The adaptive reaction responsible for tolerance may be connected with a change in receptor and other parameters of those mediator systems for which the opioid system acts as modulator [11], and also with changes in the "ensemble" of opioid peptides [14].

LITERATURE CITED

1. S. V. Zaitsev, I. N. Kurochkin, S. D. Varfolomeev, et al., Dokl. Akad. Nauk SSSR, 281, 723 (1985).
2. O. Arunlakshana and H. O. Schild, Brit. J. Pharmacol., 51, 48 (1959).
3. W. D. Bowen, S. Gentleman, M. Herkenman, and C. B. Pert, Proc. Natl. Acad. Sci USA, 78, 4818 (1981).
4. M. E. Davis, T. Akera, and T. M. Brody, Res. Comm. Chem. Path. Pharmacol., 12, 409 (1975).
5. T. Kitano and A. E. Takemori, Res. Comm. Chem. Path. Pharmacol., 18, 341 (1977).
6. H. W. Kosterlitz, S. J. Paterson, and L. E. Robson, Brit. J. Pharmacol., 73, 939 (1981).
7. W. A. Klee and R. A. Streaty, Nature, 248, 61 (1974).
8. R. Nehmad, H. Nadler, and R. Simantov, Mol. Pharmacol., 22, 389 (1982).
9. D. Overstreet and H. I. Yamamura, Life Sci., 25, 1865 (1979).
10. G. L. Peterson, Analyt. Biochem., 83, 346 (1977).
11. J.-C. Schwartz, TINS, 137 (1979).
12. S. P. Sivam, T. Nabeshima, and I. K. Ho, Prog. Neuro-Psychopharmacol. Biol. Psychiat., 6, 119 (1982).
13. R. J. Tallarida, A. Cowan, and M. W. Adler, Life Sci., 25, 637 (1979).
14. L. Terenius, Rec. Adv. Alcohol Drug Probl., 8, 1 (1984).
15. D. R. Waud and R. B. Parker, J. Pharmacol. Exp. Ther., 177, 13 (1971).
16. M. Wuster, T. Costa, and C. Gramsch, Life Sci., 33, Suppl. 1, 341 (1983).

ADEQUATE CHOICE OF FREE LIGAND CONCENTRATIONS FOR DETERMINATION OF BENZODIAZEPINE BINDING

A. M. Zharkovskii

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KEY WORDS: binding, ^{3}H-diazepam, Scatchard plot analysis.

Radioligand binding methods, which provide very valuable information about the character of interaction between substances and receptor, are now widely used for research in psychopharmacology [7, 9]. Two parameters are usually used to characterize drug-receptor interaction: the equilibrium dissociation constant (K_d) and the receptor density (B_{max}). K_d and B_{max} are obtained by analysis of the kinetics of saturation of receptors by the ligand under equilibrium conditions [1, 2]. The saturation curve is hyperbolic in shape between coordinates: abscissa – concentration of free ligand (F), ordinate – degree of specific binding (B). The dependence thus obtained is analyzed between Scatchard coordinates [8] in accordance with the equation:

$$B/F = \frac{B\max - B}{K}.$$

On a Scatchard plot intersection of the straight line with the abscissa, along which values of B are plotted, gives the maximal density of binding sites (B_{max}), and the slope of the straight line is equal to $1/K_d$. In the present investigation, using binding of ligands with benzodiazepine receptors [3] as the example, an attempt was made to demonstrate that, when determining binding parameters, errors are often introduced as a result of incorrect choice of free ligand concentrations. Analysis of the results of our own experiments and of data in the literature showed that the value of B_{max} for ^{3}H-diazepam or ^{3}H-flunitrazepam in the cerebral cortex varies within very wide limits: from 350 to 1900 fmoles/mg protein. If

Department of Pharmacology, Tartu University.

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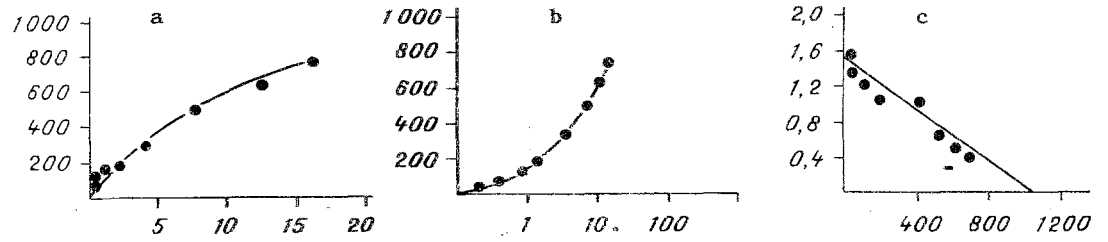

Fig. 1. Analysis of saturation kinetics of ^{3}H-diazepam binding with rat brain membranes with free ligand concentrations of between 0.25 and 16 nM. Abscissa: a, b) F (in nM); c) B (in fmoles/mg protein). Ordinate: a, b) B (in fmoles/mg protein); c) B/F·10^2. a) Between coordinates of B and F; b) the same, between coordinates of B and log F (Klotz' graph); c) the same, between Scatchard coordinates. Each point is average of 3-4 experiments.

