller, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1016 (1978); P. M. Radvin and D. Berg, *ibid.* **76**, 2072 (1979); K. Obata, *Brain Res.* **73**, 71 (1974).
2. P. I. Baccaglini and E. Cooper, *J. Physiol. (London)* **324**, 441 (1982).
3. G. Crean *et al.*, *ibid.* **331**, 87 (1982).
4. J. F. Margiotta and D. K. Berg, *Nature (London)* **296**, 152 (1982).
5. J. B. Tuttle, J. Suszkiw, M. Ard, *Brain Res.* **183**, 161 (1980).
6. R. Nishi and D. K. Berg, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5171 (1977).
7. Cranial skin from 11-day embryos was dissociated and plated. Repeated subculture at 1-week intervals allowed the contaminant myoblasts time to withdraw from the mitotic cycle, fuse, and thus be eliminated.
8. A. R. Martin and G. Pilar, *J. Physiol. (London)* **168**, 443 (1963); L. Landmesser and G. Pilar, *ibid.* **222**, 691 (1972).
9. W. Burke and B. L. Ginsborg, *ibid.* **132**, 599 (1956); S. Nishi and K. Koketsu, *J. Cell. Comp. Physiol.* **55**, 15 (1960); S. Nishi, H. Soeda, K. Koketsu, *Life Sci.* **8**, 499 (1969).
10. S. Roper, *J. Physiol. (London)* **254**, 455 (1976). The mean sensitivity of synaptic areas on intracardiac septal neurons was 504 mV per nanocoulomb, with a range to approximately 1000 mV/nC. Nonsynaptic areas averaged 109 mV/nC. Ciliary ganglion neurons in culture with muscle have areas up to at least 800 mV/nC, but correlation of these areas of high sensitivity with sites of synaptic contact was not attempted in this study.
11. K. Peper and U. J. McMahan, *Proc. R. Soc. London Ser. B* **181**, 431 (1972).
12. The contaminant myotubes probably arise from the myoblast precursor cells, which later form the extensive striated muscle system associated with avian feathers and the cutaneous musculature.
13. L. Landmesser and G. Pilar, *J. Physiol. (London)* **241**, 738 (1974).
14. G. Pilar, J. B. Tuttle, L. Landmesser, in *Disorders of the Motor Unit*, D. L. Schotland, Ed. (Wiley, New York, 1982).
15. W. A. Harris, *Annu. Rev. Physiol.* **43**, 689 (1981).
16. J. B. Tuttle, *Soc. Neurosci. Abstr.* **3**, 1529 (1977); I. McLennan and I. Hendry, *Neurosci. Lett.* **10**, 269 (1978); R. Nishi and D. Berg, *Nature (London)* **277**, 232 (1979); S. Varon, M. Manthorpe, R. Adler, *Brain Res.* **173**, 29 (1979); R. Bonyhady, I. A. Hendry, C. Hill, I. McLennan, *Neurosci. Lett.* **18**, 197 (1980).
17. R. Nishi and D. K. Berg, *J. Neurosci.* **1**, 505 (1981); T. Ebendahl, M. Belew, C.-O. Jacobson, J. Porath, *Neurosci. Lett.* **14**, 91 (1979); M. Manthorpe, S. Skaper, R. Adler, K. Landa, S. Varon, *J. Neurochem.* **34**, 69 (1980); F. Collins, *Dev. Biol.* **65**, 50 (1978).
18. L. Greene and E. Shooter, *Annu. Rev. Neurosci.* **3**, 353 (1980).
19. G. Pilar, J. B. Tuttle, K. Vaca, *J. Physiol. (London)* **321**, 175 (1981); K. Vaca, J. B. Tuttle, G. Pilar, in preparation.
20. F. Dreyer and K. Peper, *Pfluegers Arch.* **348**, 263 (1974).
21. I thank G. Pilar for advice, E. Casal for technical assistance, and B. Wolmer for secretarial aid. Supported by the U.S. Army Research Office, by grant NS-10338 from the National Institutes of Health, by the University of Connecticut Research Foundation, and by a research career development award from the National Institutes of Health.

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Number of Receptor Sites from Scatchard and Klotz Graphs: A Constructive Critique

The Scatchard plot is now perhaps the most popular method for graphical analysis of ligand-receptor binding studies. With the increasing number of receptor studies in neurobiology, endocrinology, pharmacology, immunology, and numerous other biomedical fields, it is important that this method be used and interpreted correctly. Klotz (*1*) has called attention to some potential shortcomings of the Scatchard plot method. However, it should not be concluded that there is something inherently wrong with the Scatchard plot or that the graph preferred by Klotz, in which the concentration of bound ligand is plotted against the log of the free ligand ([*B*]/log [*F*]), is intrinsically better. Clearly, the statistical informational content of the data is not altered by presentation in one or the other coordinate system: a simple algebraic manipulation will convert one format to the other. Thus identical conclusions should be drawn from both plots, provided they are interpreted correctly.

