VIDEO DIDÀCTIC i ARTICLE D'ANÀLISI DE RESULTATS PER A UNA PRÀCTICA DE LABORATORI: ESTUDI DELS RECEPTORS COLINÈRGICS MUSCARÍNICS MITJANÇANT TÈCNIQUES DE FIXACIÓ DE RADIOLLIGANDS (BINDING)

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RESUM DE L'EXPERIÈNCIA

El concepte d'equilibri químic és difícil de transmetre correctament als estudiants tant de cursos bàsics com avançats, una situació que queda ben reflectida en la frase d'en FM Harold: "El problema de les coses senzilles és que s'han d'entendre molt bé". Per això, fa quatre anys vam començar fent la pràctica experimental que es descriu en aquesta comunicació, per la qual aquest any hem filmat un vídeo introductori i tot just hem enviat un manuscrit a la revista "Biochemistry and Molecular Biology Education". L'experiment consisteix en generar una corva de saturació de la unió (binding) a membranes cerebrals de rata del radiolligand muscarínic d'alta afinitat [3H]quinuclidinyl benzilate. La sessió pràctica s'ha dissenyat per començar amb una projecció del vídeo en el laboratori de pràctiques, on s'explica el procés d'obtenció de les membranes cerebrals, així com els fonaments del protocol experimental que els estudiant faran tot seguit. Les dades experimentals, un cop obtingudes pels estudiants, s'analitzen en una segona sessió de pràctiques a l'aula d'ordinadors, per tal d'obtenir el valor de la constant d'equilibri de disociació. Aquesta proposta per estudiants de Bioquímica Farmacològica, optativa de la Llicenciatura de Bioquímica (codi 20175), que combina la projecció d'un vídeo, un experiment al laboratori i una sessió d'anàlisi de dades amb ordinador, és efectiva per a) Revisar els conceptes d'equilibri i de constants de disociació; b) Visualitzar la metodologia experimental molt senzilla del binding de radiolligands, molt utilitzada en el món del desenvolupament de nous fàrmacs, i c) Atraure l'atenció dels alumnes cap a la necessitat d'un anàlisi de dades molt acurat que, si no es fa correctament, pot amb facilitat induïr a la presa de conclusions incorrectes.

Especificar l'àmbit d'aplicació

Aquesta pràctica es duu a terme dins la programació de Bioquímica Farmacològica (codi 20175), assignatura optativa de la Llicenciatura de Bioquímica (2on cicle).

PARAULES CLAU

Equilibri, fàrmac, afinitat

1. INTRODUCCIÓ

The concept of equilibrium is hard to transmit accurately to the students in basic and even advanced courses. Whether it be because high school chemistry syllabi address it perhaps too early, or just because "equilibrium" is a misleading word whose common life meaning does not apply to chemical reactions, educators often avoid inquiring about their students' basic knowledge on this subject, fearing they may find too weak a foundation on which to build a coherent course on biochemistry or pharmacology.

Understanding what happens when equilibrium in a reaction is attained is basic to grasp concepts such as initial velocity [1] or steady state in an enzyme reaction, free energy changes in the metabolic pathways, or ligand-receptor interactions in the broad sense, including enzyme-substrate, hormone or neurotransmitter-receptor, antigen-antibody, or drug-pharmacological target. The same applies for the understanding of processes taking place out of equilibrium, which do it because there are mechanisms that work hard to keep the system away from it. ATP hydrolysis is exergonic in a cell because there is a very high ATP concentration due to the activity of its synthesizing machinery. In other words, the reaction of ATP hydrolysis works under conditions very far from equilibrium [2]. When this is not clearly settled down, chemistry magic may come to the rescue: this may be the case of the phosphate bonds of some selected high-energy compounds, frequently mentioned in textbooks.

I have addressed these basics of biochemistry with moderate success with my 6th semester students in a course on Biochemical Pharmacology devoted to ligand-receptor interaction, perhaps because advanced students that are about to graduate feel somehow reluctant to revise concepts that might have been misplaced in former courses. This is why I begun a few years ago teaching the experiment described in this paper. In essence, it consists of a standard saturation binding experiment of the very high affinity muscarinic ligand [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) to rat brain membranes. This non-selective muscarinic ligand, which allowed the first biochemical identification of muscarinic receptors [3, 4], displays an equilibrium dissociation constant of about 40 pM [5], making it excellent for the analysis of ligand depletion occurring when ligand and receptor concentrations are similar. Experimental data, which are obtained by the students, are analyzed to correct for ligand depletion, so that the actual free ligand concentration at equilibrium can be known. Overall, this proposal for advanced students has proven useful to revise the concept of equilibrium.

