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Catecholamines

Isolated *beta*-Adrenergic Binding Sites: a Potential Assay Vehicle for Catecholamines*

ROBERT J. LEFKOWITZ

Cardiac Unit, Department of Medicine, Massachusetts General Hospital, and Harvard Medical School, Boston, Massachusetts

OF the biologically active molecules which circulate in blood, few have more profound or more widespread effects than the catecholamines, norepinephrine and epinephrine. Not surprisingly, interest in their accurate measurement has led to many attempts to devise specific and sensitive assay methodology (49). Despite the sensitivity of fluorescence methods, currently, norepinephrine and epinephrine measurement remains a sophisticated technique which generally is performed only in specialized research laboratories.

The purposes of this presentation will be 4-fold: First, to review briefly the concept of competitive protein binding assays, with particular attention to physiological hormone receptors as a new type of reagent for such assays. Second, to outline current concepts of adrenergic receptors and of other potential biological binding mechanisms for catecholamines. Third, to outline some recent research which has characterized a *beta*-adrenergic binding site in membrane fractions from canine cardiac muscle. Fourth, to discuss the potential applications of such a binding site to assay of catecholamines in biological fluids.

Earlier presentations in this symposium have dealt with the theory and practice of competitive protein binding assays, as exemplified by the radioimmunoassay. These principles have not, at present, been applied successfully to the measurement of catecholamines.

The basis for all competitive protein binding assays is the availability of a binding site for the substance which is to be measured. In the case of radioimmunoassay the binding site is on an antibody. Another class of binding sites which have been used for assay purposes are those which occur on circulating binding proteins, for example, the cortisol binding protein used to measure cortisol (34) or the thyroxine binding protein used for assay of thyroxine (33). The common feature in all of these methods is the reversible interaction of a radioactively labeled molecule with a specific binding site. Unlabeled molecules with identical or very closely related structures can compete for occupancy of the binding sites. Standard curves are constructed by quantitating the amount of inhibition of binding of labeled molecules by increasing amounts of unlabeled molecule. Thus, *e.g.*, the more insulin present, the less radioactively labeled insulin which is bound to anti-insulin antibody; the more cortisol present, the less radioactively labeled cortisol which is bound to cortisol binding globulin (CBG), *etc.* From these standard curves, the amount of unlabeled substance in an unknown can be computed.

There are three important features which characterize the binding of ligands to each of these binding sites: These are 1) reversibility; 2) high affinity; and 3) specificity. The high affinity of these binding sites per-

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mits detection of very small quantities of the substances being measured. The great specificity of these interactions generally permits detection of only a single chemical structure or of very closely related structures.

These three characteristics, reversibility, high affinity, and specificity, also characterize the interaction of many hormones and drugs with cellular structures called receptors (20, 23). Until fairly recently, receptors have been purely hypothetical structures—generally envisaged as that component of a cell with which a biologically active molecule first interacts. Within the past several years, however, substantial progress has been made in demonstrating that hormone receptors are discrete, identifiable macromolecules which can be studied and purified by conventional techniques of protein chemistry (20).

The existence of intracellular cytoplasmic receptor molecules for a variety of steroid hormones has been known for some time (4). I wish to focus this discussion, however, on those hormone receptors which appear to be membrane bound. In particular, many of these are likely to be located in the plasma membrane and to be related in a regulatory fashion to the enzyme adenylate cyclase (fig. 1). This enzyme appears to be present in virtually all mammalian tissues with the exception of non-nucleated erythrocytes (38a). However, in any given tissue, only a very limited range of hormones, often only one will stimulate the cyclase. Thus, in the adrenal cortex, it is only (ACTH) adrenocorticotrophic hormone (25, 45); in the myocardium, it is catecholamines (32), glucagon (30), and thyroid hormones (29); and in adipose tissue five or six hormones (5). This great tissue specificity is felt to lie in hormone receptors. These macromolecules appear to be located in the cell membrane, as is the adenylate cyclase. Binding of a hormone or drug to its receptor is in some, as yet not understood, fashion coupled with the subsequent activation of adenylate cyclase and

