BBA 75889

ISOLATION OF A PROTEOLIPID FROM SPLEEN CAPSULE BINDING (±)-[^{3}H]NOREPINEPHRINE

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(Received November 1st, 1971)

SUMMARY

Bovine spleen capsules were extracted with chloroform–methanol (2:1, v/v) and the proteolipids were separated by column chromatography on Sephadex LH-20. Five proteolipid peaks were eluted with chloroform and one peak with chloroform–methanol (4:1, v/v). Only Peak 1 eluted between 17–22 ml of chloroform showed binding for [^{3}H]norepinephrine. The saturation curve of the binding in Peak 1 suggests that this proteolipid contains two groups of sites with different dissociation constants. For 200000 g of proteolipid 3 moles of norepinephrine are bound with high affinity and about 27 moles with low affinity. Similar findings were obtained using a partition method for the study of the binding and the competition with some adrenergic antagonists was demonstrated. The possibility that Peak 1 of proteolipid represents the adrenergic receptor of the spleen capsule is discussed.

INTRODUCTION

Although several workers, by the use of radioactive blocking agents, have tried to label adrenergic peripheral receptors and to estimate the possible number of receptor sites no serious attempts to isolate the receptor substance responsible for the binding have been made[1,2]. We have previously reported that the nerve-ending membranes isolated from basal ganglia and mesencephalic nuclei of the central nervous system had the highest binding capacity, compared to other subcellular fractions, for the adrenergic blocking agents; [^{14}C]Sy28 (*N*-α-naphtylmethyl-*N*-ethyl-β-bromoethylamine)[3], [^{14}C]Dibenamine (*N*-2-chloroethyl-dibenzylamine hydrochloride) and [^{14}C]Propanolol (1-isopropyl-amine-3-1-naphtyloxy-2-propanolol)[4].

In these studies evidence was obtained that the binding could be with proteolipid proteins (*i.e.* hydrophobic lipoproteins) which were extracted from the same tissue. However, since the drug–proteolipid complex had a tendency to dissociate in the organic solvents used, these results were not as clear cut as those obtained with labeled cholinergic blocking agents[5] and with 5-hydroxytryptamine[6], in which case the high affinity binding was clearly shown to be related to special brain proteolipids eluted with chloroform–methanol (4:1, v/v) from a Sephadex LH-20 column.

More recently our laboratory has been engaged in the isolation of the cholinergic receptor in peripheral tissues particularly the electric organs of *Electrophorus* and

Torpedo[7] and skeletal muscle[8]. In all these cases a special proteolipid, eluted in chloroform, was found to bind [^{14}C]acetylcholine and other cholinergic drugs. These findings encouraged us to try to isolate a peripheral adrenergic receptor using [^{3}H]norepinephrine and a tissue with a rich sympathetic innervation, such as the spleen capsule, with the methodology used for the cholinergic receptor. Bovine spleen was used because large amounts of tissue could be easily obtained. It will be shown here that a special proteolipid peak, extracted and purified from spleen capsule, has a high affinity for binding [^{3}H]norepinephrine and that this binding may be antagonized with some adrenergic blocking agents. Quantitative information on the characteristics of the norepinephrine–proteolipid interaction will also be presented.

METHODS

Bovine spleens were freshly obtained, packed on ice and processed in the laboratory. After sectioning into slabs the spleen capsule was separated from the parenchyma by mechanical teasing. The fragments were submitted to three consecutive washings in distilled water and blending in a Waring blender for 1 min at high speed. In each case the tissue fragments were retained on a Buchner-type of filter. Such fragments were frozen, lyophilized and stored under vacuum over a dessicant. 2 g of this material corresponding to about 15 g of fresh tissue, were homogenized in 50 ml of chloroform–methanol (2:1, v/v) in an Ultra-Turrax (Karl Kolb, Frankfurt). After standing at room temperature for 5 min the extract was filtered through Whatman No. 2 filter paper and the residue was washed with about 10 ml of chloroform–methanol (2:1, v/v). The final volume of the extract was noted and half the volume of chloroform was added. The extract was then evaporated under vacuum at room temperature to a final volume of 3 ml.

