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The Effect of pH on Interaction of Nitrobenzylthioinosine and Hydroxynitrobenzylthioinosine with the Nucleoside Transporter of Human Erythrocyte Membranes

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SUMMARY

Site-specific binding to human erythrocyte membranes of nitrobenzylthioinosine (NBMPR), un-ionized at physiological pH, was compared with that of hydroxynitrobenzylthioinosine (HNBMPR), pK_a 6.4, at graded pH values. Binding of [^{3}H]NBMPR was measured directly, and that of HNBMPR was assayed by competitive inhibition by HNBMPR of [^{3}H]NBMPR binding. K_d and B_{max} values for binding of [^{3}H]NBMPR to erythrocyte membranes were independent of pH. K_d values for the competing ligand were determined by mass law analysis of equilibrium binding data using either (a) apparent ligand concentration (dissociated plus undissociated forms of HNBMPR) or (b) the concentration of undissociated HNBMPR. K_d values for HNBMPR calculated with the apparent ligand concentration increased 10-fold as the fraction of HNBMPR molecules present in the dissociated form was increased (by pH changes) from 14 to 88%, whereas K_d values for the undissociated form of HNBMPR were independent of pH. The results presented here demonstrate that the undissociated form of HNBMPR binds more tightly to the transport-inhibitory sites of erythrocytes than NBMPR and suggest that ionization of S^6-substituted thiopurine ribonucleosides eliminates or greatly decreases their ability to interact with the binding sites.

INTRODUCTION

Nitrobenzylthioinosine is the best studied of a group of potent inhibitors of nucleoside transport in animal cells. NBMPR[1] binds with high affinity (K_d 0.1–1 nM) to transport-inhibitory sites on nucleoside transporters of erythrocytes (2, 3) and many types of cultured cells (4–6). Human erythrocytes possess about 10^4 such sites per cell, and fractional occupancy of these sites by NBMPR is proportional to inhibition of exchange diffusion of uridine (2). That the permeant and inhibitor sites are closely associated in human erythrocytes is indicated by the apparent competitive inhibition of inward transport of uridine by NBMPR (7). Although physical relationships between permeation sites and inhibitor-binding sites on nucleoside transporters of erythrocytes, or of cultured cells, have not been established, they appear to be separate entities. Permeants (uridine, adenosine) accelerate dissociation of NBMPR from the high affinity sites of membranes of erythrocytes (8) and cultured hamster fibroblasts (6), and cells that lack high affinity binding sites, but possess functional nucleoside transporters, have been identified recently (9).

Previous studies have established that nucleoside transporters do not accept anionic molecules as permeants. In cultured human lymphoblastoid cells, only the un-ionized forms of 3-deazauridine and 6-azauridine are accepted as permeants (10, 11) and in erythrocytes, 6-azauridine and orotidine (tested under conditions in which they were present as anions) do not participate as transporter substrates in the *trans* acceleration of uridine efflux (12). Since anionic forms of several nucleoside analogs are not transported, we have asked whether ionization also alters the ability of members of the S^6-substituted pentofuranoside "family" of inhibitors to bind to the transporter. HNBMPR and HNBTGR, both acidic congeners of NBMPR, are potent inhibitors of transport (12–16), and HNBTGR competitively inhibits binding of NBMPR (16) at pH 7.4, a condition in which about 90% of the HNBTGR molecules are ionized. This study compared the pH dependence of site-specific binding of [^{3}H]NBMPR (un-ionized at physiological pH) and

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[1] The abbreviations used are: NBMPR, 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; HNBMPR, 6-[(2-hydroxy-5-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; HNBTGR, 2-amino-6-[(2-hydroxy-5-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine; NBTGR, 2-amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine.

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of HNBMPR (pK_a 6.4) to erythrocyte membranes. Dissociation constants (K_d) of site-bound ligand were determined by mass law analysis of equilibrium binding of [^{3}H]NBMPR and of competitive inhibition of [^{3}H]NBMPR binding by nonisotopic HNBMPR. The results suggest that ionized molecules interact poorly, if at all, with the inhibitory sites of the nucleoside transporter of erythrocytes.