2. DESCRIPCIÓ DEL TREBALL

Materials and Methods

[3H]QNB, ethanol solution, is from Amersham Biosciences (Cat No. TRK 604, 42 Ci/mmol, 1 mCi/mL). By the time this practice is taught, the students have taken two compulsory semesters on methodologies in biochemical research, and they are familiar with the theoretical aspects of beta radiation and scintillation counting. However, as this is usually the first time they handle a tritiated compound, we review some basics on safety procedures as well as radioactivity units in an introductory session, so that they are able to calculate:

a) the concentration of the stock [3H]QNB solution:

(1 mCi/mL) x (1 mmol/42.103 mCi) = 23,81.10-6 mmol/mL = 23,8 μM

b) the transformation of dpm into mol and vice-versa:

(1 Ci / 2.22. 1012 dpm) x (1 mmol / 42 Ci) = 10.73.10-15 mmol / dpm

This laboratory practice is taught to 6 groups of 2 students at a time, handling each group a total of 30 mL of 1.2 nM and 8 mL of 1nM solutions of [3H]QNB, for experiments 1 and 2, respectively. The instructor, who must comply with local regulations on manipulation of radioactive material, has to make these solutions from the stock. This amount of radioligand is equivalent to a total tritium quantity of 7.8.105 Bq, or a concentration of 2.1.104 kBq/kg, well below the maximum limits established in Annex I of European Union Council Directive 96/29/EURATOM of 13 May 1996, according to which a practice may be exempted from the requirement to report without further consideration if either the quantity or the activity concentration of tritium does not exceed the values of 109 Bq or 106 kBq/kg [6]. However, even though the amount of tritium is very low and that its maximal emission energy (19 keV), which in air represents about 23 cm, does not require special devices against external irradiation, the students are asked to pay especial care to prevent internal irradiation derived from a possible contamination.

Other than the radioligand, the experiment requires atropine sulfate (Merck, Cat. No. 159508) and Tris-HCl buffer (10 mM, pH 7.0), containing 6 mM MgCl2. The general procedures for [3H]QNB binding and for the preparation of rat brain membranes, which are obtained previously by the instructor, are based on Sallés et al (1993) [5]. We use the brain of a pregnant rat that is occasionally killed for research purposes in order to establish primary cultures of neurons from the embryos' brains. After cleaning the meninges with buffer-soaked filter paper, cortices are dissected and white matter is trimmed off as much as possible. Then, the tissue is homogenized in 40 mL buffer using a Potter homogenizer with a motor-driven Teflon pestle. The homogenate is then centrifuged 30 min at 50,000 xg and the resulting pellet is homogenized and centrifuged again under the same conditions. After protein determination by the Bradford assay, the final pellet is resuspended at 1 mg protein/mL, transferred to 1-mL microfuge tubes and centrifuged once more. After discarding the supernatant, membrane pellets are kept at -80 oC until use. One rat brain yields enough membrane protein for 30-40 students, which is

the usual enrollment in the class.

Additional equipment includes glass micro fiber filters (Whatman GF/C), a Brandel 24-sample semi-auto harvester (or two 12-fold Millipore filtration units), scintillation cocktail (Emulsifier Safe, Packard Bioscience, Cat No. 6013389), and a liquid scintillation counter. We use a Wallac 1409 counter, which combines measured counts per minute (cpm) with built-in reference tritium spectrum information in order to take account of chemical and color quench, and therefore calculate counting efficiency to display dpm. In our hands, counting efficiency for both filter-bound and soluble ligand in the following experiments is consistently 22-23%.

The general procedure consists of the incubation of membrane protein with the radioligand in 5-mL borosilicate tubes, in a total volume of 2 mL, and in the presence or absence of excess of unlabelled muscarinic antagonist (2 μM atropine) to define non-specific binding. After 30-60 min at room temperature, time at which equilibrium has been attained, the reaction mixtures are quickly filtered through glass fiber disks in a vacuum-connected device. This may be a 12-fold Millipore filtration unit or a semi-automated Brandel harvester, which allows the simultaneous filtration of 24 samples, reducing time and variability due to manipulation by untrained hands. Filters are washed twice with 4 mL of ice-cold buffer, dried at 60-70 oC for 30 min in an oven, and counted for radioactivity.