A number of issues deserve comment.

1) When one uses a plot of bound versus free ligand or bound versus the log of the free concentration, it becomes apparent that the maximum binding capacity (B_{max}) is an extrapolated value and that one must extrapolate a very long distance (until the concentration of the free ligand equals "infinity"). Even if one reaches 80, 90, or 95 percent of the apparent upper plateau value, there is always the disturbing possibility that the curve might change its shape in the unobserved (and unobservable) region. Thus, one can never prove that one has determined the true value for B_{max}, utilizing any kind of plot or any kind of statistical analysis. This problem applies equally to the Scatchard and Lineweaver-Burk plots (*2*), although in these plots the graphical extrapolation appears to be only a short distance.

2) One can only make an estimate of B_{max} if one begins with a particular model (for example, homogeneous noninteracting sites or two, three, or more classes of independent sites, cooperativity based on a specified model) and then assumes that the model will continue to apply over the extrapolated range.

There are two distinct sources of uncertainty in the estimate of B_{max}. Random fluctuation in the observed data will result in corresponding uncertainty in the estimate of B_{max} even if we knew the exact model governing the biochemical reaction. The magnitude of this type of error will increase as the length of extrapolation increases, giving a warning when inadequate data are available. The error limits in the estimate of B_{max} may be calculated on the basis of assumptions about the error distribution for the data. More accurate limits are obtained if independent replicates of the experiment are available. Appropriate methods of calculation have been described elsewhere (*3*).

Uncertainty also results from incom-

plete knowledge of which biochemical mechanism governs the reaction. One may have inadvertently selected the wrong model (because of failure to detect curvature in the Scatchard plot, for example). If the observations are over only a limited range of free ligand concentration, it becomes difficult to distinguish between alternative models. This second form of uncertainty is more serious since it is almost impossible to quantify and is therefore often ignored.

The "model dependency" of extrapolated parameters, such as B_{max}, applies to all methods, graphical and computerized. In this respect, the Scatchard plot is no better (and no worse) than that in which $[B]$ is plotted against log $[F]$, when both are properly interpreted.

3) Proper interpretation of a graph requires that the user understand (i) the shape of the curve for any specified model and (ii) the shape of the uncertainty region around the curve (for example, 95 percent confidence region). Changing the plotting coordinate system (that is, nonlinear transformation of the data) both alters the shape of the curve and distorts the shape of the corresponding 95 percent confidence region. Small errors in the data will appear magnified near either axis of a Scatchard plot (Fig. 1A). Similarly, errors are magnified near the upper plateau when $[B]$ is plotted against log $[F]$ (Fig. 1B). Lack of appreciation of these facts may result in incorrect conclusions being drawn from either plot.

4) The biologist or biochemist does not use K (the affinity constant) and B_{max} in the same sense—or for the same purpose—as the enzymologist or physical chemist. The biologist is usually not interested in establishing the true values of affinity constants and binding capacities. These values may be biased because of failure to reach equilibrium, problems in the separation of bound and free, errors in the estimate of specific activity, erroneous assumption of an incorrect model, or a host of other factors (*4*). Rather, in the same way that the biologist can use muscle contraction, uterine weight, or behavioral changes as a measurable response to an experimental probe, he or she can also legitimately use apparent K or B_{max} values observed under defined experimental conditions.

Frequently, the biologist may observe an overall shift in the magnitude of binding, but no change in the shape of the curve. Here, it is best to characterize the data in terms of the observed range of the data, rather than basing results on extrapolated values such as B_{max}. For example, one can form the ratio of binding in the presence of a "treatment" relative to that observed for "control" for several concentrations of free ligand, and then test whether this ratio is significantly different from unity. Often, this is all that the biologist wishes to establish. The question about the *true* value for B_{max} must await the purification of the receptor to homogeneity so that the physical chemist can count the number of binding sites on the molecule (quite a different problem).