For the binding (±)-[^{3}H]norepinephrine (6.6 Ci/mmole New England Nuclear), previously purified on an alumina column[9] was used. The labeled drug was employed as such only for the lowest concentrations; for the others it was diluted with unlabeled (±)-norepinephrine (Sigma). The blocking agents Dibenamine, Propanolol and Phentolamine (2-[*N*-(*m*-hydroxyphenyl-*p*-toluidinomethyl]imidazoline) were obtained in pure form, respectively, from the local firms Szabo, Ramon and Ciba to whom we express our gratitude.

For studying the proteolipid–drug interaction two methods of binding were used:

Column chromatography

After addition of the [^{3}H]norepinephrine to the extract and standing at room temperature for 20 min this was loaded onto a Sephadex LH-20 column (2.1 cm × 18 cm) that had been equilibrated overnight with chloroform. The elution was made at room temperature with chloroform, followed by mixtures of chloroform–methanol of increasing polarity (Fig. 1). The eluate was monitored at 278 nm with an LKB Uvicord ultraviolet absorption meter at a flow rate of 0.5 ml/min and collected into fractions of variable volume (1–4 ml). In each tube the lipid phosphorus[10] and protein content[11] were determined and the radioactivity was measured in a Nuclear Chicago liquid-scintillation counter as previously described[5]. Control experiments were made at each initial concentration by loading on the column

amounts of [^{3}H]norepinephrine equivalent to those used in the binding experiments but without the extract (Table I).

Partition method

Weber *et al.*[12] have developed a method for the study of the proteolipid–drug interaction based on the differences in partition coeficient of the two components of the system. Since the proteolipid is insoluble in water and norepinephrine preferentially soluble in water, it was possible to apply the same methodology as previously used with the receptor cholinergic proteolipid of electric tissue of *Electrophorus*. In this case a fluorescent probe having an ethyltrimethylammonium end was used[12]. The method was adapted to the [^{3}H]norepinephrine binding in the following way: a sample of 2 ml of the proteolipid from peak 1 (Fig. 1) containing about 40 μg protein, was put at the bottom of a cylinder and on top 2 ml of water saturated with chloroform containing $2 \cdot 10^{-6}$ M [^{3}H]norepinephrine. After 10 min of simultaneous stirring of both phases at room temperature the equilibrium was reached and aliquots from the water phase were taken for measuring radioactivity. After putting back a similar aliquot of water, this procedure was repeated and in this way the upper phase, containing the free norepinephrine, was progressively diluted to obtain the different points of the binding curve. The theory of the method is described in detail elsewhere[12]. Essentially we used the following equation:

$$NE_i = NE_u + NE_l = [NE_b]_l \cdot V_l + [NE_f]_u \cdot \left(V_u + \frac{V_l}{S}\right) \quad (1)$$

in which NE_i is the initial amount of norepinephrine added: NE_u and NE_l the amounts of ligand in the upper and lower phases; $[NE_b]_l$ the bound concentration of norepinephrine in the lower phase, V_l and V_u the volumes of the lower and upper phases. S, the partition coefficient, is the ratio between the free concentration of norepinephrine in the upper phase $[NE_f]_u$ and in the lower phase $[NE_f]_l$:

$$S = \frac{[NE_f]_u}{[NE_f]_l} \quad (2)$$

Knowing NE_i and S the norepinephrine bound to the proteolipid may be easily calculated by measuring the radioactivity in the upper phase $[NE_f]_u$. This method becomes particularly sensitive if S is a large number in which case the ligand in the upper phase is virtually the total free ligand and that in the lower phase is the bound ligand. The partition coefficient of norepinephrine was found to be:

$$S = \frac{99}{1}$$

This value was not changed by using in the upper phase pH values varying between 4.5 and 7.0.

To study competition effects proteolipid–norepinephrine binding curves were done in the presence of different adrenergic blocking agents which were previously added to the lower phase (see Results). Such an addition did not change the partition coefficient for norepinephrine.

