Br. J. Pharmac. (1980), **68**, 563–569

COMPARISON OF PHARMACOLOGICAL AND BINDING ASSAYS FOR TEN β-ADRENOCEPTOR BLOCKING AGENTS AND TWO β-ADRENOCEPTOR AGONISTS

NICOLE BIETH, BRUNO ROUOT, JEAN SCHWARTZ & JEANNE VELLY

Institut de Pharmacologie et de Médecine Expérimentale, Faculté de Médecine,
11, rue Humann, F. 67000 Strasbourg E.R.A. 142 (CNRS) F.R.A. 6 (INSERM), France

1 The inhibition constants evaluated by binding assays for ten β-adrenoceptor blocking agents and two β-adrenoceptor agonists, were compared with the pA_2 and pD_2 values determined *in vitro* and *in vivo*.

2 There was only a limited correlation between β_1 or β_2 selectivities observed with the different methods.

3 Selectivity is generally less pronounced in binding assays than for *in vivo* and *in vitro* experiments.

Introduction

In the past few years, successful labelling of receptors by radiolabelled agonists or antagonists has enabled the study of β-adrenoceptors in many tissues from several mammalian species. These binding techniques make it possible to evaluate the affinity of β-adrenoceptor blocking agents or β-adrenoceptor agonists.

We set out to study the inhibition of [^{3}H]-dihydroalprenolol binding by a series of ten β-antagonists and two β-agonists. We investigated their affinity for the β_1 heart receptors and the β_2 receptors of the lung. We compared these results with those (some of which have been published by Miesch, Bieth, Leclerc & Schwartz, 1978), obtained by the usual pharmacological techniques.

Methods

Binding techniques; measurement of inhibition constants

Heart membrane preparation Membranes were prepared from cardiac ventricles of male Wistar rats (200 to 250 g) by a slight modification of the method of Harden, Wolfe & Molinoff (1976).

The ventricles were homogenized in 7 volumes of ice-cold buffer (5 mM Tris-HCl, 8% sucrose, 1 mM EGTA, pH 7.5) in a Polytron tissue disrupter. The homogenate was centrifuged at 10,000 *g* for 10 min in a refrigerated centrifuge and the pellet was washed by resuspension in 7 volumes of the same buffer. After a second centrifugation the pellet was suspended with the polytron in 1.72 M sucrose, 5 mM Tris-HCl buffer pH 7.5 (20 ml per g of heart). The suspension was transferred into cellulose nitrate tubes (24 ml/tube). A 15 ml layer of 0.2 M sucrose in Tris buffer pH 7.5 was placed above the layer of 1.72 M sucrose. The tubes were centrifuged at 100,000 *g* for 90 min in a Beckman SW27 swinging bucker rotor. Membranes which collected at the interface of the two sucrose layers were removed with a Pasteur pipette and diluted with 50 mM Tris-HCl buffer, pH 7.5 containing 4 mM Mg SO4 (1.5 mg protein/ml) for binding studies. Approximately 15 mg of membrane protein was obtained from 1 g of heart.

Lung membrane preparation Two techniques were used: the technique of Harden *et al.* (1976) already used for heart membranes and a technique derived from that of De Feudis & Somoza (1977). With the latter technique, the lungs were homogenized in 10 volumes ice-cold 0.32 M sucrose solution. The homogenate was centrifuged at 1000 *g* for 10 min and the pellet was discarded. The supernatant was centrifuged at 17,000 *g* for 30 min. The pellet was washed by resuspension in 5 mM Tris-HCl buffer pH 7.5 and recentrifuged at 17,000 *g* for 30 min. The final pellet was resuspended in 5 mM Tris-HCl buffer (about 3 mg protein/ml) for binding studies. Approximately 10 mg of membrane protein was obtained from 1 g of lung.