EXPERIMENTAL PROCEDURES

Preparation of erythrocyte membranes. Erythrocytes were obtained by centrifugation (1000 × *g*, 10 min, 4°) of out-dated human blood (Red Cross Society Blood Transfusion Service). The plasma and buffy coats were discarded, and erythrocytes were washed four times with 5 volumes of a medium consisting of 140 mM NaCl, 20 mM Tris/HCl buffer (pH 7.4 at 22°), 2 mM $MgCl_2$, and 0.1 mM EDTA (8). Hemoglobin-free membranes, prepared by lysis of the washed cells in 7 volumes of 5 mM KH_2PO_4 buffer (pH 7.9 at 22°) with three additional washes under the same conditions (17), were stored under liquid nitrogen. For use in binding assays, membranes were suspended in 25 volumes of ice-cold 50 mM KH_2PO_4 of the appropriate pH, and the protein content of such suspensions was determined by the Peterson modification of the Lowry method (18).

NBMPR binding. Equilibrium binding of [^{3}H]NBMPR at 22–24° was assayed at graded pH values between 5.0 and 8.0 by a modification of the filtration procedure described previously (6). Assay mixtures (final volume, 2.25 ml) contained about 10 μg of protein/ml in 50 mM KH_2PO_4. Such mixtures were incubated with [^{3}H]NBMPR at graded concentrations (0.125–4.0 nM) in the presence (10 μM) or absence of the competing ligand, NBTGR. The latter eliminated site-specific binding of NBMPR. After 30 min, membrane samples were collected on GF/C 2.4-cm glass microfiber filters (Whatman, Clifton, NJ) by fast filtration of 1-ml portions of assay mixtures on a sampling manifold (the filters were prepared by prewashing with 0.5 ml of 5 μM NBMPR, followed by 5 ml of 50 mM KH_2PO_4 at the appropriate pH). The filters were washed twice with 5 ml of ice-cold 50 mM KH_2PO_4 at the appropriate pH, and, after 48-hr incubation in a Triton-based scintillation fluid (19), were assayed for ^{3}H content. To determine final equilibrium concentrations of free [^{3}H]NBMPR in assay mixtures, 1-ml portions of assay mixtures were centrifuged (12,800 × *g*, 10 min, 4°), and 200-μl portions of the resulting supernatants were assayed for ^{3}H content. Binding constants for interaction of NBMPR with the transport-inhibitory sites were determined by linear regression analysis of mass law (Scatchard) plots of equilibrium binding data after subtraction of nonspecific binding.

TABLE 1

Effect of pH on binding of NBMPR to erythrocyte membranes

Site-specific binding of [^{3}H]NBMPR to erythrocyte membranes was determined at the pH values indicated, as described in Experimental Procedures. The binding parameters (means ± standard error) were obtained by linear regression analysis of Scatchard plots.

pH	K_d	B_{max}
	nM	*molecules/pg protein* × 10^{-4}
5.0	0.263 ± 0.014	3.86 ± 0.27
5.5	0.207 ± 0.008	4.52 ± 0.22
6.0	0.209 ± 0.002	4.27 ± 0.06
6.5	0.207 ± 0.007	4.32 ± 0.19
7.0	0.204 ± 0.005	4.18 ± 0.14
7.5	0.214 ± 0.008	4.32 ± 0.20
8.0	0.260 ± 0.012	4.21 ± 0.27

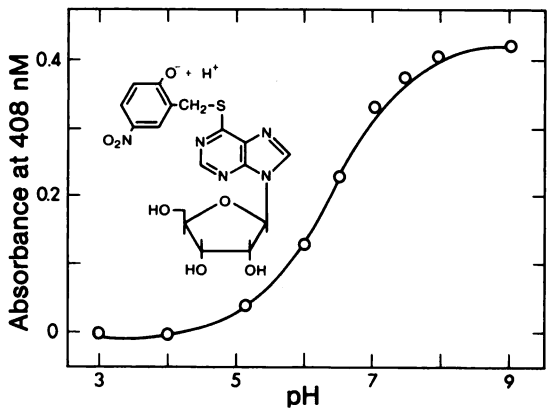

FIG. 1. *Spectrophotometric titration of HNBMPR*

A pK_a of 6.4 for HNBMPR was determined by the titration method described in Experimental Procedures.