3. RESULTATS I/O CONCLUSIONS

Table 1. Summary of experiment 1: [^{3}H]QNB binding vs. protein concentration

Tube (x2)	Atropine 200 μM	Buffer	[^{3}H]QNB 1.2 nM	Membranes 1 mg prot / mL	Radioactivity in the filters dpm ± range	Specific binding (LR) dpm	LR/L_{TOTAL} %
0	-	1 mL	1 mL	-	532 ± 47		
10	-	990 μL	1 mL	10 μL	2766 ± 525	2084	1.9 %
10'	20 μL	970 μL	1 mL	10 μL	682 ± 30		
20	-	980 μL	1 mL	20 μL	6997 ± 97	6363	5.7 %
20'	20 μL	960 μL	1 mL	20 μL	634 ± 37		
30	-	970 μL	1 mL	30 μL	11135 ± 128	10568	9.4 %
30'	20 μL	950 μL	1 mL	30 μL	567 ± 116		
40	-	960 μL	1 mL	40 μL	13354 ± 1634	12786	11.4 %
40'	20 μL	940 μL	1 mL	40 μL	569 ± 69		
50	-	950 μL	1 mL	50 μL	16183 ± 197	15355	13.7 %
50'	20 μL	930 μL	1 mL	50 μL	829 ± 38		
60	-	940 μL	1 mL	60 μL	19641 ± 188	18690	16.7 %
60'	20 μL	920 μL	1 mL	60 μL	771 ± 25		
70	-	930 μL	1 mL	70 μL	24931 ± 1470	24043	21.5 %
70'	20 μL	910 μL	1 mL	70 μL	888 ± 70		

Table 2. Design of a saturation equilibrium [^{3}H]QNB binding experiment.

Tube (x2)	Atropine 200 µM	[^{3}H]QNB 1 nM	Buffer	Membranes 0.6 mg prot/mL
5	-	10 µL	1940 µL	50 µL
5'	20 µL	10 µL	1920 µL	50 µL
10	-	20 µL	1930 µL	50 µL
10'	20 µL	20 µL	1910 µL	50 µL
20	-	40 µL	1910 µL	50 µL
20'	20 µL	40 µL	1890 µL	50 µL
30	-	60 µL	1890 µL	50 µL
30'	20 µL	60 µL	1870 µL	50 µL
50	-	100 µL	1850 µL	50 µL
50'	20 µL	100 µL	1830 µL	50 µL
100	-	200 µL	1750 µL	50 µL
100'	20 µL	200 µL	1730 µL	50 µL
250	-	500 µL	1450 µL	50 µL
250'	20 µL	500 µL	1430 µL	50 µL
500	-	1 mL	950 µL	50 µL
500'	20 µL	1 mL	930 µL	50 µL

Table 3. Results and transformation of the data in a saturation equilibrium binding experiment
The students collect experimentally results in columns 2, 4, and 5, and then calculate the concentrations of total added, free, and specifically bound ligand.

tube (x2)	Total ligand added dpm ± range	Total ligand added pM	Total ligand bound dpm ± range	Nonspecific binding dpm ± range	Specific binding LR dpm	Specific binding LR pmol / mg protein	Free ligand L dpm	Free ligand L pM
5	946 ± 74	5.1	635 ± 21	79 ± 6	556	0.20	390	2.1
10	1947 ± 21	10.4	1234 ± 58	100 ± 5	1134	0.41	813	4.4
20	4161 ± 112	22.3	2356 ± 7	113 ± 21	2243	0.80	1918	10.3
30	5914 ± 66	31.7	3295 ± 30	85 ± 35	3210	1.15	2704	14.5
50	10403 ± 351	55.8	5148 ± 21	92 ± 20	5056	1.99	5347	28.7
100	20824 ± 2468	111.7	7714 ± 109	298 ± 32	7416	2.65	13384	71.8
250	41603 ± 1264	223.2	9128 ± 51	234 ± 16	8894	3.18	32706	175.4
500	83265 ± 3787	446.7	10824 ± 288	534 ± 118	10290	3.68	72910	391.0

Table 4. Nonlinear regression analysis of [^{3}H]QNB equilibrium binding

	Uncorrected ligand depletion	Corrected ligand depletion
r^2	0.9958	0.9980
LR_{max}	12419 ± 385 dpm	11073 ± 185 dpm
K_D*	85.8 ± 7.1 pM	36.4 ± 2.0 pM

Figure 1. Total and nonspecific [^{3}H]QNB binding vs. protein concentration. Left and right panels show total (solid symbols) and nonspecific [^{3}H]QNB (open symbols), or just nonspecific binding, respectively, as a function of membrane protein in the assay. Linear regression analysis of nonspecific binding vs. protein shows that the slope is not significantly different from zero (P value = 0.0516).