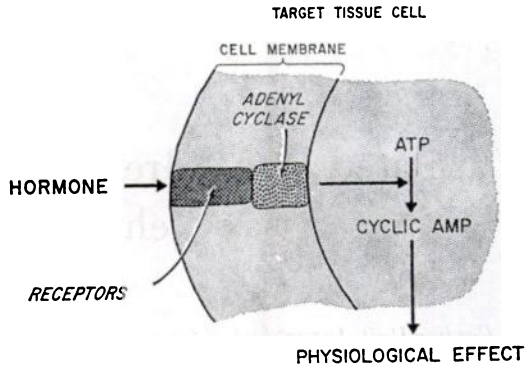


FIG. 1. Mechanism of action of hormones which stimulate adenyl cyclase. (Adapted from E. W. Sutherland and G. A. Robison: The role of cyclic 3′5′-AMP in response to catecholamines and other hormones. Pharmacol. Rev. 18: 145–161, 1966.)

an increased rate of generation of the "second messenger cyclic adenosine monophosphate (cAMP)." The receptors thus play two important roles: first a discriminatory function, based on selective binding of agonist drugs or hormones which possess an appropriate chemical structure; and second, an effector function which leads to a biological response through some mechanism such as adenyl cyclase activation, alteration of sodium permeability, *etc.*

It is the first of these functions, that of selective binding, which makes hormone receptors potentially useful as tools for hormone measurement. Binding of hormones to their receptors is a reversible process. Moreover, it is of high affinity. Thus, many of these receptors, *e.g.*, those for the polypeptide hormones, respond to circulating hormone levels in the range of 10^{-10} M to 10^{-12} M. This intrinsically high affinity of binding means that when a hormone receptor is used for competitive binding assay *in vitro*, very small amounts of hormone can be detected (26).

Hormone receptor binding is also highly specific. The specificity, moreover, is biological, *i.e.*, only those molecules capable of occupying the physiological receptor can compete for the site. This is in contrast to the specificity of radioimmunoassays which

is for an immunologically active structure. The existence of biologically inactive yet immunologically active fragments of hormones circulating in blood, makes this point of considerable relevance (36).

To date, membrane-bound hormone receptors have not been used widely as binding reagents in competitive binding assays. To a large extent, this is due to the fact that techniques for their preparation and study have only evolved over the past 2 to 3 years. Those systems in which such assays have been developed successfully are summarized in table 1. For each of these hormones, the sensitivity of the radioreceptor assay has generally been comparable to that of immunoassay.

TABLE 1

Radioreceptor assays with membrane-bound hormone receptors

Hormone Assayed	Tracer Hormone	Tissue from Which Receptors Prepared
ACTH (24, 26, 50)	ACTH-^{125}I	Adrenal cortex
HCG (7)	HCG-^{125}I	Testis
Prolactin (42, 47)	Prolactin-^{125}I	Mammary Epithelium
Growth hormone (28)	GH-^{125}I	Lymphocytes

Turning now to the question of receptors and other potential biological binding mechanisms for catecholamines, the situation is somewhat more complex. Today the concept of *alpha* and *beta*-adrenergic receptors has gained wide acceptance. Originally proposed by Ahlquist (1), this scheme was devised to explain the finding that for certain effects, such as smooth muscle contraction, norepinephrine was the most potent amine, whereas for other effects, such as smooth muscle relaxation and chronotropic and inotropic effects on the heart, isoproterenol was most potent. The former effects were said to be mediated by *alpha*-adrenergic receptors, the latter by *beta*-adrenergic receptors. However, over the years this scheme has been amended and at least two distinct populations of *beta*-receptors, $beta_1$ and $beta_2$, have been delineated (18). Even so, many actions of catecholamines, such as metabolic effects, remain difficult to classify rigorously and the situation is further complicated by considerable species variation in these patterns. Over the years a number of authors have continued to argue the possibility that only a single receptor might mediate both *alpha* and *beta* effects (17, 41) and recently this view has been supported by experimental evidence (41). In the frog heart, a single receptor type, the function of which is markedly altered by temperature, has been demonstrated by Kunos *et al.* (17). Isolation of adrenergic receptors from a variety of tissues will hopefully settle these questions. At the present time, the "*beta*-adrenergic" receptor appears, in almost all instances, to be allied closely with the adenylate cyclase system (38b). Thus, *beta*-adrenergic effects generally appear to be mediated *via* increases in cAMP. The relation of "*alpha*-adrenergic" receptors to adenylate cyclase is much less clear. Recently, it has been proposed that *alpha*-adrenergic effects might be mediated by decreases in adenylate cyclase activity, and in several systems, this has been verified experimentally (38c). This concept, however, awaits more extensive experimental testing.