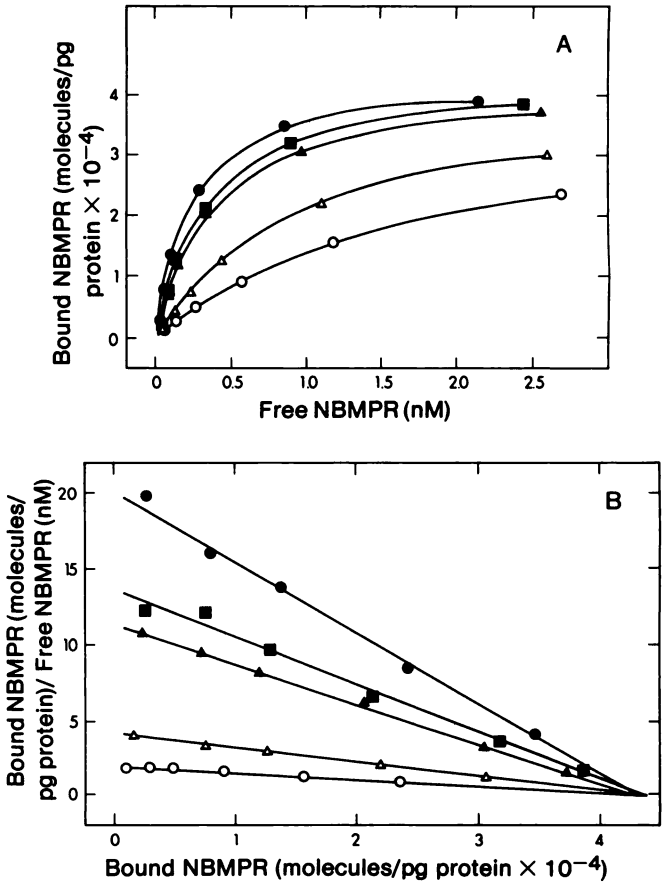

FIG. 2. *Inhibiton of site-specific binding of [^{3}H]NBMPR by HNBMPR at pH 7.5*

Suspensions of erythrocyte membranes were incubated at pH 7.5 with graded concentrations of [^{3}H]NBMPR in the absence (●) and presence of HNBMPR (■, 0.5 nM; ▲, 1.0 nM; △, 5 nM; ○, 10 nM). After 30 min, reactions were terminated by a filtration procedure, and the amount of ^{3}H associated with membranes and with medium samples was determined as described in Experimental Procedures. Nonspecific retention of [^{3}H]NBMPR, defined as the membrane-associated ^{3}H (in NBMPR molar equivalents) acquired in the presence of 10 μM NBTGR, was deducted. In Panel A, the membrane content of specifically bound [^{3}H]NBMPR is plotted against [^{3}H]NBMPR concentrations in the medium, presumably at equilibrium with the site-bound ligand. Panel B illustrates mass law analysis of equilibrium binding data by the Scatchard method. The K_d and B_{max} values (mean ± standard error) for the binding of NBMPR in this experiment were, respectively, 0.211 ± 0.008 nM and 4.27 ± 0.23 × 10^4 molecules/pg of protein.

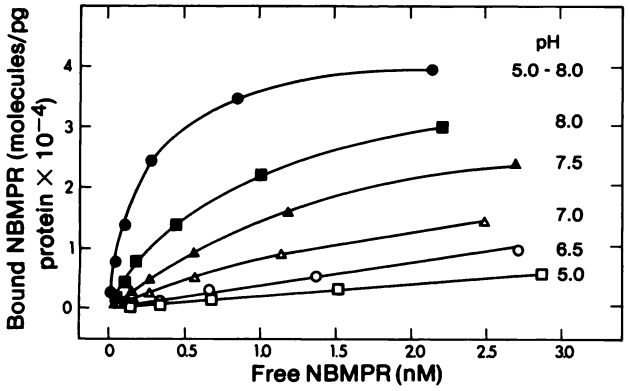

FIG. 3. *The effect of pH on inhibition of binding of [^{3}H]NBMPR by HNBMPR.*

Suspensions of erythrocyte membranes were incubated at the pH values indicated with graded concentrations of [^{3}H]NBMPR in the absence (●) and presence of 10 nM HNBMPR (■, ▲, △, ○, □). Binding assays were conducted as described in the legend of Fig. 2. The membrane content of specifically bound [^{3}H]NBMPR is plotted against the [^{3}H]NBMPR concentration of the medium (equilibrium presumed).