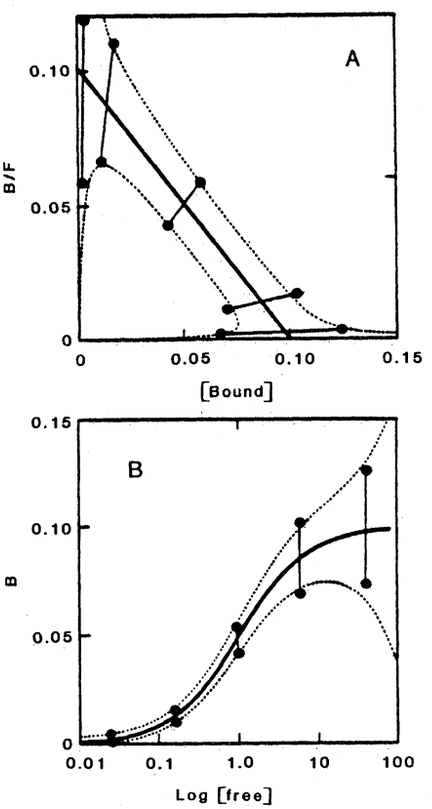

Fig. 1. Transforming the data does not change the information content. The same binding curve shown in two coordinate systems. The shape of the curve changes, but with corresponding changes in the shape of the envelope of uncertainty around the curve (enclosed by a broken line). Duplicate data points are shown in each plot. In the Scatchard plot (A), duplicates tend to lie on lines radiating from the origin. Large scatter appears as one approaches the axis for the concentration of bound ligand, especially in the presence of significant nonspecific binding. Likewise, the curve can "blow up" as one approaches the B/F axis, when there is a small but constant error in measurement of bound ligand concentration. In (B), duplicates lie on nearly vertical lines, but errors become substantial at high concentrations of free ligand because of the presence of nonspecific binding. Approximate confidence regions (broken lines) for a single new observation were generated with the assumption of 2 percent nonspecific binding with a relative 10 percent measurement error in bound concentration plus a small (0.002 unit) constant error.

Receptor studies, unlike classical enzymology, frequently deal with whole tissues and therefore numerous impurities. As a result, at large free ligand concentrations, nonspecific binding will inevitably become larger than specific saturable binding. At the extreme right-hand end of the binding isotherm (Fig. 1B) specific binding is often measured as the difference between two very large numbers. Hence, attempts to measure B_{max} by simply using large free-ligand concentrations may be unreliable. The presence of nonspecific binding magnifies all of the statistical problems discussed above. Nonspecific is better treated as an unknown parameter, to be estimated by a statistical curve-fitting technique, rather than as a fixed value to be subtracted from the observed total binding (*3*).

5) Extrapolation of a linear Scatchard plot is fraught with difficulties. Even more severe problems arise when one extrapolates nonlinear Scatchard plots, especially when one performs the extrapolation "by eye" without stipulating the exact molecular mechanism, mathematical model, or the underlying assumptions regarding the nature and magnitude of experimental errors. Such results are likely to be poorly reproducible from one investigator to another, and especially from one laboratory to another. Abuses of this nature have frequently appeared in the literature. Some laboratories have published what they claim to be "normal ranges" for Scatchard plots, including B_{max}, for various clinical studies. It is hazardous to publish the "normal range" for an extrapolated value when the rules for the extrapolation are unspecified.

6) One may avoid the issue of graphical methods altogether, by use of computerized nonlinear least-squares curve fitting (*3, 5, 6*), weighted to account for the statistical distributions of the errors. Then the results will be optimal in a statistical sense, irrespective of the choice of graphical coordinate system used for display of the data. This approach has several advantages (*3*):

- Computer analysis allows one to take account of the error distribution of the original data, thus avoiding transformation biases and artifacts.
- It provides a measure of the standard errors and confidence limits for each of the estimated parameters, and for pairs of parameters considered jointly.
- It provides several objective criteria for goodness of fit and tests for outliers.
- It facilitates comparison with alter-

native models, and selection of the "best model" on the basis of objective criteria.

- It permits simultaneous analysis of data from multiple curves or multiple experiments.
- It can provide tests of similarity of shape of two binding isotherms, and enable one to estimate the relative scaling factors for two or more binding isotherms of similar shape.

In summary, the Scatchard plot is no more and no less subject to abuse than other plots which in fact have the same informational content. Graphical methods serve well to provide subjective, preliminary understanding of the data (*6*). However, such methods sometimes oversimplify, with the paradoxical result that different graphs of the same data appear to lead to different conclusions. Such paradoxes are usually resolved with the use of an appropriate statistical analysis.

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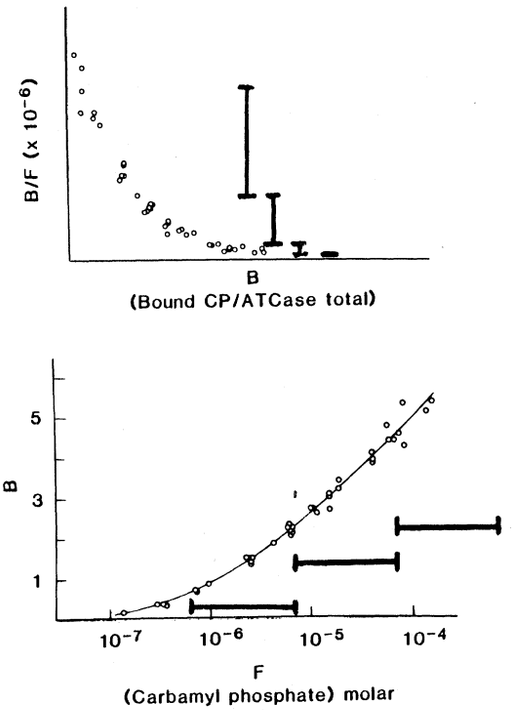

Fig. 1. Binding of carbamyl phosphate by aspartate transcarbamylase [data from (*6*)]. *B* represents normalized moles of bound ligand and *F* the concentration of free ligand. A bar, I or ⊢⊣, delineates the span of a tenfold change in concentration of free ligand. Note in the top graph the progressive diminution in the span of the bar. In contrast, in the lower graph, each tenfold increase in *F* is associated with the same bar length.