A number of biological binding mechanisms for catecholamines, other than those associated with physiological receptors have been demonstrated. These are summarized in table 2. It is apparent in each instance that the specificity of each of these is considerably different than the known *pharmacological* specificity of adrenergic receptors.

Recently, workers in several laboratories have demonstrated binding *in vitro* of tritium-labeled catecholamines to membrane preparations from a variety of tissues. This binding has a specificity distinctly different from each of those listed in table 2. Moreover, the specificity was in many ways quite parallel to that of what might have been expected of the physiological *beta*-adrener-

TABLE 2

Biological binding and uptake mechanisms for catecholamines

Mechanism	Uptake 1 (6), Neuronal Uptake	Uptake 2 (6), Extraneuronal Uptake	Nerve Storage Vesicle Uptake (48)	Adrenal Medullary Granule Uptake (15)	Metabolizing Enzymes	
					Catechol-O-methyl transferase (3)	Monoamine oxidase
Specificity (decreasing order of potency)	Metaraminol Dopamine Norepinephrine Epinephrine Mephentermine Phenethylamine Ephedrine Phenylephrine Isoproterenol DOPA Methoxamine	Metanephrine Normetanephrine Buphenine Epinephrine Phenethylamine Isoproterenol Norepinephrine Methoxamine Dopamine Metaraminol	Phenethylamine Metaraminol Dopamine Ephedrine Mephentermine	Phenethylamine Epinephrine Norepinephrine Phenylephrine Isoproterenol Ephedrine	Equally specific for all catechols no affinity for non-catechols	
Other characteristics		Operates only at high amine concentrations Inhibited by phenoxybenzamine (6, 10, 51)	Potency determined as ability to block uptake of ^{3}H-norepinephrine by splenic nerve granules (48)	Potency determined by ability to block ^{14}C-epinephrine uptake by adrenal medullary chromaffin granules (15)	1. K_M for epinephrine 1.2 $\times 10^{-4}$ M (3) 2. Present in soluble cytoplasmic fraction of tissue (3)	Present almost exclusively in mitochondrial fraction (16)

gic receptor. Such binding has been demonstrated in membranes derived from heart (19, 21, 22, 27), liver (9, 31, 46), spleen capsule (8), and erythrocytes (40).[1]

The binding site in cardiac muscle has been studied most extensively and also has been purified by affinity chromatography (21). The characteristics of binding *in vitro* of ^{3}H-norepinephrine to these sites will be described with particular reference to the potential applications of such a binding site to assay of catecholamines.

Binding can be studied with microsomal membranes prepared from canine ventricular myocardium. Binding of ^{3}H-norepinephrine to such a particulate preparation is conveniently quantified by the technique of rapid Millipore filtration and liquid scintillation counting.

The binding is reversible and non-covalent. Thus, ^{3}H-norepinephrine bound to sites in the membranes can be dissociated, *e.g.*, by 1 M HCl. This dissociated ^{3}H-norepinephrine is chromatographically unaltered and can be rebound by a fresh aliquot of membranes in a fashion identical to native ^{3}H-norepinephrine (27).

The specificity of the site for binding catecholamines and related drugs is determined by testing the ability of compounds to competitively inhibit the binding of ^{3}H-norepinephrine to sites in the particulate preparations. That the true catecholamines, which are potent *beta*-agonists, are the most potent in this regard is shown in figure 2. On a molar basis, isoproterenol was most potent. As demonstrated in figure 3, *alpha*-adrenergic amines were much less potent.