TABLE 2

Effect of pH on binding of HNBMPR to erythrocyte membranes

K_d values at the NBMPR-binding site were determined at the pH values indicated from the inhibition by HNBMPR of equilibrium binding of [^{3}H]NBMPR to erythrocyte membranes as described in Experimental Procedures. Binding data were subjected to mass law analysis by means of the Feldman equation (20) to obtain K_d values (± standard error) using HNBMPR concentrations representing dissociated + undissociated species (total [HNBMPR]) and undissociated HNBMPR. For NBMPR, the average K_d value was 0.223 ± 0.024 (± SD, n = 7) nM.

pH	K_d (total)	K_d (undissociated)
	nM	*nM*
5.5	0.098 ± 0.007	0.066 ± 0.006
6.0	0.166 ± 0.009	0.067 ± 0.006
6.5	0.261 ± 0.013	0.034 ± 0.007
7.0	0.525 ± 0.029	0.051 ± 0.014
7.5	1.01 ± 0.064	0.043 ± 0.013

The ability of HNBMPR to interact with the transport-inhibitory sites was determined at pH values between 5.0 and 8.0 by a competitive binding assay in which membranes were incubated with graded concentrations of [^{3}H]NBMPR in the presence and absence of nonisotopic HNBMPR at various concentrations (0.5–10.0 nM). Nonspecific retention of [^{3}H]NBMPR was defined as the sample content of ^{3}H retained in the presence of 10 μM NBTGR. Binding data were subjected to mass law analysis by means of the Feldman equation (20) as described previously (16) to obtain apparent K_d values for HNBMPR (the competing ligand).

Spectrophotometric titration of HNBMPR. The λ_{max} (visible range) for the ionized species of HNBMPR was determined in alkaline solution to be 408 nm. Buffered solutions of HNBMPR (23 μM) were prepared at pH 3.0 and 4.0 (20 mM citrate), 5.1, 6.0, 6.5, 7.0, 7.4, and 8.0 (20 mM phosphate), and 9.0 (20 mM borate). Measurement of absorbance (408 nm) of HNBMPR in each buffer provided a titration curve from which its pK_a could be calculated.

Chemicals. [G-^{3}H]NBMPR (16 Ci/mmol) was purchased from Moravek Biochemicals, Brea, CA and, after storage, was purified by high performance liquid chromatography on a C_{18} μBondapak column (Waters Scientific, Mississauga, Ontario) using a methanol-water gradient in which NBMPR was eluted with 50% methanol in water. NBMPR and NBTGR were prepared (13) from 6-thioinosine and 6-thioguanosine provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. HNBMPR was purchased from Calbiochem-Behring Corporation, La Jolla, CA.

RESULTS AND DISCUSSION

Binding of NBMPR to the transport-inhibitory sites of erythrocyte membranes has been well characterized in experiments conducted at physiological pH (2, 3). Jarvis *et al.* (3) have reported a mean K_d of 0.31 ± 0.29 (±SE, n = 9) nM for binding of [^{3}H]NBMPR to human erythrocytes at pH 7.4. In the experiments of Table 1, equilibrium binding of NBMPR to erythrocyte membranes was measured at graded pH values between 5.0 and 8.0 to determine if interaction of NBMPR with its binding sites is altered by changes in pH. The equilibrium binding parameters (K_d, B_{max}) were unaffected by changes in pH. Since NBMPR is un-ionized under the conditions of the experiments of Table 1, this result indicated that the binding site determinants of interaction with NBMPR are not sensitive to the imposed pH changes.