RESULTS

Chromatographic pattern

Fig. 1 shows the elution pattern obtained from the chloroform–methanol extract of the spleen capsule. In this particular experiment the final concentration of [^{3}H]norepinephrine was $1.6 \cdot 10^{-8}$ M. The proteolipid protein, as determined by the Lowry method, appears in five peaks eluted with chloroform and one peak in the chloroform–methanol (4:1, v/v), at the end of the chromatogram. The total recovery of protein was 69.8 %. This chromatographic pattern was reproducible also with higher concentrations of ligand and in the absence of norepinephrine. The lipid phosphorus was eluted in three peaks with the chloroform and one peak in the chloroform–methanol (4:1, v/v). In most cases these peaks did not coincide with those of protein and the maximum lipid P was eluted in between the protein Peaks 3 and 4 (Fig. 1). The recovery of lipid P was 88 % and in Peak 1 the lipid P/protein ratio was 0.24. Using thin-layer chromatography[13] it was shown that the main phospholipids present in peak 1 were: phosphoethanolamine, phosphoinositides and phosphatidyl-serine while phosphatidylcholine and sphingomyelin were absent.

The [^{3}H]norepinephrine appeared in a single peak with Peak 1 of protein which hence will be called the "receptor" peak. The elution of radioactivity was produced between 17–22 ml of chloroform and the recovery was of 40 %. In control experiments done with [^{3}H]norepinephrine, but without addition of the extract, all the counts were retained by the column and did not appear in any region of the chromatogram.

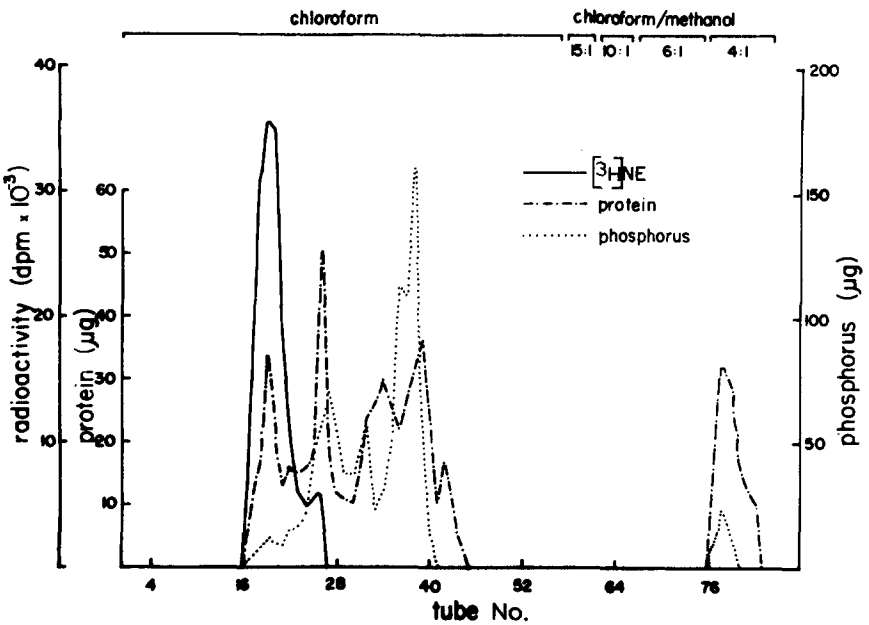

Fig. 1. Chromatographic pattern obtained from lyophilized bovine spleen capsule. The chloroform–methanol (2:1, v/v) extract was submitted to binding with $1.6 \cdot 10^{-8}$ M (±) [^{3}H]norepinephrine and then eluted through a column of Sephadex LH-20. The radioactivity appears together with Peak 1 of proteolipid protein.

Saturation curve

Binding experiments similar to that shown in Fig. 1 were done at concentrations of norepinephrine varying between $1.6 \cdot 10^{-8}$ and $5 \cdot 10^{-5}$ M using in every case the same amount of extracted proteolipid. At each point controls with [^{3}H]norepinephrine alone were carried out. Since no radioactivity was eluted in the controls the free [^{3}H]norepinephrine (NE_f) was estimated by the difference between the initial and the bound norepinephrine (Table I). Within the range of concentration used the radioactivity always appeared in Peak 1.