Summarized in table 3 are data on the potency of a variety of compounds in blocking the binding of ^{3}H-norepinephrine to these sites. The most crucial structural feature appears to be an intact catechol moiety. The catechol function has been shown previously to be of crucial importance in determining *beta*-adrenergic effectiveness (14).

beta-Adrenergic blocking compounds, such as propranolol, inhibited binding, whereas phentolamine did not (fig. 4). However, it should be noted that the concentrations of blocking agents necessary to inhibit binding

[1] Bilzekian, J. and Aurbach, G.: A beta adrenergic receptor of the turkey erythrocyte. I. Binding of catecholamine and relationship to adenylate cyclase activity. Submitted for publication.

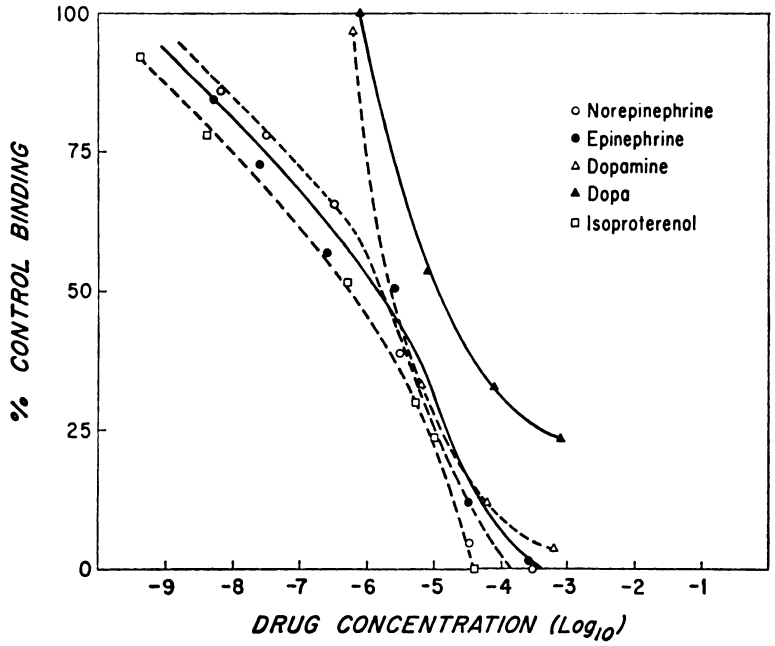


FIG. 2. Inhibition of ^{3}H-norepinephrine binding to cardiac microsomes by *beta*-adrenergic drugs. (From R. J. Lefkowitz and E. Haber: A fraction of the ventricular myocardium that has the specificity of the cardiac beta-adrenergic receptor. Proc. Nat. Acad. Sci. U.S.A. **68**: 1773–1777, 1971.)

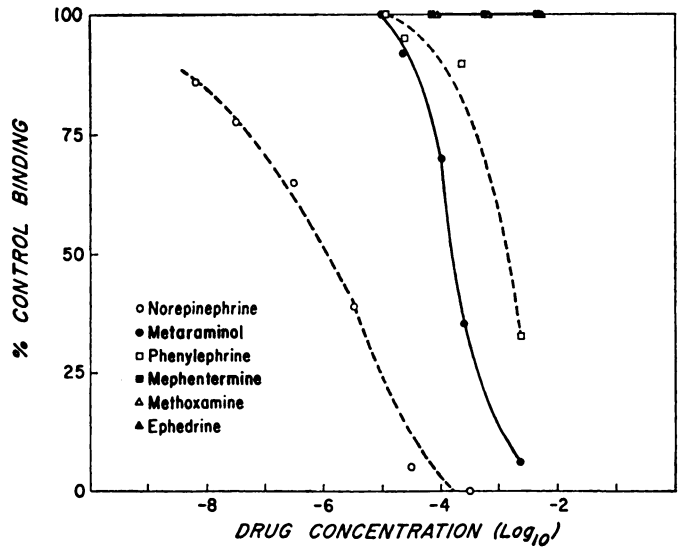


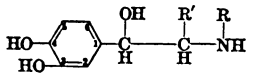
FIG. 3. Inhibition of ^{3}H-norepinephrine binding to cardiac microsomes by *alpha*-adrenergic and indirectly active amines. (From R. J. Lefkowitz and E. Haber: A fraction of the ventricular myocardium that has the specificity of the cardiac beta-adrenergic receptor. Proc. Nat. Acad. Sci. U.S.A. **68**: 1773–1777, 1971.)