Protein concentration was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

0007-1188/80/030563-07 $01.00

Binding assay [^{3}H]-dihydroalprenolol ([^{3}H]-DHA from NEN: 48 to 58 Ci/mmol) and the membrane suspension (equivalent to 0.3 mg protein for both heart and lung) were incubated for 20 min at 25°C in 50 mM Tris-HCl buffer, pH 7.5, containing 2.5 mM $MgCl_2$, giving a final volume of 500 µl. [^{3}H]-dihydroalprenolol binding in relation to concentration was studied by incubation of increasing concentrations of [^{3}H]-DHA (0.05 to 4 nM) with the same receptor concentration, with and without unlabelled alprenolol at a high concentration (10^{-5} nM). Specific binding was considered as the difference betweeen the radioactivity found in the absence and in the presence of a high concentration of alprenolol; it was usually 80% of total binding. In competittive binding experiments between [^{3}H]-DHA and β-blocking agents, the β-blocker at increasing concentrations and the [^{3}H]-DHA at a constant concentration (0.5 nM) were added to the incubation mixture before the membrane preparation.

Incubation was followed by filtration through a Skatron apparatus on Whatman GF/B paper and filters were rapidly washed with 15 ml of ice-cold Tris-HCl buffer. After drying, the radioactivity trapped by the filters was measured by counting in a Packard liquid scintillation counter with a 40% efficiency. The concentration of β-blocker inhibiting 50% of maximal specific binding of [^{3}H]-DHA is the IC_{50}. K_i values (inhibition constants) were calculated from the equation:

$$K_i = \frac{IC_{50}}{1 + \frac{([^3H]\text{-DHA})}{K_D}}$$

where [^{3}H]-DHA is the [^{3}H]-dihydroalprenolol concentration and K_D the equilibrium dissociation constant of [^{3}H]-DHA.

In vitro *and* in vivo *experiments: determination of* pA_2 *and* pD_2

For our *in vitro* experiments, we used the techniques of Horrii, Kawada, Takeda & Imai (1974) for guinea-pig atria and of Levy & Wilkenfeld (1970) for guinea-pig trachea.

In vivo studies were made on the guinea-pig and on the dog; on the guinea-pig we studied cardiac rhythm (Miesch *et al.*, 1978) and resistance to lung inflation (Konzett & Rossler, 1940); on the dog, myocardiac contractile force and vascular resistance (Willems & Boggert, 1975).

pA_2 and pD_2 were determined experimentally according to Arunlakshana & Schild (1959) and interpreted mathematically according to Miesch, Turlot, Ehrhardt & Schwartz (1977).

Results

Binding assays

[^{3}H]-dihydroalprenolol binds to β-adrenoceptor sites in the rat heart and lung. Binding was saturable and equilibrium reached a steady state after 20 min. Scatchard analysis (Scatchard, 1949) of the data showed a single class of binding sites characterized by an equilibrium dissociation constant, (K_D) of 0.8 ± 0.1 nM for the heart and 0.32 ± 0.06 nM for the lung. The maximal number of binding sites (B_{max}) was 38 ± 9 femtomoles/mg protein for the heart and 460 ± 70 for the lung. Among the ten β-blockers tested on heart preparations (Figure 1), propranolol, oxprenolol and pindolol were the most potent while sotalol, practolol and butoxamine were the least potent. In binding assays on lung preparations (Figure 2), propranolol, oxprenolol and alprenolol were the most potent and atenolol, acebutolol and practolol the least potent. Comparison of the inhibition constants observed with heart and lung preparations (Table 1) provides further evidence for the selectivity of some β-blockers for β_1 or β_2 receptors.

Acebutolol, atenolol and practolol, respectively 3, 4.5 and 9 times more potent on the heart, were β_1 selective. In contrast, sotalol and butoxamine were β_2 selective: they were respectively 8 and 5 times more potent on the lung than on the heart. Some β-blockers such as propranolol and oxprenolol, described as non-specific had inhibition constants which did not differ significantly for the heart or the lung.