HNBMPR (Fig. 1) is an acidic congener of NBMPR that is also a potent inhibitor of nucleoside transport. In experiments conducted in media buffered at pH 7.4, Paul *et al.* (13) found IC_{50} values of 15 and 69 nM for inhibition by NBMPR and HNBMPR, respectively, of a transport-dependent process[2] in human erythrocytes. Thus, nucleoside transport-inhibitory activity of HNBMPR was evident under conditions in which 90% of the HNBMPR molecules present were in the anionic form. The experiment illustrated in Fig. 2 showed that HNBMPR competes with NBMPR for binding to the transport-inhibitory sites of erythrocyte membranes at pH 7.5. An apparent K_d value (mean ± SE) of 1.01 ± 0.064 nM for HNBMPR was obtained when the data of Fig. 2 were subjected to mass law analysis by the Feldman equation (20).[3]

The effect of pH on competition between HNBMPR and [^{3}H]NBMPR for binding to erythrocyte membrane sites was examined in the experiments of Fig. 3 and Table 2. Fig. 3 describes the effect of a single concentration of HNBMPR (10 nM) on binding of [^{3}H]NBMPR to erythrocyte membranes at graded pH values between 5.0 and 8.0. Since binding of NBMPR is independent of pH, binding data for particular concentrations of NBMPR obtained in the absence of HNBMPR have been averaged for all pH values. It is apparent from the data of Figs. 2 and 3 that HNBMPR was most effective as an inhibitor of NBMPR binding at low pH values, when it was present primarily in the undissociated form. Table 2 describes the results of a series of competitive binding experiments that were conducted to determine the apparent K_d for HNBMPR at pH values between 5.5 and 7.5. Apparent K_d values were calculated by mass law analysis of binding data using HNBMPR concentrations that represented (a) both dissociated and undissociated

[2] In erythrocytes, the synthesis of [^{14}C]inosine from extracellular [^{14}C]hypoxanthine and guanosine is dependent on mediated influx of guanosine.

[3] The Feldman analysis circumvents uncertainty of the equilibrium concentration of competing ligand that arises from its depletion during binding.

species ("total" HNBMPR), and (b) only undissociated HNBMPR molecules. K_d values calculated with total HNBMPR concentrations increased from 0.098 nM at pH 5.5 to 1.01 nM at pH 7.5. The fraction of HNBMPR molecules present in the dissociated form increased from 14 to 88% as the pH was varied between 5.5 and 7.5. In contrast, K_d values calculated with concentrations of undissociated HNBMPR present in assay mixtures did not change significantly with changes in pH. Thus, the anionic form of HNBMPR exhibited greatly reduced affinity, relative to that of the undissociated form, for the NBMPR sites of erythrocyte membranes.

The results presented here indicate that ionization of S^6-substituted thiopurine ribonucleosides will greatly reduce, or eliminate, their ability to interact with the transport-inhibitory sites of erythrocytes. A parallel conclusion was reached in similar studies of the effects of ionization on interaction of permeant with the nucleoside transporter of cultured cells (10, 11). Thus, undissociated ligands apparently exhibit greater affinities for both inhibitor and permeant binding sites of nucleoside transporters of erythrocytes and cultured cells, respectively. The relationships between inhibitor and permeant binding sites of nucleoside transporters have not been established.

Previous structure-activity studies with congeners of NBMPR in erythrocytes (13) and cultured cells (14–16) showed that affinity of these ligands for the transport-inhibitory sites increases when (a) the S^6-substituent is hydrophobic, (b) the base moiety is a purine, and (c) the sugar moiety is ribose, 2′-deoxyribose, or arabinose. N^6-substituted adenine nucleosides are also potent inhibitors of nucleoside transport with high affinity for the transport-inhibitory sites (16). In demonstrating that ionized molecules of the S^6-thiopurine class of inhibitors are poor ligands for the transport-inhibitory binding sites, this study has identified an additional determinant of ligand-site interaction to be considered in the design of new inhibitors of nucleoside transport.