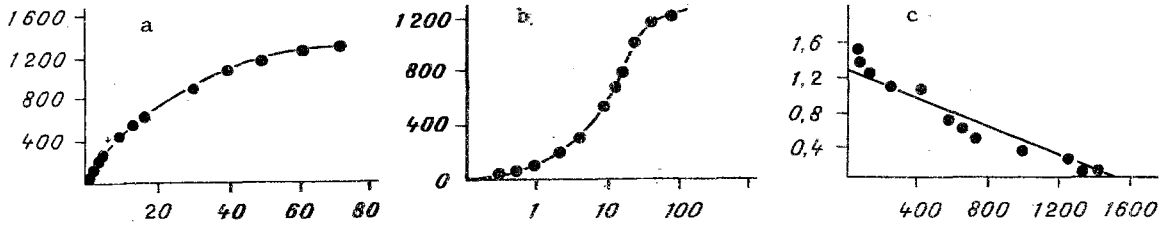

Fig. 2. Analysis of saturation kinetics of ^{3}H-diazepam binding with rat brain membranes, with free ligand concentrations of between 0.25 and 72 nM. Each point represents average of four experiments. Legend as to Fig. 1.

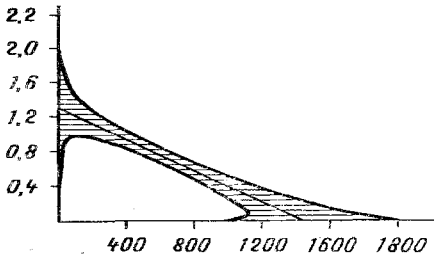

Fig. 3. Graph of saturation kinetics of ^{3}H-diazepam binding within concentration range of 0.25 to 72 nM between Scatchard coordinates and values of standard deviation (shaded area). Abscissa, B (in fmoles/mg protein); ordinate, B/F·10^2.

TABLE 1. Parameters of ^{3}H-Diazepam Binding in Absence and Presence of GABA, Obtained by Analysis of Saturation Kinetics between Scatchard Coordinates, Using Free Ligand in Concentrations of between 0.25 and 16 nM and 0.25-72 nM

Range of ligand concentrations, nM	Without GABA		In presence of GABA (10 μM)	
	B_{max}, fmoles/mg	K_d, nM	B_{max}, fmoles/mg	K_d, nM
0,25—16	918±26	7,4±0,3	1347±41,3*	4,5±0,3**
0,25—72	1519±42	9,1±0,7	1561±71,6	5,6±0,14**

Legend. Mean results of four experiments in duplicate and standard errors for them are given. *P < 0.05, **P < 0.01 (Student's t test).

saturation kinetics is studied within a relatively narrow range of radioactive ligand concentrations, the value of B_{max} as a rule is lower. This may be due to the fact that the free ligand concentration was too low to allow anything close to saturation binding.

This hypothesis was tested in the present investigation in which the kinetics of equilibrium binding of ^{3}H-diazepam was studied in different free ligand concentration ranges. The basis for analysis was taken to be Klotz' graph, which reflects the saturability of binding sites more clearly [4, 5].

EXPERIMENTAL METHODS

Experiments were carried out on male Wistar rats. The cerebral cortex of the animals was removed immediately after decapitation, homogenized in 0.32 M sucrose, and centrifuged at 1000 g for 10 min. The supernatant was centrifuged for 20 min at 40,000 g. The residue was resuspended in 50 mM Tris-HCl buffer and washed 3 times by centrifugation with intermediate rehomogenization. The residue thus obtained was frozen at −20°C, thawed 24 h later, and again washed by centrifugation 4 times in 50 mM Tris-HCl buffer with intermediate rehomogenization. The final residue was resuspended in 50 mM Tris-HCl buffer so that the final protein concentration did not exceed 0.08-0.12 mg/ml. Binding was carried out in glass test tubes into which ^{3}H-diazepam (specific radioactivity 84 Ci/mmole, from Amersham Corporation, England), the membrane suspension, and GABA were added (in separate experiments). The tubes were incubated for 60 min at 0°C, after which the contents were filtered through GF/B filters (Whatman, England) and radioactivity on the filters was determined with the LS-6800 counter (Beckman, USA). To analyze the saturation kinetics a wide range of free ligand concentrations was used: from 0.25 to 72 nM.

The results were analyzed between coordinates of B and F, B/F and B, and B and log F. The binding parameters K_d and B_{max} were calculated by linear regression analysis by the method of least average squares.

EXPERIMENTAL RESULTS

In the experiments of series I the saturation curve of ^{3}H-diazepam binding was analyzed over the free ligand concentration range from 0.25 to 16 nM, i.e., in concentrations which have most frequently been used to study benzodiazepine binding.