Since the deviation from the mean between the experiments was very small

TABLE I

NUMERICAL DATA OF THE EXPERIMENTS OF BINDING WITH VARIOUS CONCENTRATIONS OF (±)-[^{3}H]NOREPINEPHRINE

[NE_i], initial concentration of norepinephrine; [NE_f] concentration of free norepinephrine. The saturation at infinite [NE_f] was calculated by extrapolation (see description in the text). The data of the bound norepinephrine are the mean of two experiments.

$[NE_i]$ (M)	$[NE_f]$ (M)	*pmoles/μg protein of Peak 1*
$1.6 \cdot 10^{-8}$	$1.4 \cdot 10^{-8}$	0.07 ± 0.006
$3.2 \cdot 10^{-8}$	$3.0 \cdot 10^{-8}$	0.11 ± 0.008
$1.0 \cdot 10^{-7}$	$9.1 \cdot 10^{-8}$	0.50 ± 0.035
$3.0 \cdot 10^{-7}$	$2.3 \cdot 10^{-7}$	1.50 ± 0.35
$7.4 \cdot 10^{-7}$	$6.7 \cdot 10^{-7}$	4.50 ± 0.55
$1.2 \cdot 10^{-6}$	$1.1 \cdot 10^{-6}$	5.00 ± 0.50
$2.3 \cdot 10^{-6}$	$2.1 \cdot 10^{-6}$	7.50 ± 0.75
$4.4 \cdot 10^{-6}$	$4.2 \cdot 10^{-6}$	12.50 ± 0.65
$7.3 \cdot 10^{-6}$	$6.7 \cdot 10^{-6}$	20.00 ± 2.00
$1.0 \cdot 10^{-5}$	$9.3 \cdot 10^{-6}$	26.00 ± 3.50
$2.0 \cdot 10^{-5}$	$1.7 \cdot 10^{-5}$	72.00 ± 7.50
$5.0 \cdot 10^{-5}$	$4.6 \cdot 10^{-5}$	127.00 ± 6.50
Infinite		150.00

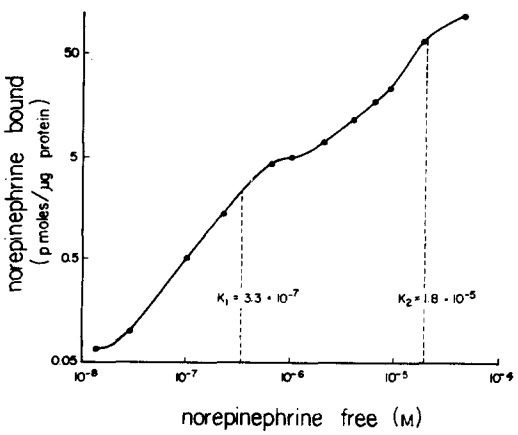

Fig. 2. Log–log plot of norepinephrine bound to the proteolipid against free norepinephrine in molar concentration [NE_f]. The apparent dissociation constants K_1 and K_2 were calculated at half the saturation of the two types of binding sites.

(Table I) it was decided to make a log–log plot of the mean values of norepinephrine bound against free norepinephrine in molar concentration (Fig. 2). The curve did not show the characteristic rectangular hyperbola expected for the saturation of a single set of binding sites. Two inflexion regions, one at $1.1 \cdot 10^{-6}$ and the other at about $4.6 \cdot 10^{-5}$ M of free norepinephrine, were observed. The variations between the experiments are indicated in Table I, last column. The curve was not extended beyond $4.6 \cdot 10^{-5}$ M free norepinephrine, at which point there was a binding of 127 pmoles/μg protein. To calculate the apparent saturation of the binding at an infinite concentration of free norepinephrine a plot of bound norepinephrine against the reciprocal of $[NE_f]$ was made. Such a plot permitted to extrapolate the binding to an apparent saturation, at which point 150 pmoles/μg of protein were bound (Table I).