REFERENCES

1. Cass, C. E., W. P. Gati, and A. R. P. Paterson. The effect of pH on interaction of tight-binding inhibitors with the nucleoside transporter of human erythrocyte membranes. *J. Cell Biol.* **99**:294a (1984).
2. Cass, C. E., L. A. Gaudette, and A. R. P. Paterson. Mediated transport of nucleosides in human erythrocytes: specific binding of the inhibitor nitrobenzylthioinosine to nucleoside transport sites in the erythrocyte membrane. *Biochim. Biophys. Acta* **345**:1–10 (1974).
3. Jarvis, S. M., J. R. Hammond, A. R. P. Paterson, and A. S. Clanachan. Species differences in nucleoside transport: a study of uridine transport and nitrobenzylthioinosine binding by mammalian erythrocytes. *Biochem. J.* **208**:2202–2208 (1982).
4. Lauzon, G. J., and A. R. P. Paterson. Binding of the nucleoside transport inhibitor nitrobenzylthioinosine to HeLa cells. *Mol. Pharmacol.* **13**:883–891 (1977).
5. Cass, C. E., N. Kolassa, Y. Uehara, E. Dahlig-Harley, and A. R. P. Paterson. Absence of binding sites for the transport inhibitor nitrobenzylthioinosine on nucleoside transport-deficient mouse lymphoma cells. *Biochim. Biophys. Acta* **649**:769–777 (1981).
6. Koren, R., C. E. Cass, and A. R. P. Paterson. The kinetics of dissociation of the inhibitor of nucleoside transport, nitrobenzylthioinosine, from the high-affinity binding sites of cultured hamster cells. *Biochem. J.* **216**:299–308 (1983).
7. Jarvis, S. M., D. McBride, and J. D. Young. Erythrocyte nucleoside transport: asymmetrical binding of nitrobenzylthioinosine to nucleoside permeation sites. *J. Physiol. (Lond.)* **324**:31–46 (1982).
8. Jarvis, S. M., S. N. Janmohamed, and J. D. Young. Kinetics of nitrobenzylthioinosine binding to the human erythrocyte nucleoside transporter. *Biochem. J.* **216**:661–667 (1983).
9. Paterson, A. R. P., E. S. Jakobs, E. R. Harley, C. E. Cass, and M. J. Robins. Inhibitors of nucleoside transport as probes and drugs, in *Development of Target-Oriented Anticancer Drugs* (Y.-C. Cheng, B. Goz, and M. Minkoff, eds.). Raven Press, New York, 41–56, (1983).
10. Dahlig-Harley, E., A. R. P. Paterson, M. J. Robins, and C. E. Cass. Transport of uridine and 3-deazauridine in cultured human lymphoblastoid cells. *Cancer Res.* **44**:161–165 (1984).
11. Belt, J. A., and A. D. Welch. Transport of uridine and 6-azauridine in human lymphoblastoid cells: specificity for the uncharged 6-azauridine molecule. *Mol. Pharmacol* **23**:153–158 (1983).
12. Cass, C. E., and A. R. P. Paterson. Mediated transport of nucleosides in human erythrocytes: accelerative exchange diffusion of uridine and thymidine and specificity toward pyrimidine nucleosides as permeants. *J. Biol. Chem.* **247**:3314–3320 (1972).
13. Paul, B., M. F. Chen, and A. R. P. Paterson. Inhibitors of nucleoside transport: a structure-activity study using human erythrocytes. *J. Med. Chem.* **18**:968–973 (1975).
14. Paterson, A. R. P., L. R. Babb, J. H. Paran, and C. E. Cass. Inhibition by nitrobenzylthioinosine of adenosine uptake by asynchronous HeLa cells. *Mol. Pharmacol.* **13**:1147–1158 (1977).
15. Paterson, A. R. P., S. R. Naik, and C. E. Cass. Inhibition of uridine uptake in HeLa cells by nitrobenzylthioinosine and related compounds. *Mol. Pharmacol.* **13**:1014–1023 (1977).
16. Paterson, A. R. P., E. S. Jakobs, E. R. Harley, N.-W. Fu, M. J. Robins, and C. E. Cass. Inhibition of nucleoside transport, in *Regulatory Function of Adenosine* (R. M. Berne, T. W. Rall, and R. Rubio, eds.). Martinus Nijhoff Publishers, The Hague, 203–220, (1983).
17. Dodge, J. T., C. Mitchell, and D. J. Hanahan. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**:119–130 (1963).
18. Peterson, G. L. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83**:346–356 (1977).
19. Pande, S. V. Liquid scintillation counting of aqueous samples using Triton-containing scintillants. *Anal. Biochem.* **74**:25–34 (1976).
20. Feldman, H. A. Mathematical theory of complex ligand-binding systems at equilibrium: some methods for parameter fitting. *Anal. Biochem.* **48**:317–338 (1972).

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