Though these results in general tallied with classical data, this was not so for pindolol which appeared to be more potent on heart receptors than on lung receptors. Moreover, with our binding techniques, we failed to show the β_2 selectivity of two β-agonists, salbutamol and terbutaline, which in our experiments seemed to have a rather more obvious affinity for the β_1 heart receptors.

This divergence from classical pharmacological data led us to test these molecules on two different lung membrane preparations obtained by the technique of Harden *et al.* (1976) and by the technique of De Feudis & Somoza (1977). Table 1 shows overall agreement between the two procedures. Pindolol was more potent on cardiac receptors with both techniques. However, the β_1 selectivity observed for salbutamol and terbutaline with the technique of Harden *et al.*, was less evident with that of De Feudis & Somoza.

Comparison of K_i *values and* pA_2 *and* pD_2

Table 2 shows, in addition to inhibition constants of the various molecules, the pA_2 of the β-antagonists

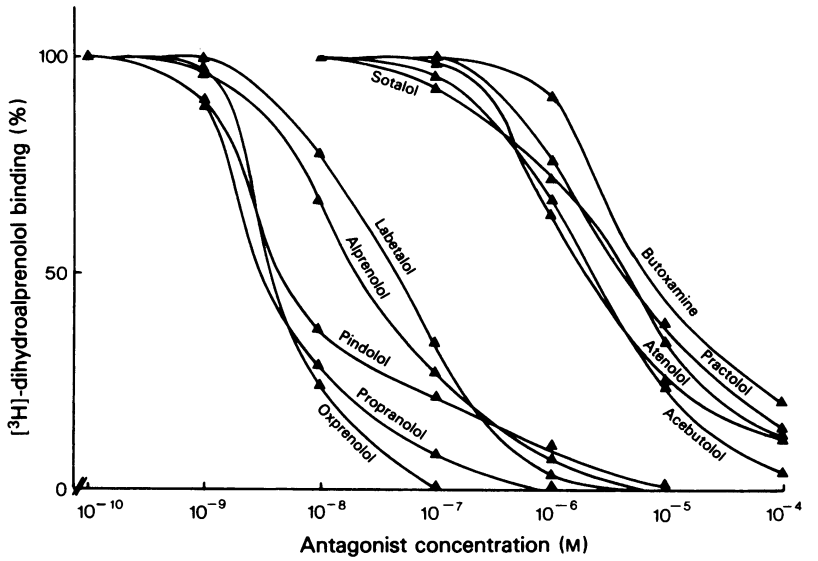

Figure 1 Inhibition of [^{3}H]-dihydroalprenolol binding to rat heart membranes by ten β-adrenoceptor blocking agents. Values given are means of three experiments conducted in duplicate.