It was determined that at this apparent saturation 1 mole of norepinephrine would bind to approx. 6660 g of proteolopid. At the first inflexion, which we consider to be the saturation of the sites of higher affinity in the proteolipid, the relation would be of 1 mole norepinephrine per 200000 g of proteolipid. Since the log $[NE_f]$ is directly proportional to the chemical potential of the ligand this type of plotting permits also to calculate the apparent dissociation constants for the two saturation regions[14]. For the high affinity one we obtained: K_1 diss. $= 3.3 \cdot 10^{-7}$ and for the lower affinity region: K_2 diss. $= 1.8 \cdot 10^{-5}$.

Competition experiments

Fig. 3 shows a control curve done with the partition method B (see Methods) and the binding obtained in the presence of the adrenergic blocking agents Phentolamine, Dibenamine and Propanolol. These drugs were added to the organic phase to a final concentration of $1 \cdot 10^{-4}$. The control curve is very similar to that obtained with the Sephadex LH-20 columns and the saturation of the high-affinity binding is oeached at approximately the same concentration of free norepinephrine. Also in this case an apparent K_1 diss. of approx. $3.0 \cdot 10^{-7}$ may be calculated. The binding curve rf the adrenergic proteolipid was exactly the same using water in the upper phase

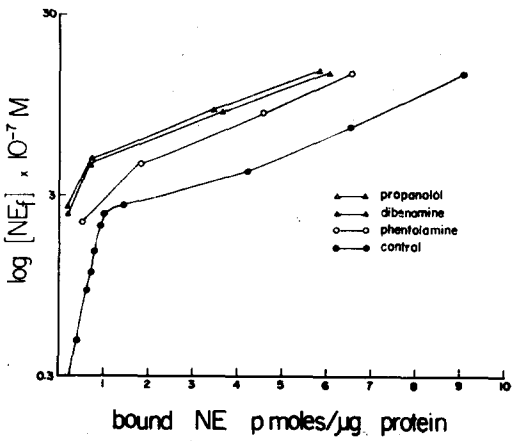

Fig. 3. Plot of the log of free norepinephrine $[NE_f]$ in the upper phase against the concentration of the norepinephrine bound in the lower phase using the method of Weber *et al.*[12]. The effect of the addition of antagonist drugs upon the binding of [^{3}H]norepinephrine by the proteolipid is shown.

TABLE II

ACTION OF ADRENERGIC ANTAGONISTS UPON THE BINDING OF [^{3}H]NOREPINEPHRINE TO THE RECEPTOR PROTEOLIPID

NE_f, free norepinephrine. Control, norepinephrine bound. Percent inhibition by the various antagonists added to the final concentration of 0.1 mM.

NE_f (*M*)	*Control* (*pmoles/μg protein*)	*Inhibition* (%)		
		Phentolamine	*Dibenamine*	*Propanolol*
$1.5 \cdot 10^{-6}$	9.0	26.7	34.4	35.6
$9.0 \cdot 10^{-7}$	7.2	37.5	50.0	52.8
$7.5 \cdot 10^{-7}$	6.5	40.0	55.1	60.0
$4.5 \cdot 10^{-7}$	4.5	60.0	84.5	86.7
$3.0 \cdot 10^{-7}$	2.2	56.0	83.0	86.4

or water with 0.1 M buffer phosphate at pH 7.0. In the presence of the various antagonists the curve is displaced upwards indicating that they have inhibited the high affinity binding sites for norepinephrine (Fig. 3). In Table II the percent inhibition caused by the blocking agents at various concentrations of free norepinephrine is indicated.

DISCUSSION

The smooth muscle of the spleen capsule is a very appropiate tissue to attempt the isolation of a peripheral adrenergic receptor. In fact it is richly innervated by the sympathetic system and it contains a high concentration of norepinephrine[15]. Most adrenergic receptors of the splenic smooth muscle are of the α type and function in the contraction of this tissue[16,17]; however, in the mouse spleen the presence of β receptors, which produce relaxation of the smooth muscle, has also been demonstrated[18].