($\sim 10^{-4}$ M) were considerably higher than those actually necessary to block adrenergic effects in a variety of preparations *in vitro*. This suggests that the blocking, as opposed to the agonist, drugs may be interacting with closely related, although not identical, portions of these receptor binding sites.

The myocardial membranes used for these

TABLE 3

Affinity of sympathomimetic amines and related compounds for cardiac beta-adrenergic binding sites in vitro

Compound	½ Max Inhibition of ^{3}H-Norepinephrine Binding						
beta-Adrenergic agents							
Isoproterenol	1.8×10^{-7} M	Yes	Yes		Yes	H	C_3H_7
Epinephrine	3×10^{-7} M	Yes	Yes		Yes	H	CH_3
Norepinephrine	5×10^{-7} M	Yes	Yes		Yes	H	H
Dopamine	3×10^{-6} M	Yes	Yes		No	H	H
DOPA	1.5×10^{-5} M	Yes	Yes		No	COOH	H
alpha-Adrenergic Agents							
Phenylephrine	6×10^{-4} M	No	Yes		Yes	H	CH_3
Methoxamine	Not active at 10^{-4} M	No	Yes	2,5,OCH_3	Yes	CH_3	H
Indirectly active							
Adrenergic agents	3×10^{-4} M	No	Yes		Yes	CH_3	H
Ephedrine	Not active at 10^{-4} M	No	No		Yes	CH_3	CH_3
Mephentermine	Not active at 10^{-4} M	No	No		No	C_3H_7	CH_3
Metabolites							
Metanephrine	Not active at 10^{-4} M	Yes	OCH_3		Yes	H	CH_3
Normetanephrine	Not active at 10^{-4} M	Yes	OCH_3		Yes	H	H
Vanillymandelic acid	Not active at 10^{-4} M	Yes	OCH_3		Yes COOH		
Homovanillic acid	Not active at 10^{-4} M	Yes	OCH_3		No COOH		
Miscellaneous							
Phenethylamine	Not active at 4×10^{-3} M	No	No		No	H	H
Dihydroximandelic acid	1×10^{-5} M	Yes	Yes		Yes COOH		
alpha-Methyl DOPA	5×10^{-6} M	Yes	Yes		No	COOH CH_3	H

binding studies also contain adenylate cyclase activity which can be stimulated by catecholamines. As shown in figure 5, the order of potency of catecholamines in stimulating this cyclase was quite parallel to that for their ability to compete for the binding sites.

As noted previously, a number of nonphysiologically active catechol compounds are effective inhibitors of binding. The limited range of hormone responsiveness of the adenylate cyclase in our preparations made it difficult to assess the effects of these compounds on adenylate cyclase activity. However, Bilzekian and Aurbach[1] have shown recently that in turkey erythrocyte membranes, there is a direct relationship between the ability of catechol compounds to inhibit binding of ^{3}H-isoproterenol and to inhibit isoproterenol stimulated cyclase activity.

Of some interest is the lack of stereospecificity of this binding. Thus, *d*- and *l*-norepinephrine compete equally well. This is in