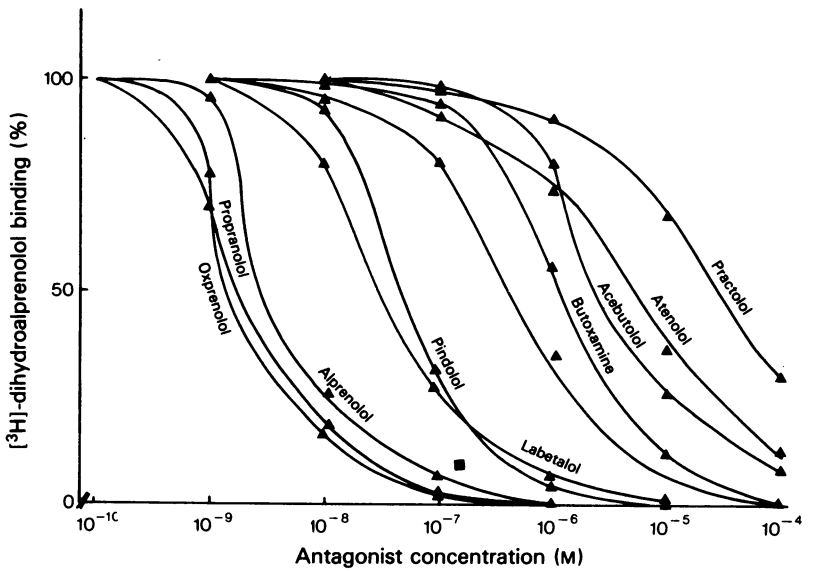

Figure 2 Inhibition of [^{3}H]-dihydroalprenolol binding to rat lung membranes by ten β-adrenoceptor blocking agents. Values given are means of three experiments conducted in duplicate.

Table 1 Inhibition of [^{3}H]-dihydroalprenolol binding to rat heart and lung membranes

	Heart K_i (*nM*) *Harden* et al. *technique*	*Lung* K_i (*nM*) *Harden* et al. *technique*	*Lung* K_i (*nM*) *De Feudis technique*
β-Antagonists			
(±)-Propranolol	2.4 ± 0.5	2.7 ± 0.5	1.2 ± 0.1
(±)-Oxprenolol	2.6 ± 0.3	1.7 ± 0.3	1.4 ± 0.2
(±)-Pindolol	2.9 ± 0.3	90 ± 15	32 ± 5
(±)-Alprenolol	9 ± 1.2	3 ± 0.5	2.4 ± 0.3
(±)-Labetalol	27 ± 5	22.5 ± 5	25 ± 0.6
(±)-Acebutolol	1200 ± 265	3700 ± 280	4000 ± 530
(±)-Atenolol	1400 ± 145	6250 ± 480	3600 ± 320
(±)-Sotalol	2400 ± 235	340 ± 50	300 ± 45
(±)-Practolol	2500 ± 210	22,000 ± 500	24,000 ± 600
(±)-Butoxamine	4200 ± 300	750 ± 70	800 ± 120
β-Agonists			
(±)-Salbutamol	520 ± 45	1000 ± 80	2150 ± 150
(±)-Terbutaline	1600 ± 130	2500 ± 205	3900 ± 320

Values given are means ± s.e. of three experiments conducted in duplicate.

Table 2 Comparison of K_i values and pA_2 and pD_2

	Heart K_i (*nM*)	*Isolated atria* pA_2	*Lung* K_i (*nM*)	*Isolated trachea* pA_2
β-Antagonists				
(±)-Propranolol	2.4 ± 0.5	8.62 ± 0.17	2.7 ± 0.5	8.47 ± 0.23
(±)-Oxprenolol	2.6 ± 0.3	8.73 ± 0.08	1.7 ± 0.3	8.46 ± 0.28
(±)-Pindolol	2.9 ± 0.3	9.19 ± 0.35	90 ± 15	8.97 ± 0.24
(±)-Alprenolol	9 ± 1.2	8.5 ± 0.27	3 ± 0.5	8.43 ± 0.37
(±)-Labetalol	27 ± 5	8.12 ± 0.26	22.5 ± 5	7.89 ± 0.25
(±)-Acebutolol	1200 ± 265	6.84 ± 0.14	3700 ± 280	5.47 ± 0.43
(±)-Atenolol	1400 ± 145	7.66 ± 0.10	6250 ± 480	6.13 ± 0.27
(±)-Sotalol	2400 ± 235	6.40 ± 0.20	340 ± 50	6.47 ± 0.46
(±)-Practolol	2500 ± 210	6.85 ± 0.12	22,000 ± 500	5.13 ± 0.13
(±)-Butoxamine	4200 ± 300	5.24 ± 0.07	750 ± 70	6.44 ± 0.37
		Isolated atria pD_2		*Isolated trachea* pD_2
β-Agonists				
(±)-Salbutamol	520 ± 45	6.45 ± 0.29	1000 ± 80	6.54 ± 0.2
(±)-Terbutaline	1600 ± 130	5.45 ± 0.25	2500 ± 205	5.8 ± 0.3

Values given are means ± s.e. of three experiments conducted in duplicate for binding studies and of six determinations for pA_2 and pD_2.

and the pD_2 of the β-agonists; the atria were used in studying β_1 activity and the trachea for β_2.