The experimental results are shown in Fig. 1. Visually the resulting curve appears to be a hyperbola. However, if we transform the graph in Fig. 1a between Klotz' coordinates (Fig. 1b), in which values of log F are plotted along the abscissa and the specifically bound ligand along the ordinate, the resulting curve has no upper part, i.e., the experimental values obtained within this concentration range are very different from near-saturation values, and, consequently, the value of B_{max} cannot be determined from these data on a Scatchard plot (Fig. 1c), for they lie outside the limits of determinacy. The same graphs are plotted on Fig. 2, but in this case the free ligand concentrations are taken over a wider concentration range (0.25-72 nM). In this case the curve between Klotz' coordinates becomes S-shaped, i.e., we obtain values of B close to saturating, and this corresponds to the model suggested by Scatchard (Fig. 2). Under these circumstances the value of B_{max} is significantly greater than that obtained with ligand concentrations between 0.25 and 16 nM (Table 1). However, when high (saturating) free ligand concentrations are used a serious problem arises, due to an increase in scatter of the data near the axes of coordinates. The great scatter of the data is the result of fluctuations of nonspecific binding, for when high concentrations of ligand are used the degree nonspecific binding rises considerably. The results of analysis of the saturation kinetics of ^{3}H-diazepam binding are shown in Fig. 3 as a Scatchard plot for ligand within a concentration range of between 0.25 and 72 nM, with standard deviations of the experimental points (the points themselves are omitted). It will be clear from Fig. 3 that the standard deviation of points lying close to the ordinate increases sharply. In this case the accuracy of the experimental data can be increased only by increasing the number of repeated experiments. The other way (perhaps more successful) is to determine values of nonspecific binding within the region of high ligand concentrations as an unknown parameter, as Munson and Rodbard [6] recommend.

Incorrect choice of free ligand concentrations may be reflected substantially in the conclusions obtained by a study of the effect of substances on binding parameters. Data on the effect of GABA on ^{3}H-diazepam binding are given in Table 1. They show that the results

obtained by the use of free ligand in concentrations of between 0.25 and 16 nM lead to the conclusion that GABA (10 μM) reduces dissociation (K_d) and increases the density of binding sites, but with a wider range of concentrations. Although GABA reduces K_d it does not affect B_{max}, in good agreement with the model of allosteric control of the benzodiazepine receptor through GABA.

The results of this investigation thus show that when determining parameters of equilibrium binding the experiments must be conducted over the widest possible range of free ligand concentrations (as Scatchard rightly pointed out), or otherwise the experimental data will not correspond to Scatchard's suggested model and cannot be analyzed by that method. The experimental points must be first represented between Klotz' coordinates, so as to make sure that the necessary range of concentrations has been correctly chosen, after which they can be analyzed between Scatchard coordinates.

LITERATURE CITED

1. S. D. Varfolomeev and S. V. Zaitsev, Kinetic Methods in Biochemical Research [in Russian], Moscow (1982).
2. J. P. Bennett, Neurotransmitter Receptor Binding, H. J. Jamamura, ed., New York (1978), pp. 57-90.
3. C. Braestrup and R. Squires, Proc. Natl. Acad. Sci. USA, 74, 3805 (1977).
4. I. M. Klotz, Science, 217, 1247 (1982).
5. I. M. Klotz, TIPS, 4, 253 (1983).
6. P. J. Munson and D. Rodbard, Analyt. Biochem., 107, 220 (1980).
7. E. Richelson, Monogr. Neur. Sci., 10, 4 (1984).
8. G. Scatchard, Ann. New York Acad. Sci., 51, 660 (1949).
9. S. H. Snyder, Handbook of Psychopharmacology, Vol. 17, New York (1983), pp. 1-12.

POSSIBILITY OF PHARMACOLOGIC INTERVENTION IN POSTICHEMIC MYOCARDIAL DAMAGE

Yu. B. Rozonov, T. V. Morozova, V. A. Markin, and Z. T. Pokazeeva

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One of the main tasks in the treatment of ischemic heart disease (IHD) is the prevention of anginal attacks. Several antianginal drugs are used for this purpose: nitroglycerin in various therapeutic forms, Ca^{++} antagonists, β-adrenoblockers, etc. However, even with the aid of these highly effective drugs, it is by no means always possible to completely prevent the development of attacks. The termination of an attack once it has arisen is therefore no less important. Each attack of angina causes definite damage to the cardiomyocytes in that region of the myocardium affected by ischemia. For instance, occlusion of the coronary artery in dogs for 15 min leads to a marked decrease in the ATP concentration in the myocardium and to inhibition of its contractile function. These changes persist for several days after restoration of the blood supply to the ischemic zone [2]. Damaged myocardial cells, depending on the intensity and duration of ischemia, may recover their function or die.

The question arises whether this pathological process, determining the fate of the myocardium after an anginal attack, can be influenced by means of drugs. The investigation described below was undertaken to study this problem.

Laboratory of Pharmacology of the Circulation, Institute of Pharmacology, Academy of Medical Sciences of the USSR. Research Institute for Testing Biological Compounds, Moscow. (Presented by Acdemician of the Academy of Medical Sciences of the USSR A. V. Val'dman.)

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