low concentrations in adult rat brain (*14*). More likely, the observations reflect sequestration of the hormone, or its metabolites, by membranes or vesicles within cell processes, in conformity with previous analytical evidence demonstrating early localization, osmotic releasability, and long retention of intravenously administered T_3* and its products in synaptosomal fractions of rat brain (*2*).

Certain conservative inferences may be drawn from these autoradiographic observations. The hormone T_3, which produces profound effects on behavior and on the autonomic nervous system, is taken up and concentrated in certain nerve cells and nerve cell aggregates of rat brain; the hormone is also differentially concentrated in the surrounding neuropil; the mechanisms involved are saturable with T_3. At 3- and 10-hour intervals after intravenous T_3* administration, there is little or no labeling of large fiber systems; further studies are required to determine whether fiber tracts may become labeled after 10 hours. The evidence is consistent with the uptake and retention of the hormone in specific neural systems and adds to a growing body of information indicating that iodothyronines serve as neuroregulators—that is, as neuromodulators, amino acid neurotransmitters, or neurotransmitter precursors (*15*) in the nervous system of the adult rat.

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22 April 1981; revised 21 September 1981

Scatchard Plots

A Scatchard plot of the binding of [^{125}I]albumin to isolated hepatocytes in a recent study (*1*) is one of a number of such graphs of experimental measurements made under conditions that differ significantly from those for which Scatchard devised his analytic method (*2*). If, for example, measurements are not made at equilibrium, or if the molarity of ligand and binding molecules is not known, a Scatchard plot of the results may be of heuristic value, but it will not yield the number and association constants of the sites on the binding macromolecule. Since Scatchard's first treatment of the binding of small to large molecules appeared 32 years ago (*3*), it may be useful to reexamine the basis for determining under which experimental conditions the use of a Scatchard plot is rigorously correct, under which some useful information may be obtained, and under which its application is virtually meaningless.

In his original article Scatchard set forth the objectives of his treatment of results of protein-binding experiments: "We want to know of each molecule or ion which can combine with a protein molecule, 'How many? How tightly? Where? Why?' The answer to the first two questions, and sometimes to the third, can be furnished by the physical chemist . . . but the answers to both of the more complicated problems . . . may be the business of the physiologist or physiological chemist . . . [and] will depend on the answers to the simpler questions, 'How many?' and 'How tightly bound?' "

Scatchard made it practical and relatively easy to extract from suitable measurements the answers to both of these "simpler questions." If the initial probability of binding a molecule of A is the same at each of n sites or groups on a protein molecule (or other macromolecule) P, the change in free energy $(\Delta G)_\nu$ for the reaction $P + \nu A = PA_\nu$ is zero if, and only if, all the components and species of the reaction are at equilibrium when a measurement is made (*4*). If binding the first molecule of A to P has a negligible effect on the tightness of binding of the second molecule of A—and so on—then

$$kc_A = \bar{\nu}/(n-\bar{\nu}) \quad (1)$$

where k is the intrinsic association constant for the reaction at a single site, c_A is the concentration of free ligand molecules, and $\bar{\nu}$ is the average number of A molecules bound to each protein molecule.

Plotting $\bar{\nu}/c_A$ against $\bar{\nu}$ forms a Scatchard plot. If the points fall on a straight line the intercept on the ordinate, where c_A and $\bar{\nu}$ approach zero, is nk, and the intercept on the abscissa, where c_A is very large, is n. The straight

0036-8075/82/0115-0312S01.00/0

line permits precise extrapolation of the experimental points to both intercepts and means that each of the n binding sites has the same intrinsic association constant k.

Curvature of a Scatchard plot implies either that there is more than one group of binding sites, with each group characterized by a different intrinsic association constant, or that the binding of each successive molecule of A alters the association constant for the next molecule of A bound (*5*). Whether groups of binding sites with different intrinsic constants or interactions between binding sites, or both, are the cause of the curvature will depend on the chemical nature of the reacting molecules.

If equilibrium between binding protein and ligand does not exist when measurements are made, the law of mass action cannot be applied and a Scatchard plot of measurements made in such a system is of limited, if any, significance.

If equilibrium has been experimentally shown to exist and a Scatchard plot yields a straight line, then n and k can be calculated from its two intercepts only if the concentration of the binding protein is known. Where the binding protein makes up an unknown proportion of a suspension of cells (*1*) or membranes, the straight lines of the Scatchard plots mean little more than that all binding sites that are accessible to ligand molecules have the same intrinsic association constant. Since we do not know what proportion of these suspensions is binding protein, let alone what the molecular weight of that protein is, the value of the intercept on the abscissa does not equal n even if the suspensions are so fine that the system may be considered a single phase—that is, a solution (*4*). Consequently, a meaningful value of k cannot be obtained by dividing the intercept on the ordinate by that on the abscissa. The only valid quantitative result to be derived from such a system is the product, nk, since the number of bound A molecules—the experimental result—will be the same whether there are 1000 binding sites with a k of 0.01 or 10 binding sites with a k of 1.0.

To be what Scatchard described, Scatchard plots must be of measurements made at equilibrium and with known concentrations of the binding macromolecule and ligand. If either of these criteria is not met, it should be made clear to the reader that a Scatchard plot of the experimental data can yield neither the maximum number of binding sites *per macromolecule* nor the association constant for the ligand studied.

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29 June 1981

Dr. Scheinberg raises a number of important points concerning the analysis of binding data, with special emphasis on one of several available methods—the Scatchard plot. We would not take issue with most of his statements, especially the requirement that analyses be based on data obtained under equilibrium conditions. In our own studies, considerable care was taken to ensure that this requirement was satisfied. Nevertheless, Scheinberg's general advice that derivation of binding constants must be based on measurements made under equilibrium conditions is quite appropriate.

We have some reservations, however, regarding Dr. Scheinberg's concerns about the calculation of the number of binding sites and their affinity. He appears to favor a more literal interpretation of the Scatchard analysis, which, as he points out, was applied originally to the binding of small ligands to macromolecules in solution. In its application to more complex systems involving cell or membrane suspensions, it is quite apparent that the horizontal intercept does not equal the value for n (number of sites of a given class per macromolecule) since the concentration of the binding entity (on which the sites are located) cannot be expressed in molar terms. Nonetheless, as long as the system is at equilibrium, the calculated concentration of bound ligand in the system can be related to some other appropriate denominator (for instance, per cell or per milligram of protein) and still provide the same general kind of information. Furthermore, we do not agree that useful affinity constants cannot be determined with such suspensions, since regardless of the units chosen for n, the ratio nk/n (the negative slope of the line) should be a valid measure of the equilibrium association constant k.

Finally, it should be noted that graphical estimation of the binding parameters from the linear portion of a Scatchard plot may overestimate the number of sites and underestimate the binding affinity if low-affinity or nonsaturable binding is also present (that is, if the plot shows upward curvature) (*1*, *2*). While methods exist to correct for this effect (*2*), they are not always satisfactory. For this reason we employed the Scatchard plot only for display of the binding data; the actual determination of the binding parameters was by computer analysis.

Alternative models for binding (consisting of up to two classes of saturable binding sites with or without nonsaturable binding) were optimized to the data by nonlinear least-squares curve fitting (*3*) and the most likely model chosen on the basis of goodness of fit with the data (*4*). Derivation of the constants by this method is inherently more accurate and objective than by visual inspection of the Scatchard plot or some other graphical method.

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