There was a good correlation between the K_i values determined on the heart preparation and the pA_2 observed on the atria (correlation coefficient $r = 0.95$, $P < 0.001$).

The comparison between the K_i values for the lung preparation and the pA_2 observed on the trachea was not so satisfying (correlation coefficient $r = 0.67$, $P < 0.05$ with the technique of Harden *et al*). When we considered K_i values determined with De Feudis & Samoza's technique, the correlation coefficient was 0.60 ($P < 0.1$).

The selectivities observed with the membrane preparations and *in vitro* on isolated organs are shown in Table 3.

Table 3 Comparison of selectivities obtained with assays on isolated organs and with binding experiments

β-Antagonists	*Antilog* ΔpA_2*	$\frac{1/K_i \text{ heart}}{1/K_i \text{ lung}}$
(±)-Propranolol	1.4	1.12
(±)-Oxprenolol	1.9	0.65
(±)-Pindolol	1.66	31
(±)-Alprenolol	1.2	0.33
(±)-Labetalol	1.7	0.83
(±)-Acebutolol	23.4	3
(±)-Atenolol	33.9	4.46
(±)-Sotalol	0.85	0.14
(±)-Practolol	52.5	8.8
(±)-Butoxamine	0.08	0.18
β-Agonists	*Antilog* ΔpD_2†	$\frac{1/K_i \text{ heart}}{1/K_i \text{ lung}}$
(±)-Salbutamol	0.81	1.92
(±)-Terbutaline	0.44	1.56

* $\Delta pA_2 = pA_2$ on atria $- pA_2$ on trachea.
† $\Delta pD_2 = pD_2$ on atria $- pD_2$ on trachea.

Comparison of in vitro *and* in vivo *data*

We studied four β-blockers *in vivo*: propranolol, pindolol, practolol and butoxamine (Table 4). In spite of the arbitrary expression of our results in apparent pA_2, there was general agreement betweeen *in vitro* studies and cardiac rhythm in the guinea-pig or contractile force in the dog (for β_1 receptors); there was also a good correlation between *in vitro* assays and resistance to lung inflation in the guinea-pig and vascular resistance in the dog (for β_2 receptors).

Propranolol did not show any selectivity. However, for practolol, *in vivo* selectivity seemed more pronounced than *in vitro*: β_1 selectivity was over 100 *in vivo* and only 50 *in vitro*.

Discussion

It is easy to determine the affinity of the β-adrenergic molecules by binding techniques which are regularly used in studying β_1 receptors. Recently, with the work of U'Prichard, Bylund & Snyder (1978) it has been shown that these techniques can also be a tool for the study of β_2 lung receptors.

In general, our results tally with those of U'Prichard *et al.* (1978) and Rugg, Barnett & Nahorski (1978). However, with binding techniques, we have observed a cardioselectivity for pindolol not revealed before with traditional pharmacological techniques. In the available literature we could find no data on pindolol binding to β_1 and β_2 receptors. Furthermore, we have noted that the selectivities observed *in vivo* or *in vitro* are more pronounced than those determined with binding techniques excepted for pindolol.