References and Notes

1. I. M. Klotz, *Science* **217**, 1247 (1982).
2. J. B. S. Haldane and K. G. Stern, *Allgemeine Chemie der Enzyme* (Wissenschaftliche Forschungs Berichte, Naturwissenschaftliche Reihe) (von Theodor Steinkopff, Dresden, Leipzig, 1932), vol. 28, p. 119.
3. P. J. Munson and D. Rodbard, *Anal. Biochem.* **107**, 220 (1980).
4. P. J. Munson, *J. Receptor Res.* **3** (No. 1), 249 (1983).
5. D. Rodbard, P. J. Munson, A. K. Thakur, *Cancer (Philadelphia)* **46**, 2907 (1980).
6. A. K. Thakur, M. L. Jaffe, D. Rodbard, *Anal. Biochem.* **107**, 279 (1980).

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Munson and Rodbard (*1*) present in summary their views of the difficulties encountered in analyzing ligand-receptor interactions. Most of their presentation is perfectly acceptable and analyzes issues not touched upon in my report (*2*), but there are some misleading statements to which I take exception.

If a set of algebraic equations can be transformed into each other, then the "statistical information content of the data" is indeed the same in terms of any one of them. Graphs, however, make their impact by a visual interaction with the observer, and they can distort in different ways the implications of a set of data. Thus some graphs can lure one into false conclusions. For example, Fig. 1 shows the same data plotted in two different coordinate systems. In each figure a tenfold change in concentration of free ligand, *F*, is represented by a bar, I or ⊢⊣, of appropriate length. It is immediately obvious that at increasing *F* there is an enormous compression of information in the top (Scatchard) graph, but a uniform distribution in the *B*–log *F* coordinate system. It is for this reason that I pointed out previously (*2*) that the former graph is more deceptive than the latter in leading people to believe that B_{max} can be established by extrapolation. For both cases, however, I also stated explicitly that "[u]nless reliable binding data can be obtained at [high] ligand concentrations . . . any [graphical] estimate [of B_{max}] will be uncertain."

It would also be desirable to distinguish between a "Scatchard graph" and a "Scatchard analysis." The latter may be used with no graph or with any graph. Scatchard analysis refers to a particular algebraic format for analyzing binding data, and depends on the assumption of a very restrictive molecular model for the receptor system. One obtains the impression from Munson and Rodbard (*1*) that an algebraic analysis of binding data must begin "with a particular model." That is not true. It has long been known (*3–5*) that a binding equation in terms of stoichiometric equilibrium constants is valid broadly, for homogeneous noninteracting sites, for two, three, or more classes of independent sites, when there is positive cooperativity between sites, when there is negative cooperativity, when there is positive and negative cooperativity, and so forth. Furthermore, from principles of algebra it has been shown that the stoichiometric binding equation can be transformed into a format that very deceptively looks like the Scatchard equation but is not; the binding constants of the former are for an ensemble of imaginary, isolated, *ghost* sites, and, except in very special circumstances, do not correspond to those of real sites. Thereafter, if a model is assumed, binding constants for the real sites may be extracted. If one uses this mathematical procedure, one recognizes that a particular model is only one solution to the general binding equation, which may or may not be in accord with knowledge of molecular structure (*5*).

It may be, as Munson and Rodbard state (*1*), that the "biologist . . . does not use *K* . . . and B_{max} in the same sense . . . as the enzymologist or physical chemist." If so, it behooves such biologists to define new symbols and new names for their new and different quantities, or confusion will be propagated in the literature.

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References and Notes

1. P. J. Munson and D. Rodbard, *Science* **220**, 979 (1983).
2. I. M. Klotz, *ibid.* **217**, 1247 (1982).
3. ———, F. M. Walker, R. B. Pivan, *J. Am. Chem. Soc.* **68**, 1486 (1946).
4. J. E. Fletcher, A. A. Spector, J. D. Ashbrook, *Biochemistry* **9**, 4580 (1970).
5. I. M. Klotz, *Trends in Pharmacological Sciences*, in press.
6. P. Suter and J. Rosenbusch, *J. Biol. Chem.* **251**, 5986 (1976).

31 March 1983