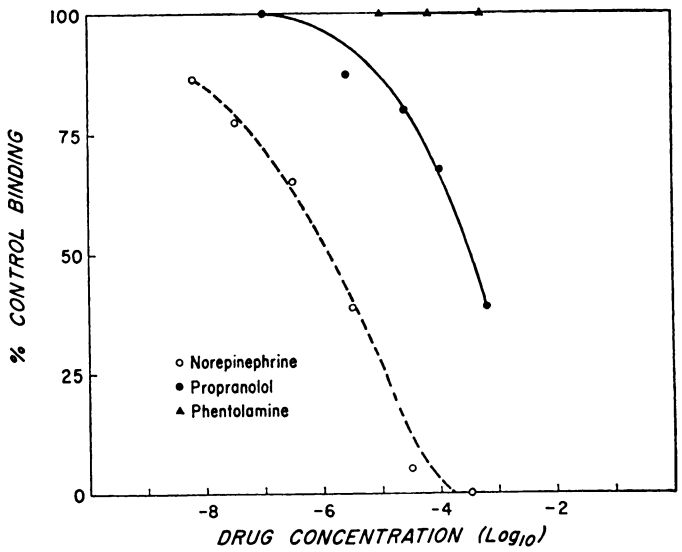


FIG. 4. Inhibition of ^{3}H-norepinephrine binding to cardiac microsomes by adrenergic blocking agents. (From R. J. Lefkowitz and E. Haber: A fraction of the ventricular myocardium that has the specificity of the cardiac beta-adrenergic receptor. Proc. Nat. Acad. Sci. U.S.A. 68: 1773-1777, 1971.)

contrast to the known stereospecificity in biological response, the *l* forms of catecholamines generally being more active than the *d*. This underscores the fact that requirements for binding on the one hand and biological activity may not be identical. Thus, biological activity, *i.e.*, the ability to induce some crucial conformation change in a receptor, may require a full or three-point contact of the hormone molecule which is only possible in one steric configuration. The less preferred conformation might be capable of only two-point contact, *etc.*

To assess the affinity of these binding sites for norepinephrine, Scatchard plots were constructed from displacement curves (fig. 6). When data is plotted in this fashion, the slope is equal to the association constant. It is clear from the figure that there are two kinetically distinct orders of sites, with K's differing by about one order of magnitude. Similar findings have been reported for catecholamine binding to membranes from liver and splenic capsule. The significance of this heterogeneity of binding sites is not clear at the present time but has been found as well to characterize the interaction of several other hormones, such as insulin[2] (13, 43) and ACTH (27), with their receptors.

Recently binding studies have been carried out with intact chick embryo myocardial cells grown in tissue culture.[3] This system is a particularly interesting one in that binding may be studied to intact cells in an environment free of nerves or blood vessels (12). The findings were virtually identical with those just described for subcellular membranes derived from canine ventricular tissue. These studies suggested that the binding sites were localized predominantly to the cell surface.

The final portion of this presentation is a consideration of the potential application of adrenergic binding sites *in vitro* to the measurement of catecholamines. How would such an assay system be set up, what would be its characteristics, and how would these compare with existing assay methods for catecholamines?

First, let us consider specificity. From the data already presented, it should be clear

[2] Kahn, R., Freychet, P. and Roth, J.: Personal communication.

[3] Lefkowitz, R. J., O'Hara, D. and Warshaw, J.: Unpublished observations.

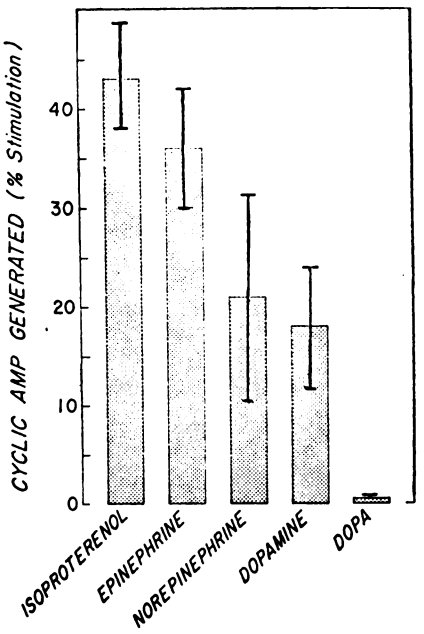


FIG. 5. Activation of adenylate cyclase in cardiac microsomes by adrenergic drugs. (From R. J. Lefkowitz, G. Sharp and E. Haber: Specific binding of beta adrenergic catecholamines by a subcellular fraction of canine ventricular myocardium. J. Biol. Chem. **248**: 342–349, 1973.)