On the other hand, we found no evidence for the bronchoselectivity of salbutamol or terbutaline, unlike U'Prichard *et al.* (1978) who reported a bronchoselectivity of 5.6 for salbutamol and 5.4 for terbutaline in the rat. For salbutamol, the inhibition constants of 1000 nM found on rat lung preparation and 520 nM on the heart preparation did not show any

Table 4 Comparison, for four β-adrenoceptor blocking agents, of β_1 and β_2 activities determined *in vivo*, *in vitro* and with binding techniques

β-Antagonists	*Binding* K_i (nM) *heart*	pA_2 *atria*	*Apparent* pA_2 *guinea-pig cardiac rhythm*	*Apparent* pA_2 *dog cardiac contractile force*	*Binding* K_i (nM) *lung*	pA_2 *trachea*	*Apparent* pA_2 *guinea-pig resistance to lung inflation*	*Apparent* pA_2 *dog vascular resistance*
Propranolol	2.4	8.62	7.35	7.04	2.7	8.47	7.41	7.16
Pindolol	2.9	9.19	7.93	8.17	90	8.97	8.19	8.07
Practolol	2500	6.85	6.54	6.46	22,000	5.13	4.60	4.39
Butoxamine	4200	5.34	—	4.70	750	6.44	5.04	5.16

bronchoselectivity; our results are comparable with those of Rugg *et al.* (1978) who, for salbutamol, found an inhibition constant of 1400 nM on a rat lung membrane preparation; they do not give any results for β_1 receptors, but Nahorski states that he was unable to demonstrate any bronchoselectivity for salbutamol or terbutaline (Nahorski, 1978).

We would like to put forward three possible explanations for these divergences:

Criticism of the binding technique

It is by no means sure that determination of K_i values by competition with a β-blocker, [^{3}H]-dihydroalprenolol, is appropriate in studying β-agonists such as salbutamol and terbutaline; in such cases, a β-agonist might be more suitable as the labelled ligand. Receptors have distinct conformations according to whether they bind with an agonist or an antagonist. Indeed, U'Prichard *et al.* (1978) reported lower values for salbutamol and terbutaline inhibition constants when [^{3}H]-adrenaline was used instead of [^{3}H]-dihydroalprenolol for competitive binding. Nevertheless, these assays require the addition to the incubation mixture of high concentrations of pyrocatechol and phentolamine, which may well interfere with the reaction.

In addition to these observations, it should be pointed out that under the electron microscope, our membrane preparations are seen to be heterogeneous and that all the techniques described in the literature lead to the same result.

Possible heterogeneity of receptors

According to Carlsson's hypothesis (Ablad, Carlsson, Carlsson, Dahlhof & Hultberg, 1974) supported by Furchgott & Wakade (1975), β_1 and β_2 receptors might be present within a given tissue in varying proportions according to the species. Miesch *et al.* (1978) found no evidence to support this hypothesis: in the guinea-pig atria assay, the shift of the dose-response curve for noradrenaline (β_1 selective) and for adrenaline (β_2 selective) is the same after IPS 339 a selective β_2 blocker. Nevertheless, Rugg *et al.* (1978) show, with inhibition of [^{3}H]-DHA binding by practolol, a selective β_1 blocker and by erythroprocaterol, a selective β_2 agonist, that β_1 and β_2 adrenoceptors may coexist in the lung; analysis of the displacement curves with the Scatchard technique indicates a majority of β_2 receptors in the rat lung (75% β_2 and 25% β_1) and a majority of β_1 receptors in the rabbit lung (60% β_1 and 40% β_2). It is only with the salbutamol experiments, that the Scatchard analysis of our curves reveals the possibility of there being two distinct types of β-receptors.

Criticism of in vitro *and* in vivo *assays*

Correlation between the inhibition constants obtained with a lung preparation and the pA_2 determined on trachea is poor.

The technique for determining the pA_2 for the β_2 receptors is reproducible; nevertheless, it is paradoxical that it may depend on isoprenaline inhibiting relaxation of the trachea previously contracted by carbachol. In the same way, the *in vivo* activity of a β_2 blocker is tested by its ability to inhibit the suppression by isoprenaline of a bronchospasm induced with 5-hydroxytryptamine.