Moran and Triggle[2] have emphasized that one of the major problems of receptor isolation is the non-specific binding of the drugs; this may not be evident from the pharmacological point of view but it may occur at the chemical or molecular level. In our case the high affinity shown by Peak 1 of the proteolipid extracted and purified by column chromatography from the spleen capsule and the lack of binding in the other proteolipid peaks suggests that we are in the presence of a specific type of binding. [^{3}H]Norepinephrine was found to bind at concentrations as low as $1 \cdot 10^{-8}$ M, at which range only a very specific type of interaction may take place. In strong support of this specificity are the findings, to be reported in another paper, in which it will be shown that (+)-norepinephrine does not interact with the adrenergic proteolipid when this is included in an artificial membrane and the conductance changes are studied.

This proteolipid seems to be unique with respect to other receptor proteolipids so far isolated in this laboratory. Control experiments were carried out using [^{3}H]norepinephrine and proteolipids extracted from a tissue known to be essentially cholinergic such as the electric organ of *Electrophorus*. No [^{3}H]norepinephrine binding was found in Peak 3 which binds acetylcholine and other cholinergic drugs[7,19]. We also observed

that unlabeled acetylcholine did not interfere with the binding of [^{3}H]norepinephrine to Peak 1 of the spleen capsule.

The amount of receptor proteolipid that may be extracted from 2 g of lyophilized tissue is extremely small. It may be calculated that it represents only about 0.0055 % of the total protein of the tissue. This consideration may be important in interpreting the results reported by Lewis and Miller[1] who postulated that the binding was mainly with the residue left after the extraction with organic solvents. In their chloroform–methanol extract they recovered about 38 % of the [^{3}H]phenoxybenzamine; however, this fraction may have had an extremely high specific activity if expressed by the amount of receptor proteolipid present in the extract. In contrast with these findings De Robertis and Fiszer[4] carried out the binding of nerve-ending membranes from brain with $1 \cdot 10^{-6}$ M [^{14}C]Dibenzamine and after chloroform–methanol extraction found 93.5 % of the counts in the organic extract and only 6.5 % in the residue. With [^{14}C]Propanolol the results were respectively, 84.5 and 14.4 %.

In the experiments described here Peak 1 of proteolipid, eluted between 17–22 ml of chloroform, contains only 80–100 μg from a total of 1400 μg proteolipid protein extracted from the original tissue. In similar studies previously done on *Electrophorus* from 1 g lyophilized tissue, equivalent to 12.5 g fresh tissue we extracted 220 μg of receptor proteolipid which appeared in Peak 3 between 40–50 ml of chloroform[19]. If one assumes a molecular weight of 200000 for the adrenergic receptor (see above) it may be calculated that there are $1.5 \cdot 10^{11}$ receptor molecules per mg dry tissue. This figure is very close to that obtained by Lewis and Miller[1] in the rat seminal vesicle using [^{3}H]phenoxybenzamine. From their data we have calculated some $2 \cdot 10^{11}$ receptor sites per mg dry tissue. Furthermore our results compare favorably with those of May *et al.*[20] in rabbit aortic strip, bound with [^{3}H]Sy28, in which $1.5 \cdot 10^{12}$ receptors per mg dry wt. were estimated.

The preoteolipid of Peak 1 has a lower content of phospholipids than the other peaks, being phosphatidylethanolamine, phosphatidyl-inositol and phosphatidylserine the main phospholipids present. In our previous work with receptor proteolipids we had evidence that it was the protein moiety the one responsible for the binding (see ref. 21), however, in the present case an accesory role for the phospholipids can not be ruled out. In our studies with [^{14}C]Dibenamine and [^{14}C]Propanolol, in various brain proteolipid peaks, we found that the binding was inversely related to the content of phospholipids[4].

Assuming that the proteolipid of Peak 1 contains a homogeneous population of molecules the shape of the curve of Fig. 2 suggests the existence of two groups of sites with different apparent dissociation constants. The affinity of the sites with K_1 diss. $= 3.3 \cdot 10^{-7}$ would be about 60 times larger than those having K_2 diss. $= 1.8 \cdot 10^{-5}$. In the cholinergic receptor extracted from the electric organ of *Electrophorus* we postulated the existence of one site of high affinity and about 10 sites of low affinity per an assumed molecular weight of 40000. In this case the difference between K_1 and K_2 was 100 times[19]. We also postulated that within the physiological range, in which acetylcholine is liberated at the synaptic junction, only the high affinity binding site would be effective. Similar considerations are applicable to the adrenergic proteolipid receptor.