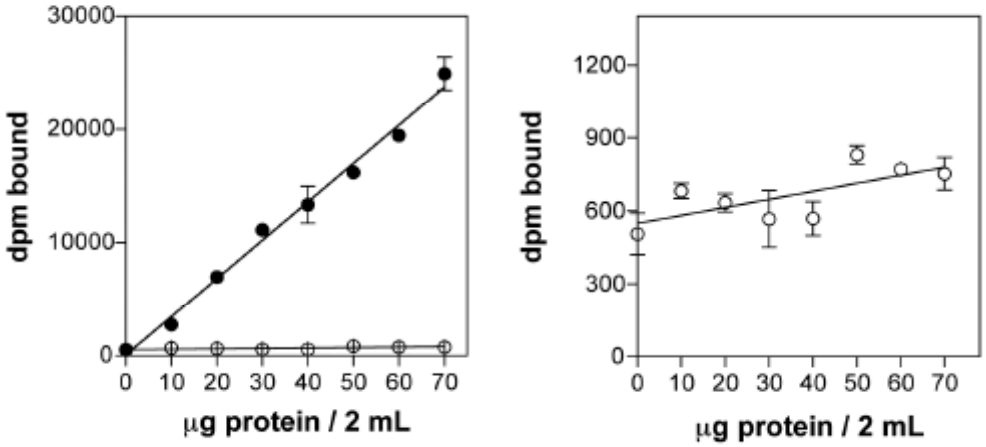
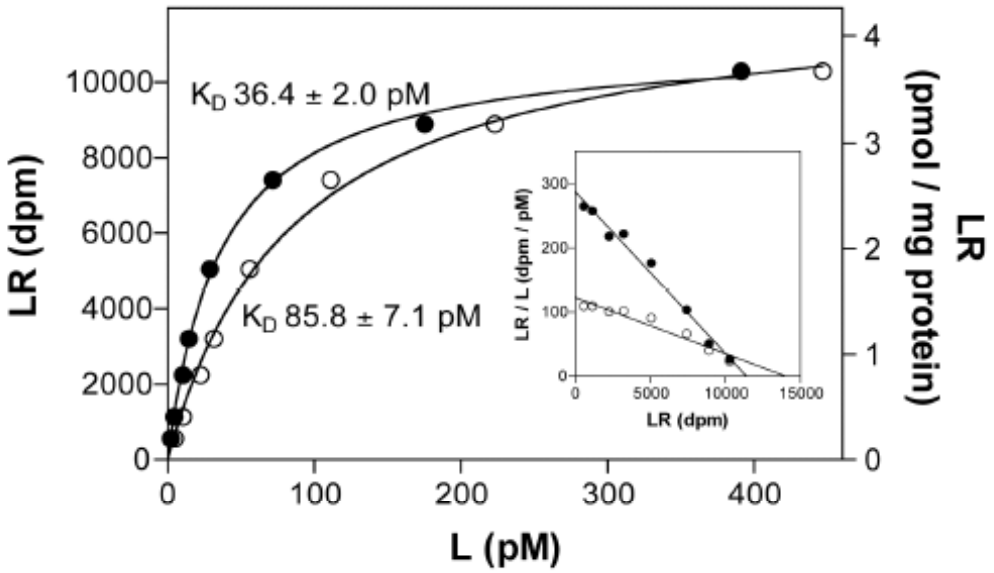


Fig. 2. Results of a saturation equilibrium [^{3}H]QNB binding experiment.
Data on specific [^{3}H]QNB binding, expressed either as dpm (left axis) or pmol bound per mg protein (right axis), are plotted against total ligand (open symbols) or actual free ligand concentrations (solid symbols), and analyzed by nonlinear regression. Inset shows the Scatchard plot of the same data to help visualize the overestimation of K_D, shown by lower slope, when ligand depletion is not taken into account.



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