In fact, we note that for the β-blockers described as highly selective such as acebutolol, atenolol, practolol and butoxamaine, the selectivity is the same whatever the technique, but their selectivity is clearer for *in vitro* assays or isolated organs than for binding assays. For example, practolol selectivity seems to decrease from *in vivo* assays to *in vitro* and still further for binding assays. It is quite possible that the biodisposability of the drugs may account for these variations better than structural differences between β_1 and β_2 receptors and perhaps other subtypes.

References

ABLAD, B., CARLSSON, B., CARLSSON, E., DAHLOF, C. & HULTBERG, EK.L. (1974). Cardiac effects of β-adrenergic receptor antagonists. *Adv. Cardiol.*, **12**, 290–302.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmac.*, **14**, 48–58.

DE FEUDIS, F.V. & SOMOZA, E. (1977). Density of GABA receptors in rat cerebral cortex measured with bicuculline methiodide in the presence of Na^+ and other inorganic ions. *Gen. Pharmac.*, **8**, 181–187.

FURCHGOTT, R.F. & WAKADE, T.D. (1975). Evidence for both β_1 and β_2 receptors in guinea-pig and tracheal smooth muscle and variation of the β_1:β_2 ratio in different animals. *Sixth Int. Congress Pharmacology.* Abstracts., No. 1508, p. 622.

HARDEN, K., WOLFE, B.B. & MOLINOFF, P.B. (1976). Binding of iodinated β-adrenergic antagonists to proteins derived from rat heart. *Mol. Pharmac.*, **12**, 1–15.

HORRII, D., KAWADA, T., TAKEDA, K. & IMAI, S. (1974). Comparison of β-adrenergic blocking activities of propranolol, isopropyl, methoxamine, sotalol, practolol, alprenolol, pindolol, oxprenolol and D-32 in the atria and trachea of the guinea-pig. *Arzneim. Forsch.* (*Drug. Res.*), **24**, 1275–1277.

KONZETT, M. und ROSSLER, R. (1940). Versuchanordnung zu Untersuchungen an der bronchial Muskulatur. *Naunyn Schmiedebergs. Arch. exp. Path. Pharmak.*, **195**, 71–74.

LEVY, B. & WILKENFELD, B.E. (1970). The actions of selective β-receptor antagonists on the guinea-pig trachea. *Eur. J. Pharmac.*, **11**, 67–74.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.

MIESCH, F., TURLOT, J. C., EHRHARDT, J. D. & SCHWARTZ, J. (1977). pA_2: modèles mathématiques et estimation statistique. *J. Pharmac. (Paris)*, **8**, 27–41.

MIESCH, F., BIETH, N., LECLERC, G. & SCHWARTZ, J. (1978). Quantification de six bêta-bloquants, Activité *in vitro* et *in vivo*. *J. Pharmac., Paris.*, **9**, 297–308.

NAHORSKI, S.R. (1978). Heterogeneity of cerebral β-adrenoceptor binding sites in various vertebrate species. *Eur. J. Pharmac.* **51**, 199–209.

RUGG, E.L., BARNETT, D.B. & NAHORSKI, S.R. (1978). Coexistence of β_1 and β_2 adrenoceptors in mammalian lung: evidence from direct binding studies. *Mol. Pharmac.*, **14**, 996–1005.

SCATCHARD, G. (1949). The attraction of protein for small molecules and ions. *Ann. N.Y. Acad. Sci.*, **51**, 660–672.

U'PRICHARD, D.C., BYLUND, D.B. & SNYDER, S.H. (1978). [^{3}H]-epinephrine and (−)[^{3}H]-dihydroalprenolol binding to β_1 and β_2 noradrenergic receptors in brain, heart and lung membranes. *J. biol. Chem.*, **253**, 5090–5102.

WILLEMS, J.L. & BOGAERT, M.G. (1975). Dopamine induced neurogenic vasodilatation in isolated perfused muscle preparation of the dog. *Naunyn Schmiedebergs Arch. Pharmac.*, **286**, 413–428.

(Received May 3, 1979.)