We found that both the α blocking agents Phentolamine and Dibenamine and the β blocking drug Propanolol, at a concentration of $1 \cdot 10^{-4}$ M are able to

displace to a great extent norepinephrine from the sites of high affinity (Table II). We are well aware that these competition experiments, done in a non polar medium, could not be compared with those done *in vivo*, with the receptor in place at the membrane of the muscle cell, in which case the blocking agents have higher affinity than norepinephrine.

The competition experiments that have been presented here (Table II) do not permit to draw conclusions about the α or β nature of the adrenergic proteolipid isolated from the spleen capsule. Since, from the pharmacological viewpoint most of spleen receptors are of the α type it is conceivable that such type of specificity may be a function of the molecular organization of the receptor within the membrane or even of the post-binding steps leading to a differential response of the smooth muscle cell. In those cases the specificity would be lost after isolation of the receptor macromolecule.

ACKNOWLEDGEMENT

The authors are indebted to Dr. G. Weber for his helpful discussion and to Dr. S. Langer for providing some drugs and technical indications. The technical assistance of Mrs. Enriqueta Flajszer is greatly appreciated.

This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) Argentina and the National Institutes of Health (5 RO1 NS 06953-05 NEUA).

One of the authors received a Career Award of CONICET.

REFERENCES

1 J. E. Lewis and J. W. Miller, *J. Pharmacol. Exp. Theral.*, 154 (1966) 46.
2 J. F. Moran and D. J. Triggle, *Fundamental Concepts in Drug–Receptor Interactions*, Academic Press, New York, 1970, p. 133.
3 S. Fiszer and E. De Robertis, *Life Sci.*, 7 (1968) 1093.
4 E. De Robertis and S. Fiszer De Plazas, *Life Sci.*, 8 (1969) 1247.
5 E. De Robertis, S. Fiszer, J. Pasquini and E. F. Soto, *J. Neurobiol.*, 1 (1969) 41.
6 S. Fiszer and E. De Robertis, *J. Neurochem.*, 16 (1969) 1201.
7 J. L. La Torre, G. S. Lunt and E. De Robertis, *Proc. Natl. Acad. Sci. U.S.*, 65 (1970) 716.
8 G. G. Lunt, E. Stefani and E. De Robertis, *J. Neurochem.*, 18 (1971) 1545.
9 S. Udenfriend, *Fluorescence Assay in Biology and Medicine*, Academic Press, New York, 1962, p. 154.
10 P. S. Chen, Jr., T. Y. Toribara and H. Warner, *Anal. Chem.*, 28 (1956) 1756.
11 O. H. Lowry, N. J. Rosebrough, A. L. Farr and E. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
12 G. Weber, D. P. Borris, E. De Robertis, F. J. Barrantes, J. L. La Torre and M. C. Llorente De Carlin, *Mol. Pharmacol.*, 7 (1971) 530.
13 V. Skipski, R. F. Peterson and M. Barclay, *Biochem. J.*, 90 (1964) 374.
14 G. Weber, in B. Pullman and M. M. Weissbluth, *Molecular Biophysics*, Academic Press, New York, 1965, p. 369.
15 U. S. Von Euler, *Pharmacol. Rev.*, 6 (1954) 15.
16 K. Ottis, J. E. Davis and H. D. Green, *Am. Physiol.*, 189 (1957) 599.
17 R. K. Bickerton, *J. Pharmacol. Exp. Therap.*, 142 (1963) 99.
18 L. S. Ignarro and E. Titus, *J. Pharmacol. Exp. Therap.*, 160 (1968) 72.
19 E. De Robertis, G. S. Lunt and J. L. La Torre, *Mol. Pharmacol.*, 7 (1971) 97.
20 M. May, J. F. Moran, H. Kimelberg and D. J. Triggle, *Mol. Pharmacol.*, 3 (1967) 28.
21 E. De Robertis, *Science*, 171 (1971) 963.