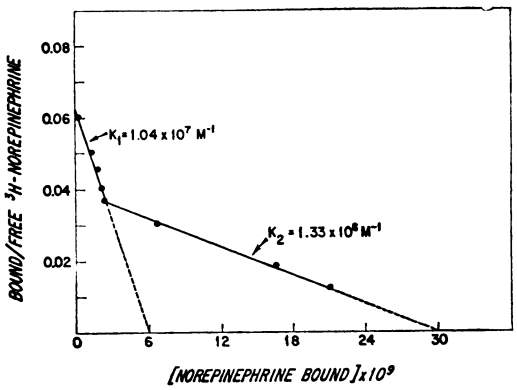


FIG. 6. Scatchard plot (G. Scatchard: The attraction of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. **51**: 660–667, 1949) for binding of norepinephrine to cardiac microsomal membranes. (From R. J. Lefkowitz, G. Sharp and E. Haber: Specific binding of beta adrenergic catecholamines by a subcellular fraction of canine ventricular myocardium. J. Biol. Chem. **248**: 342–349, 1973.)

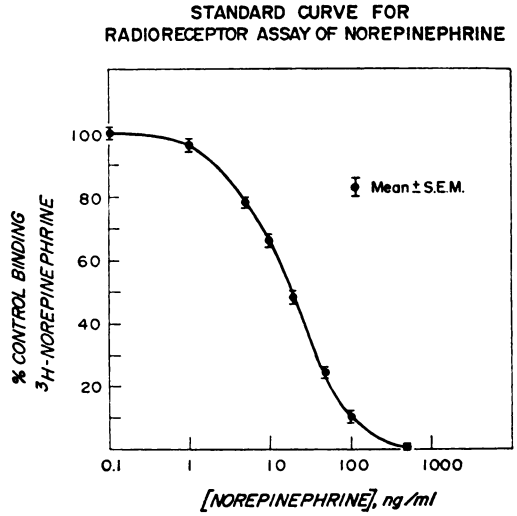


FIG. 7. Standard curve for a radioreceptor assay of norepinephrine.

that such an assay would not effectively distinguish norepinephrine from epinephrine but rather would give a "total catecholamine" value unless the individual amines were chromatographically separated prior to assay. In the case of plasma samples, about 80% of the total circulating catecholamine is norepinephrine, 20% is epinephrine, and a very small amount is dopamine (2).

A second point is that of sensitivity, which is, of course, dependent on the affinity of binding. A displacement curve for norepinephrine is shown in figure 7. It is somewhat more sensitive than those shown earlier. The increased sensitivity was obtained by using small tracer size and incubating the unlabeled norepinephrine with the membranes prior to the addition of ^{3}H-norepinephrine. Under these conditions, several nanograms of norepinephrine give a reproducible displacement.

Today the very best fluorescence assays detect as little as 1 ng or even less of norepinephrine or epinephrine (35, 37). The sensitivity of the enzymatic double isotope derivative method is in a comparable range (11). However, this sensitivity is not always achieved, and in many laboratories, 10 to 20 ng of catecholamine is the effective lower limit of detection.

If we consider the total catecholamines in plasma are about 0.5 to 1.0 ng/ml (2), then

a plasma assay based on the adrenergic binding site would have to include a step which concentrated the catecholamines from 5 to 10 ml of plasma. We have found that this can be easily and efficiently done by using a variety of strong or weak cation exchange materials in the form of small columns. After adsorption of the catecholamines from a plasma sample, the columns are eluted with molar HCl and then lyophilized. Although it is necessary to use highly purified reagents, especially the acid used for elution, these procedures can be performed conveniently with a recovery of 85 to 90% and no loss of catecholamine during the lyophilization.

To date, development of a clinically useful assay has been hampered by the presence of non-specific interfering substances of unknown type in the eluates from each of the several types of column materials which we have explored. These have included Dowex, Alumina, and an experimental resin, Biorex PC 20. However, results with other resins, such as amberlite, may be promising, and there is reason to believe that this technical problem will be solved in the future.

At such time as a radioreceptor assay for catecholamines does become available, what advantages will it have over existing methodology? First, simplicity: the binding material is stable indefinitely when frozen and 50 to 100 samples are easily assayed in 1 day. Second, rapidity: incubations are performed for 1 hr and data is available the same day. Third, the precision that accompanies use of radioisotopic methods.

It has been the purpose of this presentation to review information about isolated *beta*-adrenergic binding sites and their potential application to catecholamine measurement. Although a radioreceptor assay for catecholamines is not a reality at present, there is every reason to think that such an assay is a feasible undertaking.

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